Methyl-CpG binding domain protein 1 regulates localization and activity of Tet1 in a CXXC3 domain-dependent manner

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ABSTRACT

Cytosine modifications diversify and structure the genome thereby controlling proper development and differentiation. Here, we focus on the interplay of the 5-methylcytosine reader Mbd1 and modifier Tet1 by analyzing their dynamic subcellular localization and the formation of the Tet oxidation product 5-hydroxymethylcytosine in mammalian cells. Our results demonstrate that Mbd1 enhances Tet1-mediated 5-methylcytosine oxidation. We show that this is due to enhancing the localization of Tet1, but not of Tet2 and Tet3 at heterochromatic DNA. We find that the recruitment of Tet1 and concomitantly its catalytic activity eventually leads to the displacement of Mbd1 from methylated DNA. Finally, we demonstrate that increased Tet1 heterochromatin localization and 5-methylcytosine oxidation are dependent on the CXXC3 domain of Mbd1, which recognizes unmethylated CpG dinucleotides. The Mbd1 CXXC3 domain deletion isoform, which retains only binding to methylated CpGs, on the other hand, blocks Tet1-mediated 5-methylcytosine to 5-hydroxymethylcytosine conversion, indicating opposite biological effects of Mbd1 isoforms. Our study provides new insights on how cytosine modifications, their modifiers and readers cross-regulate themselves.

INTRODUCTION

In mammals, the fifth position of cytosine can be modified by DNA methyltransferases to 5-methylcytosine (5mC) (1,2). The majority of 5mC is present in the context of CpG dinucleotides (CpGs) (3). Constitutive heterochromatin, which is usually marked by high levels of 5mC, is highly condensed and clustered in mouse cells forming the so-called chromocenters (4,5).

The 5mC can specifically be recognized by 5mC readers, and methyl-CpG binding domain (MBD) proteins represent one such family of proteins. Until now, five members of the MBD protein family have been well characterized including Mbd1, Mbd2, Mbd3, Mbd4 and Mecp2. Except for Mbd3, all members can specifically recognize methylated CpGs (5,6). The binding of MBD proteins to methylated CpGs regulates gene expression and chromatin structure (7).

While the MBD domain mediates binding to methylated CpGs, their unmethylated counterparts can be specifically recognized by the CXXC domain protein family (8). Although members of the CXXC domain protein family share a conserved CXXC motif, which contains two cysteine-rich clusters, three types of CXXC domain proteins are further classified according to sequence similarities. Only type one can specifically recognize unmethylated CpGs, type two and type three show less or no specificity for unmethylated CpGs (9). Interestingly, Mbd1, which contains a MBD, also belongs to the CXXC domain protein family. Several isoforms of Mbd1 have been identified and the full length Mbd1 contains three CXXC domains. However, only the third CXXC domain can specifically recognize unmethylated CpGs (10–12). An increasing number of studies show that the CXXC domain proteins may act as a CpG island targeting module (8,13,14).

Recent studies showed that 5hmC, the oxidation product of ten–eleven translocation proteins (Tet) (15), is not only involved in loss of DNA methylation (16) but also acts as a stable epigenetic mark (17) involved in the regulation of gene expression (18), cellular reprogramming (19) and embryonic stem cell (ESC) differentiation (20). The unique genomic pattern of 5hmC in different tissues, cells and de-

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velopmental stages (21) indicates that Tet-mediated 5mC to 5hmC conversion is highly regulated. Indeed, several studies showed that the N-terminus of Tet1 itself (22,23), as well as post-translational modifications (24,25) and co-factors (26,27) regulate Tet1 activity. Genome wide analysis showed that Tet1 preferentially localizes to CpGs in vivo (18,22). However, the CXXC domain of Tet1 belongs to type three (9), which, as further shown by in vitro binding assays (28), has no specificity for CpGs. Accordingly, the localization of Tet1 to CpGs is more likely to be facilitated by other proteins. Previous studies showed that the CXXC domain of IDAX (29) specifically recognizes unmethylated CpGs and further recruits Tet2 to CpG sites, indicating that CXXC domain proteins might target Tet proteins to CpG sites.

Since Mbd1 has CXXC binding sites for both, methylated and unmethylated DNA (12), it is a potential candidate for targeting Tet1 to CpGs. In this study, we investigated the dynamics of Mbd1 and Tet1 by analyzing their subnuclear localization and the formation of the Tet oxidation product 5hmC. We show that Mbd1 enhances Tet1-mediated 5mC to 5hmC conversion by interacting with and facilitating its localization to methylated DNA. Subsequently, we find that catalytically active Tet1 displaces Mbd1 from methylated DNA. Finally, we show that recruitment of Tet1 by Mbd1 is not cell cycle dependent and requires the CXXC3 domain that binds unmethylated CpG. These results define the spatio-temporal network of interactions among the methylcytosine reader Mbd1, the methylcytosine modifier Tet1 and its oxidation products and the importance for regulation of chromatin organization.

**MATERIALS AND METHODS**

**Expression plasmids**

Plasmids coding for EGFP or EGFP tagged Mbd proteins were described in previous publications (30–33) and the corresponding fusion proteins are shown in Supplementary Figure S1. Mbd1 (pcDNA-Mbd1a), Flag-tagged Mbd1 with CXXC3 deletion (pFlag-Mbd1b) and pGBP-MaSat were described before (12,34). mCherry-tagged catalytic active (mCherry-Tet1CD: aa 1367–2007, H1652Y, D1654A) and inactive (mCherry-Tet1CDmut: aa 1367–2007) and the respective mutations (9), which, as further shown by binding assays (28), have no specificity for CpGs. Accordingly, the localization of Tet1 to CpGs is more likely to be facilitated by other proteins. Previous studies showed that the CXXC domain of IDAX (29) specifically recognizes unmethylated CpGs and further recruits Tet2 to CpG sites, indicating that CXXC domain proteins might target Tet proteins to CpG sites.

For construction of mCherry-tagged mouse Tet1, Np95 was replaced by Tet1 (28) in the mammalian expression vector pCAG-mCherry-Np95-IB (37) using AsISI and NotI restriction sites.

For construction of mCherry-tagged mouse Tet1CD, Tet1CD was replaced by Tet1CD in the mCherry-Tet1CD vector using AsISI and NotI restriction sites.

For construction of mCherry-tagged mouse Tet3CD, Tet3CD was replaced by Tet3CD in the mCherry-Tet3CD vector using AsISI and NotI restriction sites.

For construction of mCherry-tagged mouse Mbd1.2 (pMbd1.2G, TRD (Mbd1), aa 396–636), the sequence coding the C-terminus of Mbd1 (Mbd1 and GFP) was amplified from pFBMbd1a-GFP (31) using primers containing EcoRI and XbaI sites. Then, the fragment was inserted into pEYFP-N1 (Clontech Laboratories, Inc., CA, USA) using EcoRI and XbaI restriction sites. The primers used were:

Mbd1.2 F: 5′-ATGAATTCTAGTGCAGTTTGCCA TGAAGC-3′
Mbd1.2 R: 5′-GATCTAGAGTACTTGACAGCTCG-3′.

For construction of GFP-tagged mouse Mbd1.3 (pGmMbd1.3, Mbd1b, Mbd1ΔCXXC3), Mbd1b from pFlagMbd1b was inserted into pEGFP-C3 (Clontech Laboratories, Inc., CA, USA) using HindIII and BamHI restriction sites.

For construction of GFP-tagged mouse Mbd1.4 (pGmMbd1.4, Mbd1ΔMBD, aa 75–636), the sequence coding for Mbd1 lacking the MBD domain was amplified from pMbd1-GFP with primers containing HindIII and BamHI sites. Then, the fragment was inserted into pEGFP-C3 (Clontech Laboratories, Inc., CA, USA) using HindIII and BamHI restriction sites. The primers used were:

Mbd1.4 F: 5′-CATAGCTTACTCATCCTTGGC-3′
Mbd1.4 R: 5′-AATGGATCCAAAATTTCTCTT-3′.

For construction of GFP-tagged mouse Mbd1.5 (pGmMbd1.5, MBD (Mbd1), aa 1–98), the sequence coding for the MBD domain and NLS were amplified from pMbd1-GFP using primers containing HindIII and BamHI restriction sites. Then, the fragment was inserted into pEGFP-C3 (Clontech Laboratories, Inc., CA, USA) using HindIII and BamHI restriction sites. The primers used were:

Mbd1.5 F 5′-CATAGCTTATGCTGAGTCCTG-3′
Mbd1.5 R: 5′-AATGGATCCAGCCAACCTT-3′.

For construction of GFP-tagged mouse Mbd1.7 (pGMbMbd1.7, Mbd1 (C289,292A)), the respective mutations were introduced with the Q5® Site-Directed Mutagenesis Kit (NEB, catalog #E0554S) according to the manufacturer’s instructions. The template for polymerase chain reaction (PCR) was the plasmid containing mouse Mbd1 (pMbd1-GFP) and the primers were:

F 5′-GCCGGTCTACGTCTGCAGGAGG-3′
R 5′-CCCAGCTTTGGCTGTTGCGCTTG-3′.

Lenti-shMbd1 (pYG-21, pc3361) and lenti-shNC (negative control, pYG-22, pc3362) were cloned using lentivirus-sh-Control vector as backbone (38). Briefly, based on mouse Mbd1 mRNA sequence, shMbd1 (5′-GCTGCAAGATCCGACCTTCA-3′) was designed using BLOCK-it™ RNAi Designer software. The shNC sequence (5′-GGAATTCATTCATACGATGAC-3′) was published previously (38). The shRNA sequences together with the loop sequence (TCAAGAG), U6 promoter and restriction sites (Hpal and ClaI) were cloned using two rounds of PCRs with Lenti-U6 promoter-miR-137 vector (38) as PCR template. The primers were:

shMbd1-3:
first round forward: 5′-GAATTCGCGATCCGTACAC CAGGAAGAGGCCCATTTCCCAT-3′
first round reverse: 5′-CCAGACCTTTTACTCTTGAT GAAAAGTCGAGTCGTACGCCGAGTCATCGTC TTTCCAC-3′

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second round forward: 5’-GAATTCGGATCCGTTAAC
CAGGAAGAGGCTATTTTCCAT-3’
second round reverse: 5’-CTCCCAAGCTTATCGATA
CAAAAAAGCTGCAGATCCAGACCTTTCACTCTTGAG
TATGCATCGAATGAGATTCCCGGATCCTCGTC
GAGTATGCA-3’
shNC:
first round forward: 5’-GAATTCGGATCCGTTAAC
CAGGAAGAGGCTATTTTCCAT-3’
first round reverse: 5’-TTCGATGCATACTCTTTGAAGT
TATGCATCGAATGAGATTCCCGGATCCTCGTCT
TTTCCAC-3’
second round reverse: 5’-CTCCCAAGCTTATCGATA
CAAAAAAGGAATCTCATTCGATGCATACCTCTTGAG
GAGTATGCA-3’

The U6-shRNA cassettes were then cloned into lenti-U6-
mir137 vector via HpaI and ClaI restriction sites using the
In-fusion kit according to its instructions.

All plasmid constructs were verified by DNA sequencing
and/or western blotting analyses.

Cell culture and transfection
HEK-293 (ATCC; catalog #CRL-1573) and HEK-EBNA
(Invitrogen; catalog #620-07, Paisley PA49 RF, UK) human
cells were cultured and transfected using polyethylenimine
as previously described (39).

For generation of an RFP-PCNA C2C12 stable cell line, plasmid pEN-CAG-mRFP-PCNA was transfected into
C2C12 cells and the cells were selected with puromycin.
mRFP positive and puromycin resistant cells were used for
further experiments.

The mouse tail fibroblast (MTF) cell line was cultured us-
ing the conditions as previously described (40). Mouse wild-
type (W8) embryonic fibroblasts (MEF) (41) and mouse
Dnmt1−/− and p53−/− fibroblasts (42) were cultured as des-
ccribed before (43). These cells were transfected by nucleo-
fection as previously described (44).

J1 mouse ES cells (2) and E14 mouse ES cells (45) were
cultured under feeder-free conditions on gelatin coated cul-
ture dishes (0.2% gelatin in ddH2O) in Dulbecco’s modi-
cated Eagle’s medium (high glucose) supplemented with 16%
fetal bovine serum, 1× Minimum Essential Medium non-
essential amino acids, 1× Penicilllin/Streptomycin, 2 mM
L-Glutamine (Sigma Aldrich, St Louis, MO, USA), 0.1
mM β-mercaptoethanol, 1000 U/ml LIF (Millipore), 1 μM
PD0325901 and 3 μM CHIR99021 (Axon Medchem BV).

Confocal microscopy and image analysis
To calculate protein accumulation and colocalization, con-
fo cal Z stacks of live cells were acquired using the Ultra-
View VoX spinning disc microscopy system (PerkinElmer
Life Sciences) equipped with an oil immersion 60× Plan-
Apochromat numerical aperture 1.45 objective lens as de-
scribed before (34). Then, single mid-plane images were
used for further analysis. To calculate protein accumulation,
the mean intensity of five random regions inside and out-
side chromocenters was measured with ImageJ and the ra-
tio of the mean gray value inside chromocenters to the mean
gray value outside chromocenters was calculated. To mea-
sure the protein colocalization, H-coefficient analysis was
performed as described before (46).

To follow the localization of Tet1 and Mbd1, MEF cells
transfected with mCherry-Tet1CD and Mbd1-GFP con-
structs were used for time-lapse microscopy using the Ul-
traView VoX spinning disc microscopy system. Cells were
imaged every 90 min taking care to minimize laser exposure.

To evaluate the binding kinetics of fluorescently tagged
proteins, fluorescence recovery after photobleaching
(FRAP) was performed using the UltraView VoX spinning
disc microscopy system. Quantitative evaluation was
performed using ImageJ, and fluorescence intensity
normalization and curve fitting were performed with the
easyFRAP software as described before (44). T-half values
were extracted from the mean exponential fitting, and plots
were generated with RStudio (Version 0.99.893).

For the in vivo protein recruiting/interaction assay, Dnmt1−/− and p53−/− MEF cells were transfected with
mCherry-Tet1CD, GFP-Mbd1 with or without the GBP-
MaSat construct and 8 h after transfection, confocal Z
stacks were acquired using the UltraView VoX spinning disc
microscopy system. Then, protein accumulation tests were
performed as described above.

High content screening microscopy spot analysis
To evaluate the distribution of proteins inside the cell nu-
cleus, live cell experiments were performed using high con-
tent screening microscopy (Operetta, PerkinElmer, UK)
with a 40× 0.45 numerical aperture long working distance
objective (PerkinElmer, UK), a Xenon fiber optic as light
source, 460–490 and 560–580 nm excitation filters and 500–
550 (EGFP) and 590–640 (mCherry) emission filters, re-
spectively. Eight hours after transfection, fluorescence
signals were detected over a period of 20 or 24 h in time in-
tervals of 4 h at 37°C with 5% CO2. Harmony (Version
3.5.1, PerkinElmer, UK) software was used to perform im-
age analysis. Briefly, the cell nuclei were identified by either
Mbd or Tet signal and spots inside cell nuclei in each chan-
nel were identified. Then, the spot numbers, mean intensity
of spots and mean intensity of cell nuclei were calculated per
cell in the cell population with normal morphology, which
is defined by cell roundness (round cell) and cell size (mid-
dle size). Last, calculated intensities were further processed
and plotted using RStudio (Version 0.99.893) (Supple-
mentary Figure S5).

To calculate cell numbers with Tet1 or Mbd5 accumulating
at heterochromatic DNA, relative intensity (the ratio
of spot and nucleoplasm intensity) above a given threshold
was used for identifying the spots. Then, the percentage of
cells with high spot numbers and low spot numbers were
plotted.

To calculate the accumulation of Tet protein in het-
rochromatic DNA, the heterochromatic region was identi-
fied by Mbd1 relative intensity inside the cell nucleus (Sup-
plementary Figure S5). Then, the ratio of the Tet1 mean
intensity inside heterochromatic region and inside the cell
nucleus was plotted at the different time points.

To evaluate the effect of Tet1 protein on the localization
of Mbd1 protein, the Mbd1 signal at heterochromatic DNA
was identified by the intensity of spots (Supplementary Figure S5). The ratio of Mbd1 mean intensity inside heterochromatic regions and inside the cell nucleus was plotted against Tet1 expression and time points.

**Tet1 protein stability assay**

MEF cells were transfected with GFP-tagged Mbd1 or Mbd2 and mCherry-tagged Tet1CD constructs. Eight hours after transfection, the cells were cultured in the presence or absence of cycloheximide and imaged every 4 h by high content microscopy. The fluorescence intensity of GFP and mCherry inside the cell nucleus was calculated using the Harmony software and further plotted using R studio.

**Mbd1 knockdown and isoform-specific rescue assay**

Lentiviral particles were produced by transfection of HEK 293 cells using the pCI-VSVG and psPAX2 packaging plasmids (Addgene numbers 1733 and 12260) and pYG-21resp. pYG-22 shRNA containing plasmids. Mouse J1 ESCs were transduced using lentivirus-containing supernatants and 8 μg/ml polybrene. Twenty-four hours after transduction, the medium was replaced with virus-free ESC medium and cells were fixed at the indicated time points.

For Mbd1 isoform specific rescue, cells were transfected via nucleofection 48 h after transduction with Mbd1-GFP, GFP- Mbd1ΔCXX3 or GFP, respectively and fixed 24 h after transfection.

**Immunofluorescence and image analysis**

MEF or MTF cells were fixed for 10 min in 4% formaldehyde in phosphate buffered saline (PBS) and permeabilized for 20 min with 0.5% Triton X-100.

To detect Flag tagged Mbd1, a mouse anti-FLAG M2 (1:75; catalog #sc-2526, Santa Cruz Biotechnology, Dallas, TX, USA) primary antibody and an Alexa Fluor 594 conjugated donkey anti-mouse IgG (1:300; catalog #715-585-151, The Jackson Laboratory, Bar Harbor, ME, USA) were used.

To detect endogenous Mbd1, a mouse anti-Mbd1 B5 (1:75; catalog #sc-25261, Santa Cruz Biotechnology, Dallas, TX, USA) primary antibody and an Alexa Fluor 594 conjugated donkey anti-mouse IgG (1:300; catalog #715-585-151, The Jackson Laboratory, Bar Harbor, ME, USA) were used.

To detect endogenous Tet1 protein, MEF or MTF cells were transfected with the mCherry-Tet1CD construct. Twenty-four hours after transfection, immunostaining with a Tet1-specific antibody (24) was performed as described above. Mouse ESCs (E14) were immunostained with the same Tet1 antibody. Fixation, permeabilization, RNaseA treatment and blocking were performed as described for the 5hmC staining. Subsequently, the cells were incubated with a rat anti-Tet1 (5D8, 1:1) (24) in 2% BSA for 70 min. After three times washing with PBST, the cells were incubated with an Alexa Fluor 488 conjugated donkey anti-rat IgG (1:500, catalog #712-545-153, The Jackson Laboratory, Bar Harbor, ME USA) for 50 min at room temperature.

Then, DNA was counterstained with 4’,6-Diamidino-2-phenylindole dihydrochloride (DAPI, 1 μg/ml).

mCherry, GFP or Alexa Fluor 488 and AMCA signals were imaged using high content screening microscopy with 20 × 0.45 numerical aperture long working distance objective (PerkinElmer, UK), a Xenon fiber optic as light source, 360–400, 460–490 and 560–580 nm excitation filters and 410–480 (AMCA, DAPI), 500–550 (GFP, Alexa Fluor 488) and 590–640 (mCherry) emission filters, respectively. The images were further analyzed with Harmony software and plotted with R studio.

To test the effect of Mbdbs on 5hmC formation, we first grouped the cells by GFP/Mbdbs protein levels into low and high expressing groups. Then, in each group, cells were further binned by Tet1 protein levels. The average 5hmC intensity of single cells was plotted. To measure the effect of Mbd1 on 5hmC formation in the cells with low Tet1 protein level, cells were first grouped by Tet1 protein level and, then, in each group, cells were further binned by GFP or Mbd1 protein level into low, middle and high expressing groups. Then, single cell 5hmC intensity was plotted.

To compare Tet1 protein levels, the fluorescence intensity of ectopic Tet1 (mCherry) and anti-Tet1 antibody was measured with high content screening microscopy using the same exposure time as before (Figures 1 and 5; Supplementary Figure S2). For MTF and MEF cells, the cells were grouped as in Supplementary Figure S2 according to mCherry-Tet1CD intensity. Then, total fluorescence intensity of the whole cell nucleus of single cells was plotted according to mCherry-Tet1CD mean intensity. As a comparison, total fluorescence of whole cell nucleus of mESC was also plotted.

**Radioactive β-glucosyltransferase (BGT) assay**

MEF cells were co-transfected via nucleofection with mCherry-Tet1CD and Mbd1-GFP, GFP-Mbd1ΔCXX3 or GFP. Twenty-four hours later, cells were harvested, gDNA was isolated and the radioactive β-glucosyltransferase assay was performed as previously described (35) with the exception that 5 μg of genomic DNA was used for the reaction.
Protein interaction assay and isoform analysis

All buffers for protein interaction assays were supplemented with the protease inhibitors: AEBSF 1 mM (AppliChem; Darmstadt, Germany), E64 1 mM (Sigma-Aldrich, St Louis, MO, USA) and Pepstatin A 1 mM (Sigma-Aldrich, St Louis, MO, USA).

HEK-EBNA cells plated in p100 cell culture dishes were co-transfected with mCherry-Tet1CD and GFP-tagged subdomains of Mbd1 constructs. Forty-eight hours after transfection, the cell pellets were incubated in 500 µl of PARP buffer (2.5 mM Tris pH 8.0, 0.5 M NaCl, 50 mM glucose, 10 mM EDTA, 0.2% Tween, 0.2% NP40) for 10 min on ice. Following 10 times syringe (0.0, 5 times, followed by 10 times, 0.6) treatment and another 10 min incubation, cell lysates were centrifuged at 13 400 × g for 20 min at 4°C. After centrifugation, 25 µl of the supernatant was aliquoted for Mbd1s/GFP input (input 1) and the rest was diluted with 500 µl of PARP buffer without NaCl and was incubated with RFP binding protein coupled to sepharose beads (RFP-Trap, ChromoTek, Planegg-Martinsried, Germany) supplemented with 10 µg/ml of ethidium bromide (EtBr) for 1 h at 4°C on a rotary shaker. After incubation, the beads were washed six times with PARP buffer containing 0.25 M NaCl. Next, the proteins were denatured at 99°C in sodium dodecyl sulphate (SDS) containing buffer (1% SDS, 25 mM Tris pH 6.8, 5% glycerol, 0.005% Bromophenol blue, 50 mM Dithiothreitol) for 10 min. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins. After blotting to nitrocellulose membrane, the gel was stained with Coomassie Brilliant Blue as an input estimation for Tet1 protein (input 1). The GFP signals were detected with a rat monoclonal anti GFP (clone 3H9) primary antibody (31) (ChromoTek, catalog #029762, Planegg-Martinsried, Germany) and horseradish peroxidase conjugated goat anti-rat IgG secondary antibody (1:500, catalog #112-035-068, The Jackson Laboratory, Bar Harbor, ME, USA). To visualize the signal, ECL plus western blot detection reagent (Amersham Pharmacia Biotech, UK) was used and signals were detected on a ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA) and analyzed together with ESC lysates by 8% SDS-PAGE and western blot with anti-Mbd1 antibody as described above.

Statistical analyses and graphical representation

Independent two-group student’s t-tests were performed using RStudio (Version 0.99.893) and P-values were indicated on the plots for all experiments, except for FRAP experiments where Wilcoxon tests were used.

Boxplots represent the median with the box depicting the 25–75 percentiles and the lines and dots 95% confidence interval and outliers, respectively.

RESULTS

Mbd1 enhances Tet1-mediated 5hmC formation

We have shown before that Mbd2 and Mecp2 but not Mbd3 counteract the ability of Tet1 to modify 5mC (35). To investigate whether Mbd1 affects Tet1-mediated 5hmC formation, MTF cells were co-transfected with mCherry-tagged Tet1CD (catalytic domain of Tet1) and GFP-tagged Mbd (methyl-CpG binding domain) proteins (Mbd1, Mbd2, Mbd3, Mbd4 and Mecp2) (Supplementary Figure S1). Twenty-four hours after transfection, cells were fixed and stained with an antibody against 5hmC. As expected, 5hmC levels increased proportionally to Tet1CD protein levels in control transfected cells co-expressing GFP alone (Supplementary Figure S2). While 5hmC levels were not affected in the cells expressing low Mbd protein levels (Supplementary Figure S1, left), 5hmC signals were decreased in cells expressing high Mbd2 and Mecp2 protein levels (Supplementary Figure S2, right) (35). High expression levels of Mbd3 and Mbd4, in contrast, resulted in similar 5hmC levels as obtained for the control cell with GFP expression (Supplementary Figure S2). These results indicate that Mbd2 and Mecp2 block Tet1-mediated 5mC formation in a dose-dependent manner, while Mbd3 and Mbd4 do not affect Tet1-catalyzed 5mC oxidation. In contrast to Mecp2, Mbd2, Mbd3 and Mbd4, co-expression of Mbd1 resulted in a significantly increased 5hmC formation (Supplementary Figure S2, right). The gain in 5hmC was most pronounced in cells expressing low Tet1CD protein levels (Supplementary Figure S2, right, arrow) and, thus, we further focused on these cells (Figure 1A).

Importantly, the Tet1 protein levels of these cells are equivalent to endogenous Tet1 protein levels in mouse ESCs (Supplementary Figure S3). As shown in Figure 1B, 5hmC levels positively correlated with GFP-tagged Mbd1, but not with GFP levels. Moreover, cells expressing Mbd1 showed significantly higher 5hmC levels than cells expressing GFP. These results indicate that under physiological levels of Tet1 protein, Mbd1 enhances Tet1CD-mediated 5hmC formation. In contrast, 5hmC lev-
Figure 1. Mbd1 enhances Tet1-mediated 5hmC formation. (A) Experimental rationale. Mouse tail fibroblasts (MTF) were transfected with mCherry-Tet1CD (catalytic domain of Tet1) or mCherry-Tet1FL (full length Tet1) together with Mbd1-GFP (methyl-CpG binding domain protein 1), Mbd2-GFP or GFP. Twenty-four hours after transfection the cells were immunostained with a 5-hydroxymethylcytosine (5hmC) specific antibody. The signals of Tet1, Mbd/GFP and 5hmC were detected by high content screening microscopy and low Tet1 expressing cells were used for plotting. (B) Effect of Mbd1, Mbd2 and GFP on Tet1CD-mediated 5hmC formation. The cells were first grouped according to Tet1 protein levels, then, in each group, cells were further binned by Mbd/GFP protein levels and single cell intensities were plotted. The 5hmC intensities in the cells with high levels of Mbd/GFP and high levels of Tet1CD were used to perform student’s t-test and P-values are indicated. Experiments were repeated at least three times. Cell numbers for Tet1CD + Mbd1, Tet1CD + GFP and Tet1CD + Mbd2 are 10,817, 7,271 and 4,375, respectively. (C) Effect of Mbd1 and GFP on Tet1FL-mediated 5hmC formation in mouse embryonic fibroblast cells (MEF). Intensities of single cells are plotted and the P-value of student’s t-test is indicated. Experiments were repeated at least two times. Cell numbers for Tet1FL + Mbd1 and Tet1FL + GFP are 7,515 and 3,429, respectively. (D) Effect of Mbd1 and GFP on Tet1-mediated 5-carboxylcytosine (5caC) formation in MTF cells. MTF cells were transfected with mCherry-Tet1CD together with Mbd1-GFP or GFP. Twenty-four hours after transfection, the cells were immunostained with an 5caC antibody. The average intensity of single cells was plotted according to Tet1CD protein levels. Experiments were repeated twice and mean values were plotted. Cell numbers for Tet1CD + Mbd1 and Tet1CD + GFP are 1,770 and 582, respectively. The 5caC intensities in the cells with high levels of Tet1CD (dashed line box) were used to perform student’s t-test and the P-value is indicated. 95% confidence intervals were also indicated in the plot.
els anti-correlated with the amount of Mbd2, which is in agreement with its 5mC protection ability.

The N-terminal domain of Tet1 is dispensable for its catalytic activity but it was shown to regulate Tet activity (22,23). As mentioned earlier, Tet1 contains a CXXC domain in its N-terminus (47), which cannot bind to DNA in vitro (28). To test whether Mbd1-mediated enhancement of 5hmC formation depends on the N-terminal domain of Tet1, mouse fibroblast cells were transfected with the mCherry-tagged full length Tet1 protein together with Mbd1-GFP or GFP, respectively. Twenty-four hours after transfection, cells were fixed and stained for 5hmC as previously described (Figure 1B). Similar to Tet1CD, 5hmC levels positively correlated with Mbd1 protein levels, but not with GFP and cells expressing Mbd1 showed significantly higher 5hmC levels than cells expressing GFP (Figure 1C), indicating that the N-terminal domain of Tet1 does not affect the Mbd1-mediated enhancement of 5hmC formation.

5hmC is not the only Tet1 oxidation product as it can be further converted to 5-formylcytosine (5fC) and 5-carboxylycitosine (5caC) in an iterative reaction. To test whether the Mbd1-dependent increase in 5hmC levels was due to blocking its further conversion, MTF cells ectopically expressing mCherry-Tet1CD together with Mbd1-GFP or GFP were immunostained for 5caC. As shown in Figure 1D, 5caC levels were higher in the presence of Mbd1, indicating that Mbd1 does not block the further conversion of 5hmC to 5caC.

Taken together, these results indicate that Mbd1 enhances Tet1-mediated 5mC to 5hmC conversion and this is independent of the N-terminal domain of Tet1. Moreover, the increased 5hmC levels are not due to blocking of its further oxidation.

Mbd1 facilitates the efficient localization of Tet1 to methylated DNA

Oxidation of 5mC to 5hmC by Tet proteins involves several steps including DNA binding by a Watson–Crick polar hydrogen bond and van der Waals interactions, 5mC base flipping and 5mC to 5hmC oxidation (48,49). Therefore, the interaction between Tet1 proteins and DNA is a crucial initial step for Tet oxidation. Since Mbd1 enhances Tet1-mediated 5hmC formation, we wanted to test whether Mbd1 would affect interaction of Tet1 and DNA.

We first measured the dose and time dependency of the Tet1 oxidation reaction in in vitro experiments. At identical Tet1 protein levels, 5hmC amounts increased over time (Supplementary Figure S4). However, 5hmC levels were also proportional to Tet1 levels at both, early and late time points (Supplementary Figure S4). These results indicate that the Tet1-mediated formation of 5hmC depends on enzyme dose and oxidation time. We hypothesize that at early time points, in the presence of excess 5mC substrate, Tet1–DNA interactions predominate relative to later time points, where most of the substrate is oxidized. Thus, we performed live cell experiments 8 h after transfection to test the localization of Tet1 proteins.

In mouse cells, Mbd1 and Mbd2 proteins are mostly localized at highly methylated pericentric heterochromatin (HC). Confocal microscopy analysis showed that Tet1 proteins colocalize with Mbd1 at heterochromatic DNA, but not with Mbd2 (Figure 2A). To quantify the colocalization of Tet1 and Mbd proteins, H-coefficient (46) analysis was performed. As shown in Figure 2B, the colocalization between Tet1 and Mbd1 was significantly higher than that between Tet1 and Mbd2. In addition, we measured the accumulation of Tet1 proteins to heterochromatic DNA by quantifying the fluorescence signal of Tet1 proteins inside and outside heterochromatin. We found that co-transfection of Mbd1 but not Mbd2, significantly increased the accumulation of Tet1 proteins to heterochromatic DNA (Figure 2C). Altogether, these data show that Mbd1 enhances Tet1CD localization to heterochromatic regions. To further validate this, we performed high content microscopy screening spot analysis (Supplementary Figure S5A and B). Since Mbd1 protein is mostly accumulated at heterochromatin, one spot would represent one heterochromatic region in the cell. The results showed that the accumulation of Tet1CD at heterochromatic DNA, as well as 5hmC levels correlate with the amount of Mbd1 proteins (Supplementary Figure S6A and 7), indicating that Mbd1 enhances Tet1-mediated 5hmC formation by facilitating its localization to heterochromatic DNA in a dose-dependent manner. Similar to the catalytic domain of Tet1, Tet1 enhances the heterochromatin accumulation of Tet1FL (Supplementary Figure S6B and 7), indicating that the N-terminal domain of Tet1 is dispensable for its Mbd1-mediated subnuclear localization.

Since Tet1-mediated 5mC oxidation depends on enzyme dose and Mbd1 enhances this process, we further tested whether Mbd1 could stabilize Tet1 proteins from degradation. To this end, we analyzed Tet1 stability in the presence of Mbd1 or, as a control, Mbd2. As shown in Supplementary Figure S8, Mbd1 did not enhance the stability of Tet1CD protein, therefore this cannot be the cause for increased 5hmC.

In mammalian cells, three Tets have been reported. Since we observed that Mbd1 enhanced Tet1CD localization at heterochromatic DNA, we further tested whether Mbd1 could also enhance Tet2 and Tet3 localization at heterochromatic DNA at an early time point (8 h after transfection). To this end, we measured the accumulation of Tet2CD and Tet3CD in the presence of Mbd1. As shown in Supplementary Figure S9, the accumulation of Tet1CD was significantly higher than the accumulation of Tet2CD and Tet3CD in the presence of Mbd1, indicating that the Mbd1 enhanced heterochromatin localization of Tet protein is specific for Tet1.

The accumulation of Tet1 proteins to heterochromatic DNA prompted us to investigate whether Tet1 DNA binding kinetics are affected. To this end, we performed fluorescence recovery after photobleaching (FRAP) analysis to measure the mobility of Tet1 proteins. We co-transfected MTF cells with mCherry-Tet1CD and Mbd2-GFP or Mbd1-GFP constructs. Eight hours after transfection, FRAP measurements were performed. Compared to freely nuclear distributed Tet1CD, heterochromatin accumulated Tet1 had much slower recovery kinetics (Figure 2D) and, thus, decreased mobility. Hence, we hypothesize that the prolonged retention time of Tet1 proteins at hetero-
Figure 2. Mbd1 facilitates the localization of Tet1 to heterochromatic DNA. (A–C) Localization of Tet1CD and Mbd1 or Mbd2 in MTF cells. (A) MTF cells were transfected with Tet1CD together with GFP-tagged Mbd1 or Mbd2 constructs. Eight hours after transfection, Tet1 and Mbd1/Mbd2 signals were detected by confocal microscopy and images were used for H-coefficient colocalization (B) and protein accumulation (C) analysis. To calculate protein accumulation at heterochromatin, mean fluorescence intensities inside and outside heterochromatin were measured with ImageJ and the ratio of the mean gray value inside and outside heterochromatin was plotted. Scale bars: 5 μm. The cell number and P-values of the student’s t-test are indicated. Two independent experiments were performed. (D) Fluorescence recovery after photobleaching (FRAP) analysis of Tet1 protein mobility. MEF cells were transfected with constructs coding for mCherry-Tet1CD together with Mbd1-GFP or GFP. The mCherry-Tet1CD signal at heterochromatin regions was photobleached with a 488 nm laser. The mean recovery curve was plotted. Cell numbers and P-value from Wilcoxon test are indicated on the plot. Two independent experiments were performed. (E) Accumulation of Tet1CD and Tet1CDmut (catalytic mutant of Tet1) to heterochromatic DNA. Shown are quantification results of the accumulation of Tet1 proteins at heterochromatic DNA using images from high content screening time-lapse microscopy. To better visualize the accumulation changes, the median accumulation value of Tet1CD at an early time point (8 h after transfection) is indicated and P-values of the student’s t-test are indicated. Two independent experiments were performed. For each time point, more than 100 cells were analyzed.
heterochromatic DNA allows an increased number of oxidation reactions.

The crystal structure of Tet2 in complex with DNA revealed that Tet2 proteins contain three iron binding sites, which are conserved in all three Tet proteins (48,49). Since the oxidation ability of Tet1 proteins is abrogated through mutations of two iron binding sites (50), we tested whether mutation of these two sites affects the accumulation of Tet1 to heterochromatic DNA. To this end, cells were transfected with a catalytic mutant of Tet1 (H1625Y and D1654A, Tet1CDmut) (50) and its accumulation to heterochromatic DNA was quantified as described earlier (Supplementary Figure S5A and C). At an early time point after transfection, the accumulation of Tet1CDmut at heterochromatic DNA is similar to the accumulation of Tet1CD (Figure 2E; Supplementary Figures S7 and 10). While for Tet1CDmut, the extent of heterochromatin accumulation (defined by the Mbd1 signal) remained stable over time (Figure 2E), the accumulation of the catalytically active Tet1 protein significantly decreased with progressive oxidation time (Figure 2E). Since Tet proteins have a substrate preference for 5mC (51), we suggest that once the oxidation reaction is completed, Tet1 proteins no longer bind to heterochromatic DNA resulting in decreased accumulation of Tet1CD at heterochromatic DNA. Since the two iron binding sites are indispensable for Tet1-mediated 5mC oxidation, we suggest that the accumulation of Tet1 to heterochromatic DNA and the subsequent 5mC oxidation are two distinct steps, whereby iron binding sites are not involved in the protein accumulation at heterochromatin. Taken together, these results indicate that Mbd1 enhances Tet1-mediated 5hmC formation by promoting its accumulation to heterochromatin DNA. This accumulation is per se independent of Tet1 catalytic activity.

Cell cycle-independent Tet1 accumulation at heterochromatic DNA

Mbd1 has been shown to recruit the histone lysine N-methyltransferase SETDB1 to the large subunit of the chromatin assembly factor 1 to form a S-phase-specific complex (52), suggesting a role for Mbd1 during DNA replication. To test whether the subnuclear localization of Mbd1 to heterochromatic DNA is cell cycle dependent, we transfected cells stably expressing the cell cycle S-phase marker RFP-PCNA (proliferating cell nuclear antigen) with Mbd1-GFP and performed the spot analysis as described earlier (Supplementary Figure S5A and B). The distribution of PCNA is cell cycle dependent. In G1 or G2-phase, PCNA distributes homogenously in the cell nucleus, while in S-phase it localizes to DNA replication foci. Especially in late S-phase, PCNA accumulates at heterochromatin (53,54). While about 75% of the cell population presented Mbd1 accumulated at heterochromatin, only about 25% of all cells showed PCNA accumulating at heterochromatin (Supplementary Figure S11). These results indicate that Mbd1 accumulation at heterochromatin is independent of the cells being in late S-phase.

Since we had shown that Mbd1 enhances the localization of Tet1 to heterochromatic DNA (Figure 2), we tested whether this enhancement is cell cycle dependent. To this end, MEF cells were transfected with CFP-PCNA, Mbd1-GFP and mCherry-Tet1CD, and the subnuclear localization of all three proteins was analyzed by confocal microscopy. In late S-phase, when PCNA accumulates at heterochromatin, colocalization with Tet1CD and Mbd1 was observed. In G1 or G2-phase, however, when PCNA distributes homogenously throughout the cell nucleus, Tet1 and Mbd1 were still bound to heterochromatic DNA (Figure 3A), indicating that Mbd1-enhanced accumulation of Tet1 to heterochromatin is not cell cycle dependent. To further analyze the distribution of Tet1 and Mbd1 throughout the cell cycle, time-lapse microscopy was performed with cells expressing mCherry-Tet1CD and Mbd1-GFP. Live cell imaging showed that before and after cell division, Tet1 and Mbd1 colocalize with each other (Figure 3B and Supplementary Video 1), indicating that the accumulation of Tet1 to heterochromatin in the presence of Mbd1 is not cell cycle dependent.

The interaction of Mbd1 and Tet1 enhances the localization of Tet1 to heterochromatic DNA

In MEF cells, about 80% of the major satellite DNA is methylated (43) and allows Mbd1 to bind and localize to heterochromatic regions mainly via its MBD domain (12). However, besides its MBD domain, the specific binding ability of the CXXC3 domain to unmethylated CpGs allows Mbd1 to localize to heterochromatic DNA in Dnmt1−/−
cells (12), which were shown to have <20% methylation of the major satellite DNA (43). To test whether Mbd1-facilitated accumulation of Tet1 to heterochromatin is 5mC dependent, Dnmt1−/− MEF cells, ectopically expressing Tet1CD and Mbd1 were analyzed by confocal microscopy. As control, cells co-expressing Mbd2 and Tet1CD were used. The low level of residual DNA methylation in these cells lead to a very substantial decrease of the Mbd1 protein localization at major satellite DNA and an almost complete abrogation of the localization of Mbd2 (Figure 4A). As shown in Figure 4B (left, bar plot), Tet1 proteins did not accumulate to heterochromatic DNA in Dnmt1−/− cells expressing Mbd1 and Mbd2, respectively. Live cell microscopy experiments showed that the CXXC3 domain is necessary for localizing Mbd1 proteins to heterochromatic DNA in Dnmt1−/− cells (Supplementary Figure S12A). However, the efficiency of CXXC3-mediated localization of Mbd1 to heterochromatic DNA in Dnmt1−/− cells is much lower compared to that of the Mbd1 in wildtype cells (Supplementary Figure S12A and B). The levels of Mbd1 proteins accumulated at heterochromatic DNA in Dnmt1−/− cells might not be sufficient for Tet1 recruitment. These results indicate that Mbd1 enhances the localization of Tet1 to heterochromatin in a 5mC-dependent manner.

Previous studies have shown that a GFP-binding protein (GBP) fused to a polydactyl zinc finger major satellite DNA binding protein can target GFP-tagged proteins to major satellite heterochromatic DNA (Figure 4C) (34). To test whether artificially heterochromatin-tethered Mbd proteins can affect the subnuclear localization of Tet1, we subjected Dnmt1−/− cells that were triple transfected with mCherry-Tet1CD, GBP-MaSat and GFP-tagged Mbd1 or Mbd2 constructs to live cell microscopy. Compared to cells without GBP-MaSat transfection, GFP-tagged Mbd1 or Mbd2 were successfully targeted to heterochromatic DNA by GBP-MaSat, as assessed by an increased protein amount at heterochromatic DNA (Figure 4D). For cells co-expressing GFP-Mbd2 and mCherry-Tet1CD, we observed no colocalization of both proteins, while GFP-Mbd1 and mCherry-Tet1CD highly colocalized at heterochromatic DNA upon co-expression with GBP-MaSat (Figure 4D). Those results indicate that heterochromatin-tethered Mbd1 but not Mbd2 can recruit Tet1CD in Dnmt1−/− cells.

To further clarify whether the observed recruitment of Tet1CD by Mbd1 is mediated by direct protein–protein interactions, HEK cells expressing mCherry-Tet1CD and GFP-tagged deletion mutants of Mbd1 were subjected to co-immunoprecipitation (co-IP) experiments. To disrupt potential protein–DNA interactions, ethidium bromide was added to the interaction buffer (55). Although Tet1CD interacted with all deletion mutants of Mbd1, the strongest interaction was observed in the presence of the MBD domain (Figure 4E). Since Mbd1 and Tet1 are DNA binding proteins, purified proteins were used for in vitro co-IPs, to further exclude a DNA-bridged interaction. The interaction between Tet1 and Mbd1 could still be detected (Supplementary Figure S12C), indicating that Mbd1 physically interacts with Tet1 in vitro. To further validate these interactions, we ectopically expressed GFP tagged Mbd1 mutants to major satellite heterochromatic DNA as described above (Figure 4D) and analyzed colocalization of co-expressed mCherry-Tet1CD. The results showed that Tet1CD had a stronger interaction with the Mbd1 mutant containing the MBD domain (Supplementary Figure S12D), which is in agreement with the co-IP data.

Taken together, these results indicate that direct protein–protein interactions between Mbd1 and Tet1 favor the recruitment of Tet1 to heterochromatic DNA.

The CXXC3 domain of Mbd1 is necessary for the recruitment of Tet1 to heterochromatic DNA

In murine cells, different Mbd1 isoforms lacking the CXXC3 domain were found alongside with full length Mbd1 (10,11). The CXXC3 domain of Mbd1 is able to bind unmethylated CpG sites, which allows Mbd1 to specifically bind to unmethylated CpG-rich DNA. To test whether the ability to bind unmethylated CpG sites affects the localization of Tet1 proteins to heterochromatic DNA, MEF cells expressing mCherry-Tet1CD and GFP-tagged Mbd1 or Mbd1ΔCXXC3 (Figure 5A) were used for live cell microscopy experiments. Spot analysis showed more cells with Tet1 at heterochromatin in the presence of Mbd1 compared with Mbd1ΔCXXC3 (Supplementary Figure S13A, upper row). Moreover, as shown in Figure 5B and C, accumulation of Tet1 to heterochromatic DNA was increased in the presence of overexpressed Mbd1 compared to Mbd1ΔCXXC3 (Figure 5B and C; Supplementary Figure S13A, lower row). Similar results were obtained with full length Tet1 protein (Supplementary Figure S13B). In summary, these results indicate that the CXXC3 domain of Mbd1 is necessary for an enhanced localization of Tet1 to heterochromatic DNA.

Next, we tested the effect of the CXXC3 domain on Tet1-mediated 5hmC formation. To this end, MEF cells overexpressing Tet1CD and Mbd1 or Mbd1ΔCXX3 were used for in situ 5hmC stainings. In contrast to what we measured in Mbd1-transfected cells, 5hmC signal was lower in cells overexpressing Mbd1ΔCXXC3 (Figure 5D). Similar to Mbd2 (Figure 1B), 5hmC levels decreased with increasing Mbd1ΔCXXC3 levels (Figure 5D), indicating that Mbd1 proteins lacking the CXXC3 domain protect from Tet1-mediated 5mC to 5hmC conversion and CXXC3 is necessary to enhance Tet1-mediated 5hmC formation. To exclude any effects of Mbd1 on chromatin accessibility resulting in a changed 5hmC antibody access to DNA, we radioactively labeled and quantified 5hmC in genomic DNA of MEF cells, ectopically expressing mCherry-Tet1CD and GFP-tagged Mbd1 or Mbd1ΔCXXC3 (Supplementary Figure S14). The results of this radioactive assay perfectly agreed with the antibody based 5hmC detection.

Previous studies showed that point mutations of CXXC3 alone can abolish the binding of Mbd1 to unmethylated CpGs (56). Hence, we generated CXXC3 point mutants (Supplementary Figure S15A) and tested their effect on Tet1-mediated 5hmC formation as before (Figure 5). First, by spot analysis, we showed that the number of cells with Mbd signal localized at heterochromatin in Dnmt1 knock-out cells was higher in the presence of Mbd1 when comparing with Mbd1(C289,292A) (Supplementary Figure S15B and C). This indicated that the double mutations in CXXC3 domain indeed abolished the binding ability of Mbd1 to unmethylated heterochromatic DNA. Then, we measured the
Figure 4. The interaction of Mbd1 and Tet1 enhances Tet1 localization to heterochromatic DNA. (A) Dnmt1<sup>−/−</sup> MEF (p53<sup>−/−</sup> and Dnmt1<sup>−/−</sup>) cells were transfected with mCherry-Tet1CD and Mbd1-GFP or Mbd2-GFP. Eight hours after transfection, the cells were imaged using confocal microscopy. The left panel shows the schematic of the experiment and the right panel shows exemplary cells with Tet1 and Mbd1/Mbd2 expression. (B) Accumulation of Mbd1/Mbd2 and Tet1 proteins to heterochromatic DNA measured as in Figure 2. (C) Dnmt1<sup>−/−</sup> MEF cells were triple-transfected with mCherry-Tet1CD, GBP-MaSat (GFP binding protein, polydactyl zinc finger major satellite binder) and Mbd1-GFP or Mbd2-GFP plasmids. Eight hours after transfection, cells were imaged using confocal microscopy. The left panel shows the schematic of the experiment and how GBP-MaSat targets GFP-tagged proteins to heterochromatic DNA. The right panel shows exemplary cells with Tet1 and Mbd1/Mbd2 expression in the presence of GBP-MaSat. (D) Accumulation of Tet1 and Mbd1/2 proteins to heterochromatic DNA. Cell number and P-value of t-test are indicated. (E) Interaction between Tet1CD and subdomains of Mbd1. HEK (human embryonic kidney) cells co-expressing mCherry-Tet1CD and GFP tagged Mbd1 were used for co-immunoprecipitation assays using immobilized RFP binding protein. The input and bound GFP signals on the membrane were detected using a GFP-specific antibody. Left panels are input for mCherry and GFP fusion proteins and rightmost panel shows interaction bands. The middle panel depicts the structures of Mbd1 fusions. The right panel summarizes the results of two independent biological replicates.
Figure 5. The CXXC3 domain of Mbd1 is necessary for Mbd1 enhanced 5hmC formation. (A) Structure of full length Mbd1 and Mbd1ΔCXXC3. (B) Examples of Tet1CD localization in the presence of Mbd1 and Mbd1ΔCXXC3. MEF cells were transfected with mCherry-Tet1CD and Mbd1-GFP or GFP-Mbd1ΔCXXC3. Eight hours after transfection, the cells were imaged using confocal microscopy. Scale bars: 5 μm. (C) The effect of the CXXC3 on Tet1 accumulation to heterochromatic DNA. The ratio of mean intensity inside and outside heterochromatic DNA was plotted. Cell number and P-value of student's t-test are indicated on the plot. (D) Effect of the CXXC3 on Tet1-mediated 5hmC formation. MEF cells were transfected with mCherry-Tet1CD and Mbd1-GFP or Flag-Mbd1ΔCXXC3. Twenty-four hours after transfection, cells were stained for 5hmC. Then, cells with low Tet1CD level were used for plotting. The cells were first subgrouped by Tet1 level and then, in each group, cells were further binned by Mbd1/Mbd1ΔCXXC3 protein level. Single cell 5hmC intensity was plotted. The 5hmC intensity in the cells with high levels of Mbd1s and high levels of Tet1CD were used to perform student’s t-test and P-values are indicated. Experiments were repeated at least three times. Cell numbers for Tet1CD + Mbd1 and Tet1CD + Mbd1ΔCXXC3 are 16757 and 2589, respectively.

accumulation of Tet1 to heterochromatic DNA. In agreement with the data from the deletion of CXXC3 domain, we found lower Tet1CD recruitment to heterochromatic DNA in the presence of Mbd1(C289,292A) (Supplementary Figure S15D). Finally, in situ staining showed that, comparing with Mbd1, the 5hmC levels were significantly lower in the presence of Mbd1(C289,292A) (Supplementary Figure S15E) further validating the results from the CXXC3 deletion.

Taking together, the results of both, the CXXC3 point and deletion mutants, confirmed that the binding ability of Mbd1 to unmethylated CpGs is necessary to enhance Tet1-mediated 5hmC formation.
Mbd1 isoform-dependent regulation of Tet-mediated 5hmC formation in mouse ESCs

Previous studies showed that 5hmC is highly enriched in mouse J1 ESCs due to Tet1 and Tet2 (57). In addition, Mbd1 is also present in mouse ESCs (58). To further test the effect of Mbd1 on Tet1-mediated 5hmC formation, we performed Mbd1 RNAi knockdown experiments and determined the endogenous 5hmC levels.

By western blotting, we observed that in mouse ESCs the short isoform of Mbd1 (without the CXXC3 domain) is more abundant than the long isoform (including the CXXC3 domain) (Figure 6A). After knocking down both isoforms of Mbd1 in mESCs, we observed an increase in 5hmC (Figure 6B and C; Supplementary Figure S16A and B), suggesting that, in mouse ESCs, the more abundant Mbd1ΔCXXC3 isoform, negatively regulates Tet1-mediated 5hmC formation. These results agree with the data from ectopic expression of Mbd1 and Tet1 in mouse fibroblasts, whereby we found that the Mbd1ΔCXXC3 isoform negatively impacts on Tet1-mediated 5hmC formation (Figure 5).

Subsequently, we performed isoform-specific rescue assays by ectopically expressing either of the isoforms of Mbd1 (full length and ΔCXXC3) in the Mbd1 knockdown mouse ESCs. We observed increased 5hmC levels when the cells were rescued with full length Mbd1 and a minor decrease of 5hmC with Mbd1ΔCXXC3 isoform (Figure 6D).

These results indicate that in mouse ESCs, Mbd1 regulates endogenous Tet1-mediated 5hmC formation in an isoform-dependent manner and agree with our prior isoform-specific ectopic expression data.

Tet1 and its oxidation products displace Mbd1 from heterochromatic DNA

As Mbd1 recruitment of Tet1 to heterochromatin affected Tet1 mobility (Figure 2), we analyzed next whether the converse was also true, i.e. whether Tet1 affects the mobility of Mbd1. To this end, we performed FRAP analysis. First, we tested the mobility of Mbd1 and Mbd1ΔCXXC3 in the absence of Tet1. Compared to Mbd1, Mbd1ΔCXXC3 showed faster recovery kinetics (Figure 7A), indicating that the CXXC3 domain provides an additional binding mode of Mbd1 to heterochromatic DNA.

Then, a selected heterochromatic region in cells co-expressing mCherry-tagged Tet1CD and GFP-tagged Mbd1 or Mbd1ΔCXXC3 was photobleached using a 488 nm laser, which was shown to simultaneously bleach mCherry-tagged proteins (59). In the presence of Tet1CD, the recovery kinetics of Mbd1 was faster (Figure 7A and B, right), indicating that binding of Mbd1 to heterochromatin DNA is partially lost. In contrast, the binding kinetics of Mbd1ΔCXXC3 were not affected by Tet1 proteins (Figure 7A and B, left). As previously mentioned, Mbd1 can either bind to methylated CpG sites via its MBD domain or to unmethylated CpG sites via its CXXC3 domain, or both. Since we observed increased 5hmC levels, the MBD domain of Mbd1 might be displaced. The CXXC3 targeting sites could provide DNA docking sites for Mbd1 in the presence of Tet1 proteins with the interaction between the MBD domain of Mbd1 (as well as other Mbd1 domains) and Tet1, recruiting Tet1 to heterochromatic DNA for 5mC oxidation (Figure 7C).

Interestingly, similar binding kinetics of Tet1CD were observed with Mbd1 and Mbd1ΔCXXC3 (Figure 7B), indicating that once Tet1 binds to DNA, the interaction of Tet proteins and DNA is unaffected by Mbd1. Unlike Mbd1, which has at least two DNA binding domains, Tet1CD might only have one DNA binding site, resulting in a more simplistic protein–DNA interaction mode.

As previously mentioned, the accumulation of Tet1 to heterochromatin DNA is independent of its catalytic activity (Figure 2). To test whether the catalytic mutant of Tet1 would affect Mbd1 DNA binding, we measured the mobility of Mbd1 in the presence of Tet1CDmut and Tet1CD. While Tet1CD and Tet1CDmut had similar binding kinetics at heterochromatic DNA (Supplementary Figure S17, right), we observed an increased mobility of Mbd1 to heterochromatin DNA in the presence of Tet1CD and Tet1CDmut (Supplementary Figure S17, left), with the highest mobility in the presence of the catalytically active Tet1 variant. These results indicate that the catalytic activity of the Tet1 protein is not necessary but promotes displacement of Mbd1 from heterochromatic DNA (Supplementary Figure S17).

Previous studies have shown that Mbd1 cannot bind to 5hmC (60) and here we show that Tet1 increases the mobility of Mbd1. The initial displacement of Mbd1 from heterochromatin DNA by Tet1 might be independent of the catalytic activity of Tet proteins. However, the catalytic activity of Tet1, which leads to depletion of the Mbd1 binding substrate 5mC, enhances the fraction of mobile Mbd1 proteins to a larger extent than the catalytically inactive Tet1 variant. To further test the effect of Tet1 and its oxidation products on Mbd1 localization, high content image analysis was performed. Since we showed that the oxidation of Tet1 is dose and time dependent, we first tested the effect of different Tet1 protein levels on the localization of Mbd1 proteins. We analyzed the distribution of Mbd1 in cells with low and high Tet1 expression levels using images from in situ 5hmC staining (Figure 5). The homogeneous (Mbd1 freely distributed in the cell nucleus) or heterogeneous (Mbd1 accumulated at heterochromatic DNA) distributions of Mbd1 were determined by the coefficient of variation (CV) of the Mbd1 signal, which is similar as the signal standard deviation measurement (43). As mentioned earlier (Figure 2), Mbd1 accumulates at methylated heterochromatin DNA. Decreased accumulation of Mbd1 to heterochromatin DNA results in a decreased CV. The results showed that high Tet1 expression leads to increased 5hmC formation and a decreased Mbd1 CV (Supplementary Figure S18A), indicating that Mbd1 loses DNA binding sites due to 5hmC formation. However, high levels of catalytically inactive Tet1 can also decrease the localization of Mbd1 to heterochromatin DNA but with less efficiency (P-values $2.57 \times 10^{-10}$ versus $3.67 \times 10^{-3}$) (Supplementary Figure S18A). These results indicate that high levels of Tet1 protein itself can decrease the DNA binding ability of Mbd1 to heterochromatin DNA and this is enhanced by the Tet1 oxidation products.

To test the effect of oxidation timing on localization of Mbd1 proteins, live cell microscopy of cells expressing Mbd1-GFP and mCherry-Tet1CD/CDmut or only
Figure 6. Mbd1 regulates 5hmC formation in mouse ESCs. (A) Endogenous Mbd1 isoforms in mouse ESCs compared to Mbd1 isoforms ectopically expressed in HEK cells and analyzed by western blotting with anti-Mbd1 rabbit polyclonal antibody. The first (upper) Mbd1 band in mouse ES cells corresponds to the long isoform of Mbd1, as it shows the same mobility as the ectopically expressed untagged Mbd1 (upper red line). The second (lower) Mbd1 band in ESCs corresponds to the CXXC3 deletion isoform as it migrates similarly to the ectopically expressed Mbd1\DeltaCXXC3. The minor difference in migration is due to the addition of the Flag tag (~1.1 kDa, lower red line/arrow) in this construct. M: protein size marker. Exposure times are different between the lanes to compensate for the different protein levels. (B) Effect of Mbd1 knockdown on 5hmC formation in J1 ESCs. About 24, 48 and 72 h after lentivirus infection, the mouse ESCs were fixed and stained with Mbd1 (left) or 5hmC (right) antibodies. Then, the fluorescence signals were detected using high content screening microscopy and fluorescence intensities were further calculated using the Harmony software. The red line indicates the median fluorescence intensity of either Mbd1 or 5hmC for wild-type mouse ESCs. For each time point, at least 900 cells were used for analysis and \( P \)-values from \( t \)-tests are indicated on the plot. (C) Exemplary images for Mbd1 and 5hmC staining 72 h (24 and 48 h are shown in Supplementary Figure S16) after Mbd1 knockdown. Scale bars: 5 \( \mu m \). (D) Effect of Mbd1 isoform specific rescue on 5hmC formation in mouse ESCs. A scheme of the rescue assay is shown on the left, 5hmC quantification results are shown on the right hand side. For each time point, 70 cells were used for analysis and \( P \)-values from \( t \)-tests are indicated on the plot.
Figure 7. Tet1 displaces Mbd1 binding to heterochromatic DNA. (A) Mobility of Mbd1 and Mbd1ΔCXXC3. MEF cells were transfected with Mbd1-GFP or GFP-Mbd1ΔCXXC3. Eight hours after transfection, the mobility of Mbd proteins was measured by FRAP. The mean values are plotted as a recovery curve and the cell number is indicated on the plot. T-half values were extracted from mean exponential fitting. Solid and dashed lines indicate recovery curve of Mbd1ΔCXXC3 and Mbd1, respectively. (B) Mobility of Mbd1 (right) and Mbd1ΔCXXC3 (left) in the presence of Tet1CD. MEF cells co-expressing mCherry-Tet1CD and GFP-Mbd1 or GFP-Mbd1ΔCXXC3 were used for FRAP analysis. One heterochromatic region was photobleached with a 488 nm laser, and after photobleaching the intensities of GFP and mCherry were measured. The mean values are plotted as a recovery curve and cell numbers are indicated on the plot. T-half values were extracted from the mean exponential fitting. T-half values for single cells are shown as box plots and P-values (Wilcoxon test) are indicated. At least two independent experiments were performed. (C) Scheme summarizing the Mbd1 and Tet1 binding modes to heterochromatic DNA. Black and white circles indicate methylated and unmethylated CpG, respectively.
mCherry were used for spot analysis. Eight hours after transfection, GFP and mCherry signals were detected using high content screening microscopy and spot analysis was performed as described before. To exclude Tet1 dose effects, only cells with low Tet1CD expression were used to quantify the accumulation of Mbd1 at heterochromatic DNA. As shown in Supplementary Figure S18B, the accumulation of Mbd1 at heterochromatic DNA anti-correlated with Tet1CD expression and the effect was enhanced over the oxidation time. The catalytically inactive mCherry-Tet1CD or mCherry, in contrast, did not affect the accumulation of Mbd1 at heterochromatic DNA, indicating that, at low levels of Tet1CD/CDmut, Mbd1 looses DNA binding sites due to Tet1-dependent 5hmC formation, which correlates with its oxidation time. This result is in agreement with live cell experiments using confocal microscopy (Supplementary Video 2).

Altogether, these results show that Mbd1 can be displaced from heterochromatic DNA by high levels of Tet1 protein and its oxidation product further enhances the Mbd1 displacement. However, at low levels of Tet1 protein, its oxidation product eventually causes the displacement Mbd1 from heterochromatic DNA.

**DISCUSSION**

The dynamics of cytosine modification create a more diverse genome and regulate cellular differentiation by controlling gene expression. For proper development, the maintenance and removal of cytosine modifications have to be thoroughly regulated. Methylation of cytosines is catalyzed by Dnmts and regulation of this process has been studied extensively (61). In contrast, the regulation of methylcytosine modifiers, Tet proteins, is still highly debated and poorly understood. 5mC can be specifically recognized by methylcytosine readers and recognition of 5mC further regulates gene expression and chromatin structure (7). Methylcytosine readers Mbd2 and Mecp2 block Tet1-mediated 5mC to 5hmC conversion and further repress transcriptional noise (35). Here, we show that the MBD protein family member Mbd1, in contrast, regulates Tet1-mediated 5mC to 5hmC conversion in an isoform-dependent manner. Isoforms containing the CXXC3 domain enhance Tet1-mediated 5mC to 5hmC conversion while the ones lacking the CXXC3 domain block this process. The CXXC3 domain of Mbd1 could provide a docking site for Mbd1, which further recruits Tet1 to methylated DNA for 5mC oxidation. In summary, in stark contrast with other 5mC readers, we show here that Mbd1 enhances Tet1 activity by targeting it to heterochromatin, which contains methylated DNA. As this effect is dependent on the Mbd1-specific binding to unmethylated CpGs, we propose that this allows targeting Tet1 to CpG dense regions while not simultaneously blocking methylated CpGs from Tet1 catalytic activity.

Our study provides new insights on how methylcytosines, their readers and modifiers regulate each other. Tet1-mediated 5hmC formation is a dose and oxidation time-dependent process (Figure 8). The formation of 5hmC can be enhanced by either facilitating Tet1 binding to DNA, or by prolonging the oxidation reaction time (Figure 8). We show here that the accumulation of Mbd1 to heterochromatic DNA and its unmethylated CpG binding ability allow Mbd1 to recruit Tet1 proteins, which bind to methylated CpGs and further oxidize 5mC to 5hmC (Figure 8). However, during the oxidation process, more and more 5mC is converted to 5hmC or even further to 5fC and 5caC and it is conceivable that a progressive reduction of Tet1 protein binding to heterochromatic DNA occurs due to the absence of their substrates (Figure 8). In parallel Mbd1 proteins are displaced from heterochromatic DNA, which might be explained by the fact that 5hmC is not the preferred binding substrate for Mbd1 (Figure 8 and Supplementary Video 2).

Mbd1 has already been showed to be involved in several biological processes, such as maintaining of inactive X-chromosome (62,63) and repression of HOXA gene cluster (64), which are all correlated with DNA methylation. It would be very interesting to assess whether these processes are Mbd1 isoform dependent and whether Mbd1 regulates these processes by affecting Tet1-mediated 5mC oxidation.

Mice lacking Mbd1 develop normally and appear healthy throughout life, but are impaired in spatial learning, have decreased neurogenesis and reduced long-term potentiation in the dentate gyrus of the hippocampus (65). Similar to Mbd1 knockout mice, Tet1 knockout mice develop normally but exhibit abnormal hippocampal long-term depression and impaired memory extinction due to hypermethylation of genes targeted by Tet1 (66,67). The coinciding phenotypes of Tet1 and Mbd1 knockout mice suggest that both might be involved in neural cell function and share genomic targets. Here, we show that Mbd1 enhances Tet1-mediated 5mC to 5hmC formation, thus we suggest that Mbd1 might affect neural cell function by affecting the removal of DNA methylation marks.

Although MBD1 contains a conserved MBD domain, it was shown to have different sequence binding preferences (56,68) and also different target genes in prostate cancer cells (69) compared to MECP2. Overexpression of MBD1 in several cancer cells lines and tissues was observed (49,70).
Since aberrant DNA methylation is a hallmark of cancer, MBD1 isoform-dependent regulation of Tet-mediated 5mC to 5hmC conversion might further contribute to the regulation of cancer-related gene expression.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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**Author contributions:** P.Z., C.R., F.D.H., B.B., A.F. and S.J.P. performed experiments. P.Z., C.R. and F.D.H. analyzed the data. U.A.N., Y.G. and X.Z. provided materials and expertise. P.Z., C.R. and M.C.C. conceived the project and wrote the manuscript.

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Supplementary figures and videos

Figure S1 Illustration of protein structures.

Plasmid construction details are given in the methods part for all plasmids not published before. Amino acid coordinates are indicated above each structure (without GFP, mCherry, Flag and CFP).
Figure S2 Effect of Mbd on Tet1-mediated 5hmC formation.

MTF cells were transfected with mCherry-Tet1CD/CDmut and GFP-tagged Mbd constructs. 24 hours after transfection, immunostaining with a 5hmC antibody was performed. Tet1, Mbd, and 5hmC signals were detected with high content screening microscopy and further analyzed using the Harmony software. To analyze the effect of Mbd on 5hmC formation, we first grouped the cells according to their Mbd protein level in low and high expressing groups. Then, in each group, the cells were further binned by their Tet1CD protein level. The average 5hmC intensity of single cells was plotted. Shown are mean values of at least three independent experiments. 95% confidence intervals are indicated in the plot. Cell number (Mbd low/Mbd high): Tet1CD+Mbd1 4701/1390; Tet1CD+GFP 2496/1043; Tet1CD+Mbd1 1779/690; Tet1CD+Mbd3 2666/1462; Tet1CD+Mecp2 2051/764; Tet1CD+Mbd2 3631/2177; Tet1CDmut+GFP 1349/771.
Figure S3 Comparison of ectopic and endogenous Tet1 protein levels.

(A) MEF or MTF cells were transfected with an mCherry-Tet1CD construct. 24 h after transfection, immunostaining with a Tet1 specific antibody was performed. In parallel, mouse embryonic stem cells (mESC, E14) were also immunostained with the same Tet1 antibody. DNA was counterstained with DAPI. The fluorescence intensity of ectopic Tet1 (mCherry) and anti Tet1 antibody were measured with high content screening microscopy using same exposure time as before (Fig. 1, Fig. S2, Fig.5). (B) For MTF and MEF cells, the cells were grouped exactly the same as in Figure S2 according to their mCherry-Tet1CD intensity. Then, in each group, total fluorescence intensities of whole cell nucleus were plotted (left). As a comparison, total fluorescence of whole cell nucleus of ESCs was also plotted (right). Cell numbers for MEF, MTF and mESC are 5188, 9712 and 21935, respectively. (C) Comparison of endogenous Tet1 protein levels among MEF, MTF and mESC. For MEF and MTF cells, the total fluorescence intensities of whole cell nucleus in cells without mCherry-Tet1CD (intensity below 20, and confirmed by microscopy images) were plotted. Median values of endogenous Tet1 protein are indicated below the plot. Cell numbers for MEF, MTF and mESC are 133597, 193690 and 21935, respectively.
Figure S4 Tet1 dose and oxidation time dependent 5hmC formation.

MEF cells were transfected with an mCherry-Tet1CD construct. The cells were fixed at eight hours or 24 hours after transfection and immunostained against 5hmC. Shown are mean values of 5hmC intensity and 95% confidence intervals. The results showed that although with similar Tet1 levels, the 5hmC intensity is much lower at eight hours compared with 24 hours. However, the amount of 5hmC is proportional to Tet1 levels. Cell numbers for eight hours and 24 hours are 111 and 366, respectively.
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A

- **Input Image**
  - mcherry-Tet1CD
  - Mbd1-GFP

- **Find nuclei by cherry signal (nucleus mask)**
  - Method 1
  - Method 2

- **Find spot (heterochromatin)**
  - Method 1
  - Method 2

- **Select population by cherry signal**

- **Calculate nucleus intensity**
- **Count spot number**
- **Calculate spot intensity**

**Spot finding methods**
- GFP/mcherry signal
- Method 1
- Method 2

- **Distribution of Tet1CD spot numbers**
- **Distribution of Mbd1 spot numbers**

- **Identify spot by method 1 in each channel**

B

C

- **Tet1CD accumulation analysis**
  - Cell population with high number spots
  - Heterochromatin (HC) mask
  - Mean HC intensity
  - Nucleus intensity (HC mask)

D

- **Mbd1 accumulation analysis**
  - Cell population with high number spots
  - Heterochromatin (HC) mask
  - Mean HC intensity
  - Nucleus intensity (HC mask)
**Figure S5 Spot analysis.** (A) Workflow of image analysis and fluorescence intensity calculation. Live cell imaging was performed using high content screening microscopy and images were analyzed using the Harmony software (see methods). Methods to identify spots are listed in the right panel. (B) To calculate cell numbers with Tet1 or Mbd1 accumulating at heterochromatic DNA, the relative intensity (the ratio of spot and nucleoplasm intensity) above a given threshold was used for identifying the spots. One spot corresponds to one heterochromatin region. Shown are exemplary frequency distributions of spot numbers per cell in the Tet1CD (left) and the GFP (right) channel. According to the spot number distribution, a threshold (grey vertical dashed line) was used to group cells with high number of spots or low number of spots. Then, the percentage of cells with high spot numbers and low spot numbers was plotted. Red and green bar plots are cell number percentage with high spot numbers (dark red or dark green) or low spot numbers (light red or light green), respectively. (C) Schemes describing methods used to calculate accumulation of Tet1CD at heterochromatic DNA. The cells with high spot numbers in the Mbd1 channel were used to calculate Tet1CD or Tet1CDmut accumulation. To calculate the accumulation of Tet protein at heterochromatic DNA, the heterochromatic region was identified by Mbd1 relative intensity (the ratio of spot and nucleoplasm intensity above a given threshold). Then, the ratio of the Tet1 mean intensity inside heterochromatic region and whole cell nucleus was plotted. (D) Schemes describing methods used to calculate accumulation of Mbd1 at heterochromatin. To evaluate the effect of Tet1 protein on the localization of Mbd1 protein, the Mbd1 signal at heterochromatic DNA was identified by the intensity of spots to avoid losing cells, which showed low value of relative spot intensity. The ratio of Mbd1 mean intensity inside heterochromatic region and inside the cell nucleus was plotted according to Tet1 expression and separated by time-points, represented by different colors.
Figure S6 Mbd1 enhances Tet1CD and Tet1FL mediated 5hmC formation.

(A) The effect of Mbd1 on the accumulation of Tet1 at heterochromatin. Mouse tail fibroblasts were co-transfected with mCherry-Tet1CD and GFP tagged Mbd1 or GFP constructs. Eight hours after transfection, high content live cell imaging was performed (upper row). Relative intensity (ratio of spot and nucleoplasm intensity above given threshold) was used to identify heterochromatic regions (Fig. S5). The number of heterochromatin spots per cells was plotted. Red and green bars indicate Tet1 and Mbd1 signals, respectively. Dark and light color (red and green) indicate the cells with high spot numbers and low spots numbers, respectively. The results showed that the majority of Mbd1 transfected cells showed Mbd1 accumulation at heterochromatin, which was unaffected by Mbd1 expression levels (green bar, left). Compared with Mbd1, we did not observe any accumulation of GFP (green bar, right). However, the distribution of Tet1CD protein is highly dependent on Mbd1 expression levels. Compared with cells co-
expressing GFP (red bar, right), more cells showed Tet1CD accumulating at heterochromatin in the presence of Mbd1 (red bar, left). Moreover, with increased expression of Mbd1, the number of cells with Tet1 accumulated at heterochromatin also increased (red bar, left), indicating that the accumulation of Tet1 is dependent on Mbd1 protein levels. Lower plots show the effect of Mbd1 on Tet1-mediated 5hmC formation. Mouse embryonic fibroblast cells were transfected with mCherry-Tet1CD together with Mbd1-GFP or GFP constructs. 24 hours after transfection, immunofluorescence was performed. Cells were first grouped according to Tet1 protein levels. Then, in each group, cells were further binned by Mbd1/GFP protein levels. The 5hmC values of single cells were plotted. In agreement with increased accumulation of Tet1CD in the presence of Mbd1, we observed that 5hmC formation could also be enhanced by Mbd1 protein. Experiments were repeated at least two times. Cell numbers for Tet1CD+Mbd1 and Tet1CD+GFP are 16757 and 574, respectively. (B) The effect of Mbd1 on the accumulation of Tet1FL at heterochromatin. Details are as in (A). Full length Tet1 accumulation at heterochromatin is, as for the catalytic domain alone, dependent on Mbd1 presence.
Figure S7 Validation of the spot analysis quantification method.

(A) The images were exported from the Harmony software and the cell numbers were counted by eye according to Mbd/GFP and Tet1 signal distribution either with or without spots. (B) Exemplary images for localization of Tet1CD in the presence of Mbd1 or Mbd2. Red circles: Tet1 colocalizes with Mbd1/2 at chromocenters. Green circles: Tet1 does not colocalize with Mbd1/2 at chromocenters. Insets show enhanced contrast images for better visualization. Scale bar 50 µm.
Figure S8 Effect of Mbd1 on stability of Tet1 proteins.

MEF cells were transfected with GFP tagged Mbd1 and mCherry tagged Tet1CD. Eight hours after transfection, the cells were cultured in the presence or absence of cycloheximide (CHX) and imaged every four hours by high content microscopy. The fluorescence intensity of GFP and mCherry inside the cell nucleus were calculated using the Harmony software and further plotted using Rstudio. To better visualize the data, we plotted the normalized fluorescence intensities for the last time-point as boxplot (A). For each treatment and each time-point, the total fluorescence intensities of different time-points were normalized to the first time-point. The p values from t-tests are indicated in the plot. (B) As control for the effect of Mbd1 on the stability of Tet1CD proteins, we used cells expressing Mbd2 and Tet1CD. For each treatment and each time-point, the total fluorescence intensities of different time-points were normalized to the first time-point (as 1) and last time-point (as 0). For each time-point and each treatment, median fluorescence intensities of at least 500 cells were plotted. Dashed line marks the T-half.
Figure S9 Accumulation of Tet1, Tet2 and Tet3 in the presence of Mbd1.
MEF cells were transfected with GFP tagged Mbd1 and mCherry tagged Tet1, Tet2 or Tet3. Eight hours after transfection, the cells were imaged with confocal microscopy and protein accumulation analyses were performed. Shown are the quantification results of Tet1, Tet2 and Tet3 accumulation at heterochromatin (left) and exemplary images (right). Cell numbers used for accumulation analysis and p values from t-test are indicated on the plot. Scale bar: 5 µm.
Figure S10 Mbd1 enhances Tet1CD accumulation at heterochromatic DNA independent of the catalytic activity of Tet1.

Mouse fibroblast cells were transfected with mCherry-Tet1CD or mCherry-Tet1CDmut together with GFP tagged Mbd1 or GFP constructs. Eight hours after transfection, high content live cell imaging was performed. Relative spot intensity in the Mbd1 channel (ratio of spot to whole cell nucleus intensity above given threshold) was used to identify the heterochromatin and the number of spots (heterochromatic region) per cell are plotted. The Tet1CD/CDmut spot numbers of single cells were plotted according to Mbd1 spot numbers of the same cell. Cell numbers for Tet1CD+Mbd1 and Tet1CD+Mbd2 are 2527 and 2121, respectively.
Figure S11 Cell cycle independent heterochromatin localization of Mbd1.

C2C12 cells stably expressing RFP-PCNA were transfected with Mbd1-GFP. Eight hours after transfection, signals of Mbd1 and PCNA were detected by high content microscopy and spot analysis was performed. Late S-phase cells with high number of spots were determined by the PCNA signal. The upper panel shows the percentage of cells with high or low number of spots. Red and green bars indicate PCNA and Mbd1 signals, respectively. The lower panel shows examples of PCNA and Mbd1 distribution. Cells are labeled as either in G1/G2 or in S-phase according to the PCNA distribution. PCNA: proliferating cell nuclear antigen. Scale bar: 20 µm
Figure S12 The interaction of Mbd1 and Tet1 enhances Tet1 localization to heterochromatic DNA.

(A) Distribution of Mbd1 and its subdomains in Dnmt1−/− MEF cells. Dnmt1−/− MEF cells were transfected with GFP tagged Mbd1 and its subdomains. Eight hours after transfection, spot analysis of GFP signals was performed. Shown are the quantification results of the spot analysis. Cell percentage with high (dark green) and low (light green) number of spots per cell were plotted. Similar amounts of cells with Mbd signal localized at heterochromatic DNA were observed after Mbd1 and Mbd1ΔMBD expression, whereas Mbd1ΔCXXC3 and MBD expressing cells showed less heterochromatic Mbd signal. N=cell number.

(B) Comparison of Mbd1 distribution in Dnmt1−/− and wild type (WT) mouse embryonic fibroblasts cells.

(C) Interaction of Mbd1 and Tet1CD using purified proteins. mCherry-Tet1CD and Mbd1-GFP or GFP were purified from Sf9 cells and further used for co-immunoprecipitation assay. Immobilized mCherry-Tet1CD was incubated with similar amounts of Mbd1-GFP or GFP (by GFP intensity measurement) on a rotary shaker. After incubation, the beads were washed and proteins were separated via SDS-PAGE. After blotting to a nitrocellulose membrane, the gel was stained with Coomassie Brilliant Blue as an input estimation for Tet1 protein. Then, GFP signals were detected using a GFP antibody.

(D) Dnmt1−/− MEF cells were triple-transfected with mCherry-Tet1CD, GBP-MaSat (GFP binding protein, polydactyl zinc finger major satellite DNA binding protein) and GFP-tagged Mbd plasmids. Eight hours after transfection, cells were imaged using a confocal microscope. The left panel shows the schematic of the experiment and how GBP-MaSat targets GFP-tagged proteins to heterochromatic DNA. The right panel shows the accumulation of Tet1 and Mbd/GFP proteins to heterochromatic DNA. The p values of t-test are indicated. Cell numbers for Mbd1, Mbd1ΔCXXC3, Mbd1ΔMBD and GFP are 31, 26, 16, and 32, respectively.
Figure S13 The CXXC3 domain of Mbd1 is necessary for Mbd1 enhanced 5hmC formation.

(A) Spot analysis of Tet1CD and Mbd1 distribution. Mouse embryonic fibroblast cells were co-transfected with mCherry-Tet1CD and Mbd1-GFP or GFP-Mbd1ΔCXXC3. Eight hours after transfection, high content live cell microscopy analysis was performed. The upper row shows spot number analysis of Tet1CD, Mbd1 and Mbd1ΔCXXC3. Green bar plots show the percentage of cells with high number of spots (dark green) and low number of spots (light green). Red bar plots show the percentage of cells with high number of spots.
(dark red) and low number of spots (light red). N=cell number. Lower row shows the accumulation of Tet1CD in the presence of Mbd1 and Mbd1ΔCXXC3. To measure the accumulation of Tet1CD at heterochromatic (HC) DNA, the ratio of mean fluorescence intensity at heterochromatin to the whole nucleus was plotted. To better visualize Tet1CD accumulation, the median accumulation value of Tet1CD in the presence of Mbd1 is indicated as a red line. Student’s t-tests were performed and the p value is indicated on the plot. For each time-point, more than 400 cells were analyzed.

(B) Spot analysis of Tet1FL and Mbd1 distribution. Mouse embryonic fibroblast cells were transfected with mCherry-Tet1FL together with Mbd1-GFP or GFP-Mbd1ΔCXXC3. The spot number analysis and accumulation analysis of Tet1FL were performed as described above. For each time-point, more than 100 cells were analyzed.
Figure S14 Radioactive beta-glucosyltransferase (BGT) assay to analyze 5hmC levels.

(A) Schematic representation of the experimental protocol. MEF cells were co-transfected with mCherry-Tet1CD and Mbd1-GFP, GFP-Mbd1ΔCXXC3 or GFP. 24 hours after transfection cells were harvested, gDNA was isolated and sheared to <2000 bp size (B). To ensure equal starting DNA amount in the reactions, 3 µg of gDNA after shearing were blotted onto a membrane and stained with methylene blue (C). Radioactively labeled glucose was enzymatically transferred to 5hmC by the beta-glucosyltransferase enzyme (BGT). After the reaction, gDNA was cleaned up and the radioactivity was measured. Relative 5hmC levels of two biological and two technical replicates are shown (D). Cells transfected with mCherry-Tet1CD and Mbd1-GFP showed higher level of 5hmC compared to GFP-Mbd1ΔCXXC3 or GFP expressing cells.
Figure S15 The unmethylated CpG binding ability of Mbd1 is necessary for Mbd1 enhanced 5hmC formation.

(A) Structure of full length Mbd1 and Mbd1(C289,292A). (B) Distribution of Mbd1FL and Mbd1(C289,292A) in Dnmt1 knockout cell. Percentages of cells with high (dark green) and low (light green) number of spots per cell were plotted. (C) Examples of Tet1CD localization in the presence of Mbd1 and Mbd1(C289,292A). MEF cells were transfected with mCherry-Tet1CD and Mbd1-GFP or GFP-Mbd1ΔCXXC3. Eight hours after transfection, the cells were imaged using confocal microscopy. Scale bars: 5 µm. (D) The effect of the CXXC3 point mutations on Tet1 accumulation to heterochromatic DNA. The ratio of mean intensity inside and outside heterochromatic DNA was plotted. Cell numbers and the p value of student’s t-test are indicated on the plot. (E) Effect of the CXXC3 mutation on Tet1 mediated 5hmC formation. MEF cells were transfected with mCherry-Tet1CD and GFP tagged Mbd1 or Mbd1(C289,292A). 24 hours after transfection, cells were stained for 5hmC. Then, cells with low Tet1CD level were used for plotting. The cells were first subgrouped by Tet1 level and then, in each group, cells were further binned by Mbd1/Mbd1(C289, 292A) protein level. Single cell 5hmC intensity was plotted. The 5hmC intensity in the cells with high levels of Mbd1 and high levels of Tet1CD were used to perform student’s t-test and p values are indicated. Experiments were repeated at least three times. Cell numbers for Tet1CD + Mbd1 and Tet1CD + Mbd1(C289, 292A) are 5,974 and 7,794, respectively.
Figure S16 Mbd1 knock down validation and 5hmC staining.

(A) Validation of the Mbd1 knock down after 72 hours in mouse ESCs via western blotting. Endogenous Mbd1 was detected with an Mbd1 specific antibody recognizing both isoforms (Mbd1 and Mbd1ΔCXXC3). Actin was used as a loading control. (B) Shown are images taken at 24 and 48 hours after Mbd1 knockdown (72 hours time point is shown in Fig 6C). Scale bars: 5 μm.
**Figure S17 Effect of Tet1CD and Tet1CDmut on the mobility of Mbd1.**

MEF cells were transfected with mCherry-Tet1CD/CDmut and Mbd-GFP. Eight hours after transfection fluorescence recovery after photobleaching analysis was performed. Mean values of single cell recovery intensity are plotted. T-half values for single cell were plotted as box plots and \( p \) value from Wilcoxon test were indicated in the plot. N=cell number.
**Figure S18 Effect of Tet1CD protein and its oxidation product 5hmC on Mbd1 localization.**

(A) The effect of Tet1CD protein on Mbd1 localization. *In situ* staining images were used for Mbd1 signal variation analysis (upper row). Representative example of live cells with Mbd1 and Tet1CD (with high and low Tet1CD) levels (middle row). Scale bar = 5 μm. The lower panel shows quantification results of the coefficient of variation (CV) analysis. The CV is defined as the ratio of GFP signal standard deviation to GFP mean intensity. Dark and light red represent high and low Tet1CD/CDmut protein level, respectively. Mean value of single cell results are plotted and cell numbers and *p*-values of *t*-tests are indicated on the plot.

(B) The effect of 5hmC on Mbd1 localization. Spot analysis was performed on cells expressing Mbd1-GFP together with mCherry-Tet1CD, mCherry-Tet1CDmut or mCherry using high content screening microscopy. The accumulation of Mbd1 at heterochromatin was shown as ratio of Mbd1 mean intensity at heterochromatin to Mbd1 mean intensity in whole nucleus (Fig S5D). The accumulation of Mbd1 was plotted according to Tet1 expression and separated by time-points. The linear regression lines are indicated on the plot. Cell number for Mbd1-GFP+mCherry-Tet1CD, Mbd1-GFP+mCherry-Tet1CDmut and Mbd1-GFP+mCherry are 1716, 508 and 306, respectively.

**Video 1 Cell cycle independent Mbd1 and Tet1 localization to heterochromatic DNA.**

MEF cells were transfected with mCherry-Tet1CD and Mbd1-GFP and time-lapse confocal microscopy imaging was performed. Tet1CD (red), Mbd1 (green) and merged images are shown on the left, middle and right, respectively. Channel labels (Tet1CD, Mbd1) are indicated on top and time is indicated on the right bottom corner.

**Video 2 Oxidation time dependent Mbd1 and Tet1 accumulation at heterochromatic DNA.**

MEF cells were transfected with mCherry-Tet1CD (catalytic domain of Tet1) and GFP-Mbd1 (methyl-CpG binding domain protein 1) and time-lapse microscopy imaging was performed using confocal microscopy. Images of Tet1 and Mbd1 are shown on the left and the right, respectively. Channel labels (Tet1CD, Mbd1) are indicated on the bottom and time is indicated on the top.