

TET1 modulates H4K16 acetylation by controlling auto-acetylation of hMOF to affect gene regulation and DNA repair function

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Received March 31, 2016; Revised September 30, 2016; Accepted October 04, 2016

ABSTRACT

The Ten Eleven Translocation 1 (TET1) protein is a DNA demethylase that regulates gene expression through altering status of DNA methylation. However, recent studies have demonstrated that TET1 could modulate transcriptional expression independent of its DNA demethylation activity; yet, the detailed mechanisms underlying TET1's role in such transcriptional regulation remain not well understood. Here, we uncovered that Tet1 formed a chromatin complex with histone acetyltransferase Mof and scaffold protein Sin3a in mouse embryonic stem cells by integrative genomic analysis using publicly available ChIP-seq data sets and a series of *in vitro* biochemical studies in human cell lines. Mechanistically, the TET1 facilitated chromatin affinity and enzymatic activity of hMOF against acetylation of histone H4 at lysine 16 via preventing auto-acetylation of hMOF, to regulate expression of the downstream genes, including DNA repair genes. We found that Tet1 knockout MEF cells exhibited an accumulation of DNA damage and genomic instability and Tet1 deficient mice were more sensitive to x-ray exposure. Taken together, our findings reveal that TET1 forms a complex with hMOF to modulate its function and the level of H4K16Ac ultimately affect gene expression and DNA repair.

INTRODUCTION

The Ten Eleven Translocation 1 (TET1) protein, a member of TET family, is a key enzyme in DNA demethylation

(1). However, a recent study revealed that Tet1, in addition to its transcriptional regulatory function through its catalytic activity in DNA demethylation, possesses both activator and repressor functions in the regulation of a certain subset of genes in mouse embryonic stem cells (mESCs) (2). This observation was further supported by a study in which changes of transcriptional expression induced by overexpression of TET1 were highly similar to those induced by its demethylation-enzymatically-dead mutant in differentiated cell lines, suggesting that TET1 could regulate gene expression through a DNA methylation-independent manner (3). The repressive role of TET1 in transcriptional regulation has been proposed to derive from its interaction with polycomb repressive complex 2 (PRC2) to form a histone modifying complex, thereby modifying chromatin repressive mark (H3K27me3) in mESCs (4). However, the interaction between TET1 and PRC2 complex has, so far, been presented in embryonic stem cells (ESCs), but not in differentiated cells such as fibroblasts and HEK293T cells (5), indicating that TET1/PRC2 complex may act to repress gene expression in an ESCs-specific manner. On the other hand, SIN3A (homolog of Sin3 in yeast), a key component in multiple regulatory complexes, is involved in both transcriptional repression and activation through recruitment of diverse transcriptional factors or chromatin remodeling machinery at target promoters (6,7). A recent study has shown that TET1 interacts with SIN3A in both mESCs and HEK293T cells and presents highly overlapping binding profile on a genome-wide scale (2), implying TET1 may associate with SIN3A to regulate gene expression in both ESCs and differentiated cells. However, the exact mechanisms underlying the functional nature of TET1 and its associated protein complexes in regulating its target gene expression remain to be unveiled.

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Recently, it was demonstrated that there are dysfunctional DNA repair mechanisms and increased mutation frequencies in TET1-deficient non-Hodgkin B cell lymphoma (B-NHL), indicating that TET1 may function as a tumor suppressor (8). This observation, in line with a previous study in which there were decreased foci of MLH1 and delayed removal of RAD51 in mouse Tet1-knockout primordial germ cells (9), indicates that TET1 plays an important role in DNA repair in mammalian cells. However, the underlying mechanisms of TET1 functions in DNA repair in response to DSBs are largely unknown.

Homologous recombination repair (HRR) and non-homologous end joining (NHEJ) are two mechanisms of DNA repair pathway in response to DNA double strand breaks (DSBs). Some DNA repair genes, such as *RAD50*, *BRCA1*, *RAD51* and *TP53BP1*, act as tumor suppressors and are frequently mutated or aberrantly downregulated in human cancers, resulting in impaired DNA repair in response to DSBs, which is recognized as one of the hallmarks of tumorigenesis (10,11). However, Whole Genome Bisulfite Sequencing (WGBS) data analysis in the Tet1-deficient primordial germ cells showed that the methylation levels of most DNA repair genes had no obvious alteration (9), indicating that Tet1 possibly affects expression of DNA repair genes through a mechanism independent of its DNA demethylation function.

H4K16ac is a well-known targeted epigenetic site for post-translational modification in transcriptional activation (12). Hypo-acetylation of H4K16 plays important roles in DNA repair in response to DSBs through facilitating recruitment of some DNA repair factors as a platform, such as 53BP1, or affecting chromatin conformation (13). Human MOF (hMOF, also known as KAT8), a member of the MYST (Moz-Ybf2/Sas3-Sas2-Tip60) family of HATs, specifically modifies H4K16ac and is frequently downregulated in various types of cancers, including medulloblastomas, breast carcinomas, colorectal carcinoma, gastric cancer, and renal cell carcinoma (14,15). Studies have shown that depletion of hMOF renders both a global reduction of H4K16ac and DNA repair defects in budding yeast and mammalian cells (16,17). In addition, overexpression of hMOF reverses silencing of certain tumor suppressor genes induced by H4K16 deacetylation (18). Mechanically, N-terminal half of MOF regulates MOF's substrates binding and enzymatic activity against H4K16 residue to be involved in X chromosome dosage compensation in *Drosophila* (19).

In this study, we first revealed, through integrative genomic analysis using publicly available ChIP-seq data sets, that significantly overlapped distribution of TET1, Sin3a, Mof, and H4K16ac was observed in mESCs. By employing *in vitro* biochemical studies in human cell lines, we further demonstrated that TET1, hMOF and SIN3A interacted with each other. Furthermore, we demonstrated that TET1 specifically modulates H4K16ac through a mechanism in which the C-terminus of TET1 prevents auto-acetylation of hMOF and subsequently facilitates its chromatin affinity and enzymatic activity, to involve in DNA repair function. This mechanism was verified by observations in which Tet1-knockout MEF cells had an accumulation of DNA damage and genomic instability, and Tet1-deficient mice were more

sensitive to X-ray exposure. Therefore, we uncovered the TET1's role in which TET1 forms a complex with hMOF to modulate its function and the level of H4K16Ac ultimately affect gene expression and DNA repair.

MATERIALS AND METHODS

Mice

Tet1^{+/-} mice were obtained from the Jackson Laboratory (Cat# 017358). For genotyping of Tet1^{+/-} mice, the forward primer AACTGATTCCCTTCGTGCAG, and the reverse primer TTAAAGCATGGGTGGGAGTC were used. The expected band size for homozygote mutant was 650bp, 850bp for the wild type strain, and 650bp and 850bp double bands for the heterozygote strain.

X-ray irradiation

Irradiations were performed at the Chinese Academy of Sciences (Beijing) using an x-ray machine (RS-2000 PRO Biological system). WT mice and Tet1^{+/-} mice were irradiated with a single whole-body dose of 3 Gy X-ray at 60 days of age. The irradiation was operated at 160-kV constant potential and 25 mA (0.3 mm Cu filter) at a dose rate equal to 1.136 Gy min⁻¹ for a total of 2.64 min. The cage was cleaned with 75% ethanol when irradiation cycle was completed. The coat condition of WT mice and Tet1^{+/-} mice with irradiation or sham-irradiated were scored after four months of irradiation.

Cell culture, plasmids, and siRNA oligonucleotides

Mouse Tet1^{-/-} MEF cells were a generous gift from Dr. Guoliang Xu, SIBS of Chinese Academy of Sciences, Shanghai. All cells were cultured in DMEM media (Hyclone, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone, USA) and penicillin-streptomycin (Invitrogen, USA). RNA interference (RNAi) experiments were performed using Dharmacon siGENOME SMARTpool siRNA duplexes (Thermo Fisher Scientific, USA) against *TET1* (D-014635), *TET2* (L-013776) and *TET3* (L-022722), respectively. Cells were transfected with a siRNA complex at a final concentration of 20 nM using RNAiMAX transfection reagent (Invitrogen). TET1shRNA Lentiviral particles (sc-154204) were purchased from Santa Cruz. *TET1* cDNA was purchased from Origene (RC218608), cDNA for *hMOF* (NM_032188), *SIN3A* (NM_001145357), and *Histone H4* (NM_003541) were generated from cDNA library and confirmed by DNA sequencing, and then subcloned into pcDNA3.0 vector, followed by sequencing validation. Antibodies used in this study were purchased from different commercial companies as detailed in Supplemental Materials.

GST pull-down assay

GST-tagged proteins, His-SIN3A and His-hMOF were induced using IPTG in BL21 bacterial cells and were further purified following the manual of Glutathione-Sepharose 4B (GE Health, USA) and His-tag Purification Resin (Roche, USA). GST or GST-fusion protein was incubated with the

whole cell lysates or His-hMOF as indicated in results and prepared glutathione-sepharose beads at 4°C for overnight. After centrifugation at $1,500 \times g$ for 10 min, the pellets were washed for four times with 100 bead volumes of NETN buffer (0.5% NP-40, 0.1mM EDTA, 20 mM Tris-HCl, pH 7.4, 300 mM NaCl). The pellets were eluted by heating at 100°C for 10 min in SDS-PAGE loading buffer.

In vitro acetylation assay

GST-TET1-F3 and GST-H4 were expressed and purified from *E.coli* according to the GE purification protocol. Then, 5 μ g of substrate was incubated with 3 μ g of His-hMOF and GST-TET1-F3 in reaction buffer with or without acetyl-CoA at 30°C for 1 h.

Immunofluorescence staining

Cells grown on a glass coverslips were fixed in 4% paraformaldehyde/PBS for 15 min. at RT and then permeabilized with PBS containing 0.1% Triton X-100 (PBST) for 15 min at RT. After blocking in PBS with 5% BSA/PBST for 30 min, cells were incubated with anti-H4K16ac in 5% BSA/PBST for 1 h at RT. Cells were washed three times with PBST and then incubated with secondary antibodies, Alexa Fluor 546 (Goat anti-mouse IgG, 1:500) and/or Alexa Fluor 488 (Goat anti-rat IgG, 1:200) in 5% BSA/PBST at RT for 1 h. After three times washes in PBST, slides were mounted with clear nail polish and analyzed with an inverted microscope (Leica, Germany).

Neutral comet assay

Briefly, Tet1^{-/-} and wild type MEF cells were mixed respectively with low melting-point agarose gel gently at 37°C and then smoothly spread on glass slides. After the mixture solidified at RT, cells were lysed in buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1% N-lauroylsarcosine, pH 9.5) and subjected to electrophoresis in TBE buffer (890 mM Tris-base, 890 mM boric acid, 20 mM EDTA) at 21 V for 30 min. The nuclei on gel were stained with propidium iodide (PI) and visualized by microscopy. Images were captured and measured by a comet assay analysis system. The extent of DSBs damage was represented by the parameter of tail moment and the length in the tail of comet.

HRR and NHEJ reporters

HRR was detected by DR-GFP reporter system. The DSBs can be repaired by HRR between the two GFP mutant genes, resulting in the restoration of a functional GFP gene and the expression of GFP proteins. Therefore, quantification of the percentage of GFP-positive cells after expression of I-SceI in DR-GFP cells can be used to measure the efficiency of HR-mediated DSBs repair. Specifically, DR-GFP cells were seeded at 3×10^5 cells per 60mm dish and transfected with 2.5 μ g pCBASceI plasmid DNA. Cells were analyzed using FACS. For each analysis, 20,000 cells were processed, and each experiment was repeated three times.

NHEJ was detected by EJ5-GFP system, which contained a promoter that was separated from a GFP coding region by a puro gene. The puro gene was flanked by

two I-SceI sites that are in the same orientation. Once the puro gene was removed by NHEJ repair of the two I-SceI-induced DSBs, the promoter was rejoined to the rest of the coding cassette to restore the GFP gene. Therefore, analysis of the percentage of GFP-positive cells after co-transfection of I-SceI could be used to measure NHEJ activity. Specifically, 3×10^5 cells were co-transfected with 2 μ g of pCBASceI in containing EJ5-GFP. Cells were analyzed using FACS. For each analysis, 20,000 cells were processed, and each experiment was repeated three times.

Sucrose gradient

Nuclear extracts were applied to a 15–30% (w/v) sucrose gradient, and centrifuged in a SW41 rotor for 5.5 h at 41,000 rpm at 4°C. Equivalent volumes from 16 fractions were separated by SDS-PAGE and analyzed by immunoblotting.

ChIP-seq data preparation

For integrated genomic analysis, we collected 219 ChIP-seq data sets of 103 DNA binding proteins and 14 data sets of eight histone modification markers in mESCs from GEO and ENCODE (See Supplementary Table S1). Further analysis details are provided in Supplemental Methods.

RESULTS

Integrative genomic analysis reveals similar binding patterns between Tet1, Sin3a, Mof and H4K16ac in mESCs

Previous studies have generated a considerable number of ChIP-seq data sets of DNA binding proteins (DBPs) from mESCs, which are available in GEO and ENCODE databases. We retrieved all 219 available ChIP-seq data sets corresponding to 103 different DBPs in mESCs to investigate the co-occupancy between Tet1 and the rest of the DBPs (Supplementary Table S1). In hierarchical clustering, pair-wise correlation analysis between Tet1 and other DBPs demonstrated that Tet1 could be grouped into one sub-cluster with 13 DBPs in the 2000 bp transcriptional start site (TSS) flanking region (defined as promoter) (Figure 1A and Supplementary Figure S1). Next, the same correlation analysis of each component in this sub-cluster with eight types of available histone modifications in mESCs derived from ENCODE database showed that although Tet1, with other five DBPs (Kdm2a, Mof, Dpy30, Sin3a and Lsd1), were mostly related together, they do have certain degree of overlapping with H4K16ac, H3K4me3, H3K9ac and H3K27ac (range of co-efficiency from 0.54 to 0.80) (Figure 1B). Given that Mof is the only histone acetyltransferase in the sub-cluster, which specifically modifies H4K16ac, we decided to focus on the investigation of the co-efficiency among the three histone acetylation markers, Tet1, Mof, and Sin3a by correlation analysis. In addition, Tip60, and histone methylation markers, H3K4me1 and H3K4me3, have been simultaneously included for such analysis as negative controls. Our results revealed that Tet1, Sin3a, Mof, and H4K16ac were clustered together with the highest correlation co-efficiency (Figure 1C). Furthermore, ChIP-seq signals enrichment analysis revealed that Tet1,

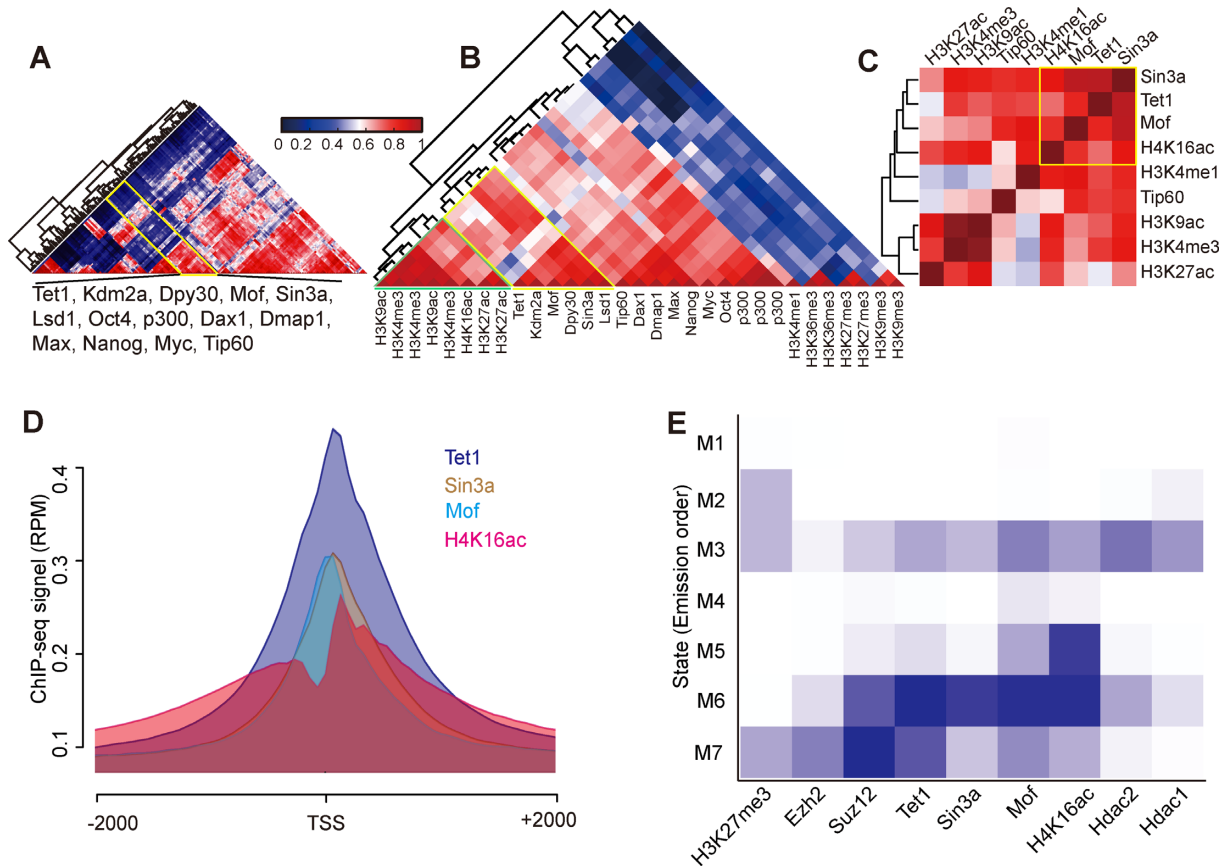


Figure 1. Integrative genomic analysis of published ChIP-seq data sets in mESCs show highly similar binding patterns between Tet1 and H4K16ac. (A) Pearson correlation between Tet1 and other 102 DBPs (Supplementary Table S1) in the 2000 bp TSS flanking regions. DeepTools was employed to calculate the correlation of ChIP-seq data. Color bar represents correlation coefficient. (B) Further correlation between Tet1, other 13 DBPs (Kdm2a, Dpy30, Mof, Sin3a, Lsd1, Oct4, p300, Dax1, Dmap1, Max, Nanog, Myc and Tip60) and 6 histone modifications (H3K4me3, H3K9me3, H3K9ac, H3K27ac, H3K36me3 and H4K16ac) in 2000 bp TSS flanking regions. (C) Correlation of Tet1, Sin3a, Mof, H4K16ac, H3K27ac, H3K9ac and H3K4me3. (D) The bindings of Tet1, Sin3a, Mof and H4K16ac are commonly enriched in the TSS regions. (E) Seven distinct distribution among Tet1/Sin3a/Mof complex, PRC2 complex and Sin3a/Hdac1/Hdac2 complex were generated by ChromHMM. (F) The enriched pathways in cluster M6, including DNA repair pathways marked as red color.

Sin3a, Mof, and H4K16ac had highly overlapping distribution patterns around promoter regions (Figure 1D and Supplementary Figure S2A). Enrichment analysis showed high signals around Tet1-centered peaks (Supplementary Figure S2B). Of 9,375 overlapped peaks among Tet1, Sin3a, Mof and H4K16ac in genome-wide, 71%, 17% and 12% distributed on the promoters, genobody, and intergenic regions, respectively (Supplementary Figure S2C). In addition, as shown in Supplementary Figure S2D, enriched signals centered on corresponding overlapped peaks were colocalized in genomic regions of promoters, genobody, and intergenic regions respectively. Taken together, these observations imply that TET1, SIN3A and hMOF have highly similar binding patterns with H4K16ac at the genomic level.

To differentiate the binding profiles among TET1/SIN3A/hMOF, PRC2 and SIN3A/HDAC in genomic level, we analyzed ChIP-seq datasets of Suz12, Ezh2, Sin3a, Tet1, Mof, Hdac1 and Hdac2, as well as their associated histone markers H3K27me3 and H4K16ac by employing ChromHMM software, which supports the comparison of models with different number of ChIP-seq datasets based on correlations in their emission parameters

(See Supplementary Methods). Simply, we initially tried ChromHMM analysis with default value through training several models in parallel mode ranging from 3 states to 15 states with transition parameters. We decided to use seven states for further analyses since it captured all the key interactions among these nine components, and because other numbers of states did not capture sufficiently distinct interactions (Supplementary Figure S3A). Our results showed that Tet1/Sin3a/Mof, PRC2 and Sin3a/Hdac1/Hdac2 were enriched in states of M6, M7 and M3, respectively (Figure 1E, Supplementary Figure S3B and S3C). Taken together, these data led us to hypothesize that TET1/SIN3A/hMOF may form a complex targeted chromatin marker H4K16ac which distinguished from either PRC2 complex or SIN3A/HDAC complex.

TET1 forms a chromatin complex with SIN3A and hMOF

To investigate whether TET1 formed a chromatin complex with hMOF and SIN3A, we initially obtained different fractions of nuclear protein extracts in HEK293T cells separated by size fractionation using sucrose gradient centrifugation. As shown in our data, TET1, hMOF, and

SIN3A were simultaneously enriched in pools 3, 4 and 5, suggesting they may be complexed with each other (Figure 2A). Next, we performed immunoprecipitations (IPs) analysis of chromatin-bound protein using TET1 or SIN3A antibody in HEK293T cells. Our data showed that both TET1 and SIN3A interacted with hMOF (Figure 2B). In order to identify which region of TET1 interacted with hMOF and SIN3A, we constructed three fragment plasmids of TET1, described as Flag-TET1-F1, Flag-TET1-F2 and Flag-TET1-F3, which respectively contained CXXC domain, Cysteine-rich domain and DSBH (double stranded β -helix) conserved domain (Figure 2C). Our Co-IP data showed that hMOF and SIN3A only interacted with Flag-F3 (Figure 2D). Consistently, the interactions were confirmed by His-pulldown assays using proteins overexpressed in, and purified from *Escherichia coli* cells (Figure 2E). Taken together, our results suggest that TET1 forms a chromatin complex with hMOF and SIN3A via its C-terminal region.

TET1-depletion results in a significant reduction of H4K16ac level

To address whether TET1 modulates the level of H4K16ac, we analyzed alterations of several histone modifications in TET1-depleted cells. Western blot analysis showed that H4K16 was hypo-acetylated in Tet1-knockout mice embryonic fibroblast (Tet1^{-/-} MEF) cells compared to in wild-type Tet1^{+/+} MEFs. We observed a similar hypo-acetylation status in TET1-knockdown HCT116 and HeLa cells, respectively. However, the other histone markers, such as H3K4me2, H3K4me3, H3K27me3, H3K9ac and H3K14ac, demonstrated insignificant alterations (Figure 3A and Supplementary Figure S4A). Immunofluorescence staining further confirmed a significant reduction of H4K16ac in Tet1^{-/-} MEF cells, TET1-knockdown HCT116 and TET1-knockdown HeLa cells compared to their respective controls (Figure 3B). Our results indicated that depletion of TET1 resulted in a significant reduction of H4K16ac level.

TET1 modulates H4K16ac via affecting auto-acetylation of hMOF

Previous studies showed that auto-acetylation of hMOF down-regulates its recruitment and enzymatic activity against H4K16ac. Therefore, we speculated that TET1 could control HAT activity of hMOF against H4K16 residue via affecting auto-acetylation of hMOF. To test this hypothesis, we expressed and purified His-hMOF, GST-TET1-F3 and GST-H4 in BL21 cells (Figure 4A). We initially determined that GST-TET1-F3 interacted with His-hMOF directly via a pull-down assay (Supplementary Figure S5A). Next, we determined that GST-TET1-F3 could increase enzymatic activity of His-hMOF against H4K16 residue *in vitro* (Figure 4B). Further, we performed *in vitro* acetylation assay using His-hMOF and GST-TET1-F3 to detect acetylation of hMOF by an acetylated lysine specific antibody. Interestingly, GST-TET1-F3, but not GST-TET1-F1 and GST-TET1-F2, could prevent auto-acetylation of His-hMOF (Figure 4C and Supplementary

Figure S5B). Moreover, we expressed recombinant protein of hMOF mutant in its major auto-acetylated site lysine 274. The result of *in vitro* acetylation assays showed that GST-TET1-F3 could not significantly affect the acetylated level of His-hMOF K274R (Supplementary Figure S5C), suggesting that auto-acetylated site of hMOF K274 may be blocked by TET1-F3. Importantly, we found that hMOF binding on chromatin was decreased in TET1-knockdown HEK293T cells in chromatin fraction (Figure 4D), whereas the total protein of hMOF did not change (Supplementary Figure S5D). These results indicate that TET1 controls auto-acetylation of hMOF to affect its chromatin affinity and enzymatic activity against H4K16 residue.

TET1 regulates some important DNA repair genes via modulating H4K16ac on the promoters of the DNA repair genes

We found that TET1-depletion induced transcriptional repression of the important genes in DNA repair in response to DSBs, including *RAD50*, *BRCA1*, *RAD51* and *TP53BP1*, via RT-PCR and western blot analysis (Figure 5A and B). To determine whether TET1 was required for regulation of DNA repair genes through modifying H4K16ac, we measured the mRNA levels of *RAD50*, *BRCA1*, *RAD51* and *TP53BP1* in HA-hMOF overexpressed HEK393T cells, with or without TET1 knockdown, via RT-qPCR. We found that depletion of TET1 blocked the increased expression of *RAD50*, *BRCA1*, *RAD51* and *TP53BP1* in hMOF-overexpressed cells (Figure 5C). Next, we further determined the effect of H4K16ac enrichment at the promoter of *RAD50*, *BRCA1*, *RAD51* and *TP53BP1* in HA-hMOF-overexpressed HEK293T cells, with or without TET1 knockdown, via ChIP-qPCR. The results showed that depletion of TET1 blocked the increased H4K16ac enrichment at the promoter of *RAD50*, *BRCA1*, *RAD51* and *TP53BP1* in both hMOF-overexpressed HEK293T cells (Figure 5D). We also observed these DNA repair genes, including *Brcal*, *Brcal2*, *Rad51*, *RAD50*, *Trp53* and *Mlh1*, were co-occupied with binding of Tet1, Sin3a, Mof and H4K16ac as shown in Supplementary Figure S6. Collectively, these results suggest that TET1 could regulate some important DNA repair genes, including *RAD50*, *BRCA1*, *RAD51* and *TP53BP1* via modulating H4K16ac on the promoters of the DNA repair genes.

TET1 depletion results in an accumulation of DNA damage and genomic instability

To determine the role of TET1 in DNA repair, we performed a comet assay and measured the percentage of DNA present in comet tail and the tail moment to determine the extent of DNA damage in Tet1^{-/-} MEF cells, respectively. Our results showed both of these parameters were increased approximately by 2-folds in the Tet1^{-/-} MEF cells than those in WT cells (Figure 6A–C), indicating that Tet1^{-/-} MEF cells had a higher level of DSBs. We also measured foci formation of DSBs maker γ H2AX by immunofluorescence staining. Consistent with our comet assay, we found that the number of γ H2AX foci increased two-fold in Tet1^{-/-} MEF cells compared to WT cells (Figure 6D and E). In addition, western blot analysis indicated that the

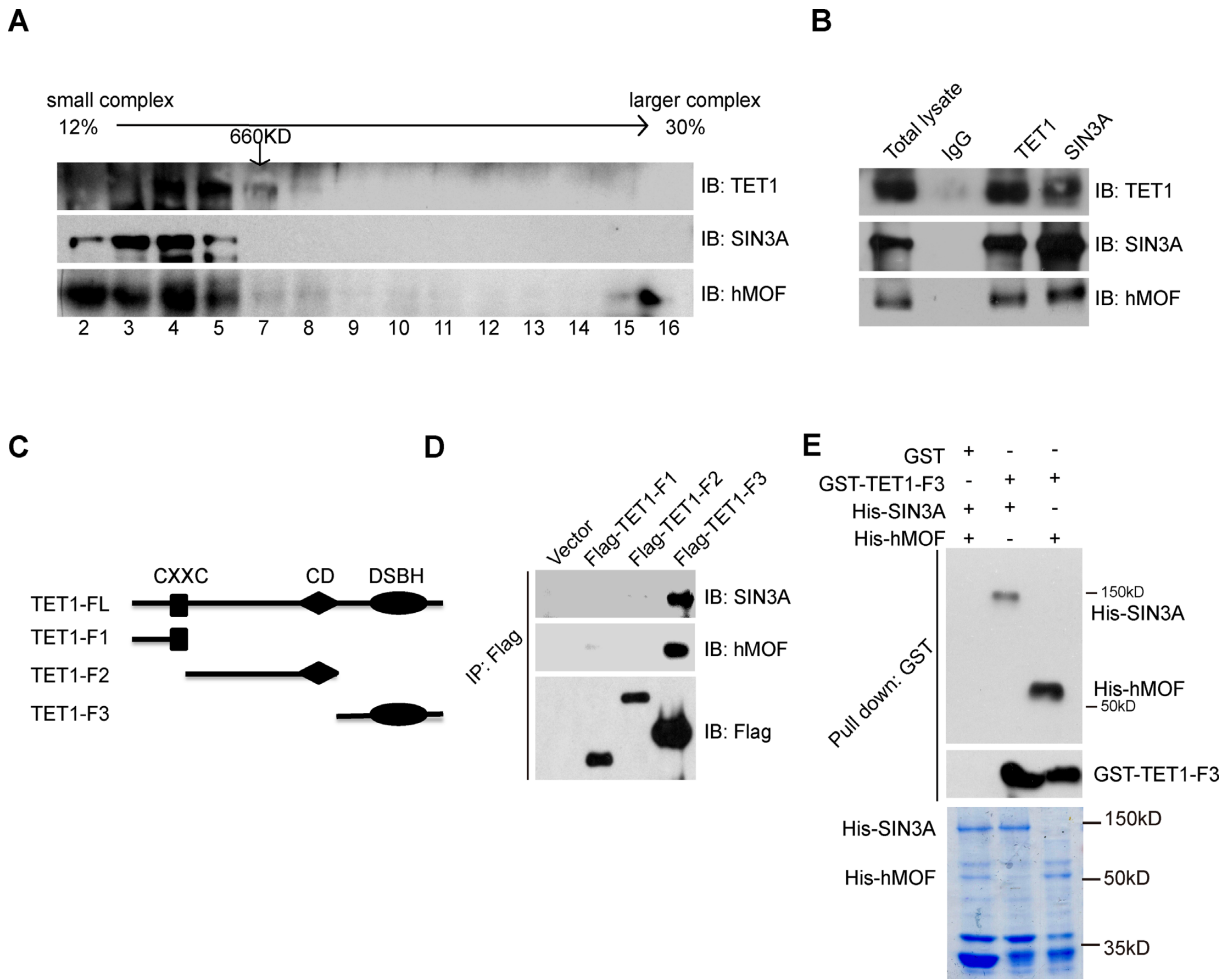


Figure 2. TET1 forms a chromatin complex with hMOF, and SIN3A. (A) Nuclear extracts from HEK293T cells were added to a 12–30% sucrose gradient, and fractions were assayed by immunoblotting. The fraction numbers and 660 kDa molecular mass standard are given across the top. The larger fraction numbers indicate the fraction with smaller molecular weight. (B) TET1 and SIN3A were shown interacting with hMOF using nuclear protein immunoprecipitations (IPs) in TET1 overexpressing HEK293T cells. (C) Schematic representation of TET1 fragments, including Flag-F1, Flag-F2, and Flag-F3 (CXXC: binding CpG islands; CD: Cysteine-rich domain; DSBH: double stranded β -helix). (D) HEK293T cells were transiently transfected with Flag-TET1-F1, Flag-TET1-F2 and Flag-TET1-F3, respectively. Nuclear protein IPs were performed using a Flag-tag antibody, followed by western blot analysis using indicated antibodies. (E) GST and GST-F3 were expressed in BL21 cells and purified following pGEX-GST-vector’s manual. His-SIN3A and His-hMOF1 were also expressed in BL21 and purified and. Pull down assays were performed using a GST-tag antibody.

level of γ H2AX increased in TET1-knockdown cells, but not in TET2- or TET3-knockdown HEK293T cells (Supplementary Figure S7). Specificity and inhibitory efficiency of TET1, TET2 and TET3 siRNA via semi-quantitative PCR is presented in Supplementary Figure S7B. We also determined the number of 53BP1 foci and found a 2-fold increase in the number of 53BP1 foci in Tet1^{-/-} MEF cells, which could be recruited to DNA damage site in response to DNA damage (Figure 6F and G). DAPI staining and statistical analysis further showed that the percentage of micronuclei in Tet1^{-/-} MEF cells were 2-folds higher than that in WT cells (Figure 6H and I). Next, C57 wild type mice (WT) and Tet1 heterozygous mutant mice (Tet1^{+/-} mice) were subjected to x-ray radiation. The coat-state rating scale results showed that there was a severe deterioration of the coat in Tet1^{+/-} mice compared with WT mice after four months of X-ray radiation (Figure 6J and K), suggesting that Tet1^{+/-} mice had defects in DNA repair mechanisms in

response to DSBs. These results indicate that loss of TET1 leads to severe DNA damage and the defects in DNA repair and genomic instability.

TET1 facilitates HRR and NHEJ via modulating auto-acetylation of hMOF

Homologous recombination repair and non-homologous end joining are two types of DNA repair mechanisms in response to DSBs. To determine the extent of HRR and NHEJ repair frequencies in TET1-depleted cells, we employed GFP-based chromosomal reporter assays with two stable cell lines, DR-GFP-U2OS and EJ5-GFP-HEK293, in which a defective GFP gene is functionally restored to WT cells upon I-SceI transfection (20). Our results showed that TET1-depletion resulted in 25% decrease of GFP positive cell numbers in HRR reporter assay (Figure 7A), and 50% reduction in NHEJ reporter assay, and neither of these outcomes was observed in TET2- and TET3-knockdown

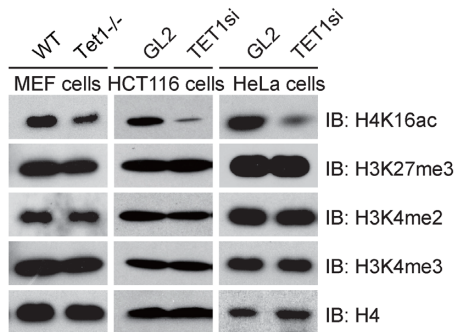
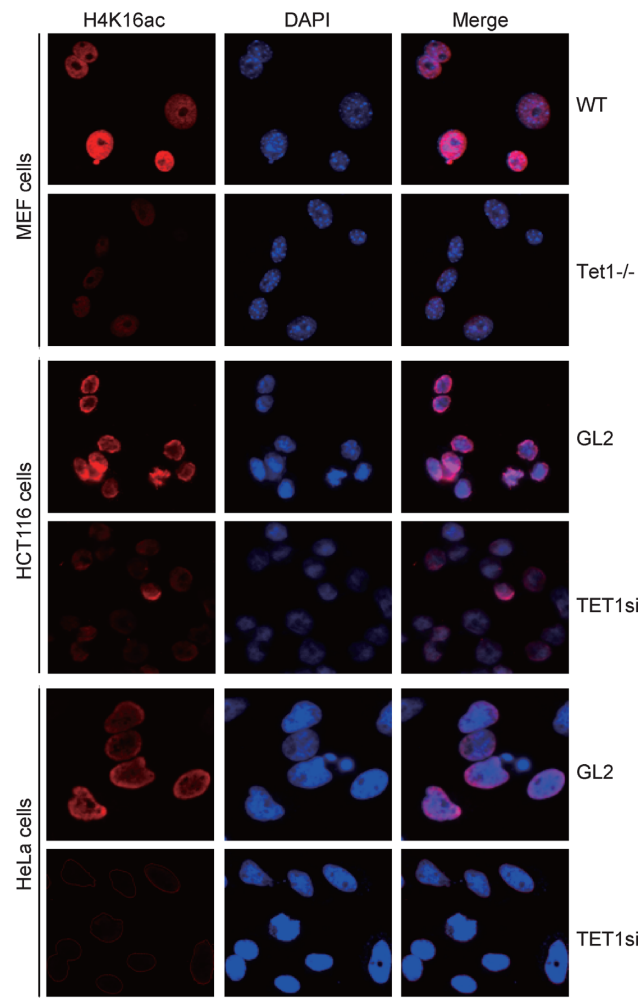
A**B**

Figure 3. TET1 deficiency results in a reduction of H4K16ac level. (A) Western blot analysis of histone modifications in Tet1^{-/-} MEF cells, TET1-knockdown HCT116 cells and TET1-knockdown HeLa cells using specific antibodies as indicated. (B) Immunofluorescence staining of Tet1^{-/-} MEF cells, TET1 knockdown HCT116 cells and TET1-knockdown HeLa cells with H4K16ac antibody. Nuclei were stained with DAPI.

cells (Figure 7B). We performed immunoprecipitations and determined that hMOF could interact with TET1, but not TET2 or TET3 (Supplementary Figure S8A). Interestingly, overexpressing hMOF K274R mutation, but not hMOF, can rescue the defects of HRR and NHEJ in TET1-depleted cells (Figure 7C and D). To examine whether this regulatory mechanism played a role in cell proliferation in response to DSBs, we generated HEK293T cells with stable knockdown of TET1 expression by shRNA. Inhibitory efficiency of TET1 shRNA is shown in Supplementary Figure S8B. We found that TET1 depleted cells, exhibited more sensitivity to x-ray exposure or bleomycin compared to TET1 WT cells (Figure 7E and F). Collectively, our results indicate that depletion of TET1, but not TET2 or TET3, results in the defective HRR and NHEJ in response to DSBs and genomic instability.

DISCUSSION

In this study, we revealed that TET1 can function as a core component of HAT complex as supported by their co-occupancy of common targets across the genome, associa-

tion with each other by co-IP, and co-migration in size fractionation assays. Further, we revealed a molecular mechanism in which TET1 prevents auto-acetylation of hMOF and then facilitates its chromatin affinity and enzymatic activity against H4K16. Importantly, we observed a phenotype in which Tet1 deficient mice present more sensitive to X-ray exposure, suggesting a defect of DNA repair. Here, our results provide a potential mechanism for understanding the roles of TET1 in transcription regulation, DNA repair and genomic stability. With this work, a novel role of TET1 is proposed as shown in Figure 7G.

Previous reports showed that TET1 and modified 5-hmC correlated with 'bivalent' chromatin markers with both repression (H3K27me3) and activation (H3K4me3) chromatin markers in mESCs (4,21). The fact that TET1 contributes to silencing some genes by facilitating recruitment of PRC2 complex (4), supports the hypothesis that TET1 represses gene expression by forming a TET1/PRC2 repressor complex that targets H3K27me3. However, the following two lines of evidence imply that involvement of TET1/PRC2 in transcriptional repression may be ESCs-

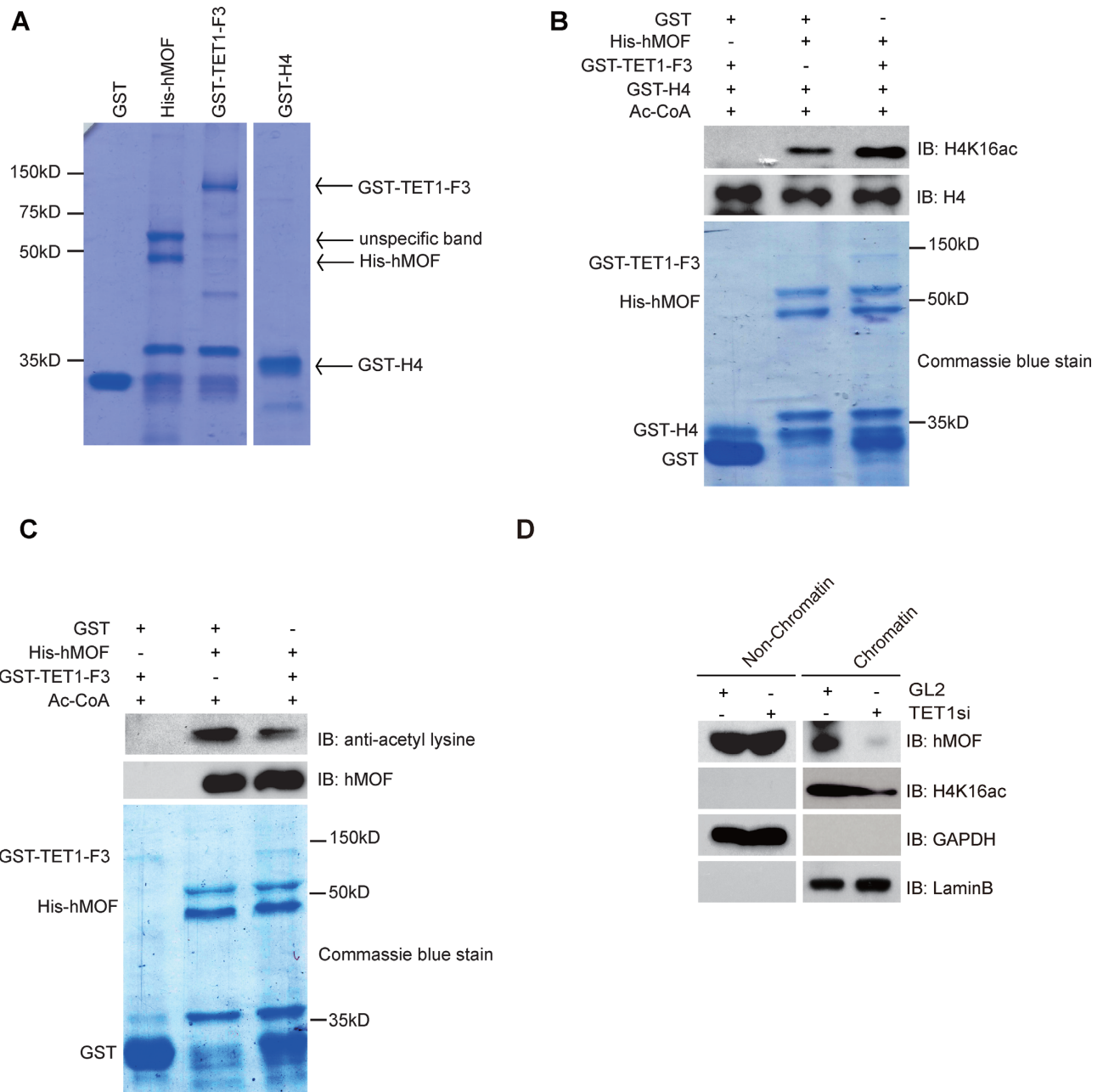


Figure 4. TET1 modulates H4K16ac via affecting auto-acetylation of hMOF. (A) Expressed and purified GST, His-hMOF, GST-TET1-F3 and GST-H4 were shown by Coomassie blue stain. (B) His-hMOF with or without GST-TET1-F3 were incubated with GST-H4 in HAT buffer at 30°C for 1 h. Acetylation of signals were detected using anti-H4K16ac antibody. Equal amounts of input were measured by Coomassie blue stain. (C) His-hMOF with or without GST-TET1-F3 were used for autoacetylation of hMOF. Acetylation of signals were detected using anti-acetyl lysine antibody. Equal amounts of input were measured by Coomassie blue stain. (D) Western blot analysis of the fractions in TET1-knockdown HEK293T cells using antibodies as indicated.

specific: (i) correlation between 5hmC and H3K27me3 is unique to ESCs, and is not present in differentiated fibroblasts or adult tissues and (ii) interaction between TET1 and the components of PRC2 complex was only observed in ESCs, but not in fibroblasts or HEK293T cells (5). Our observation, in which co-occupancy between Tet1 and Suz12/Ezh2/H3K27me3 present in M7 (mainly involved in cell differentiation and cell fate functions) (Supplementary Figure S3C), further supports the notion that Tet1/PRC2

complex may play an important role in cell differentiation and cell fate in mESCs. Additionally, we observed high co-occupancy of Tet1/Mof/Sin3a/H4K16ac at DNA repair genes in M6, whereas binding of H3K27me3 was absent (Figure 1). Our results, combined with a previous report in which TET1 was shown to interact with SIN3A in HEK293 cells and their shared binding on TSS region are H3K27me3-binding negative (5), exclude the possibility that TET1 downregulates these DNA repair genes by

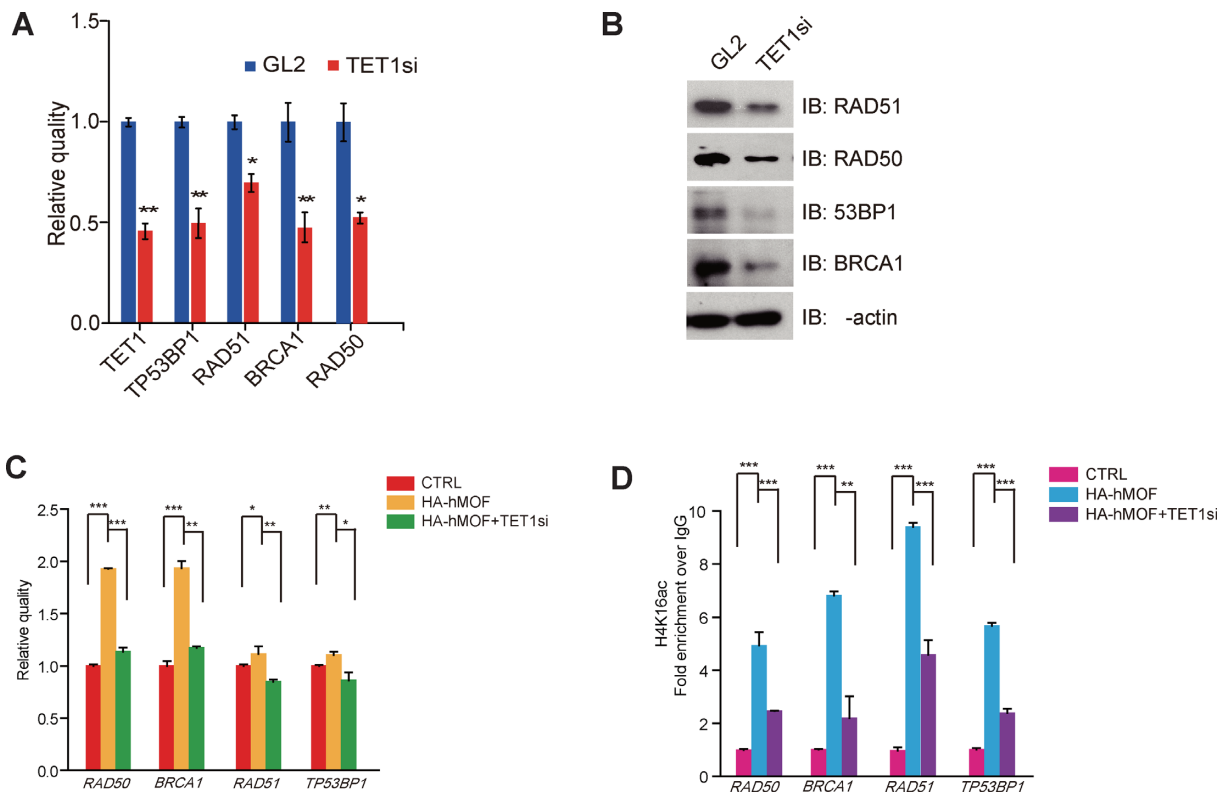


Figure 5. TET1 regulates some important DNA repair genes via modulating H4K16ac marker on the promoters of the DNA repair genes. (A) RT-qPCR analysis of mRNA levels in DNA repair genes (*RAD50*, *BRCA1*, *RAD51* and *TP53BP1*) in TET1-knockdown cells ($*P < 0.05$, $**P < 0.01$). (B) Western blot analysis of 53BP1, RAD51, RAD50 and BRCA1 protein expression in TET1-knockdown HEK293T cells. (C) RT-qPCR analysis of *TP53BP1*, *RAD51*, *RAD50* and *BRCA1* mRNA levels in HA-hMOF overexpressed HEK293T cells, with or without TET1-knockdown, as indicated ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (D) ChIP-qPCR analysis of H4K16ac binding at the promoter of *RAD50*, *BRCA1*, *RAD51* and *TP53BP1* gene loci in HA-hMOF-overexpressed HEK293T cells, with or without TET1-knockdown, as indicated ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). *P* values were calculated by unpaired Student's *t* test.

TET1/PRC2 complex through the activation of repressive histone marker H3K27me3 in differentiated cells.

Recent studies demonstrate that TET1 acts as a stable component of O-GlcNAc transferase (OGT) in ESCs, and promotes histone O-GlcNAcylation and H3K4me3 states for gene activation (22). However, several reports argue that TET2 and TET3, but not TET1, interact with OGT to activate gene expression in HEK293T cells (23,24), raising the possibility that TET1/OGT complex might be involved in transcriptional activation exclusively in ESCs. Our results in several differentiated cells lines revealed that TET1 knockdown resulted in hypo-acetylation of H4K16 only, but did not affect levels of H3K27me3, H3K4me2, nor H3K4me3, suggesting that TET1 complex mainly modulates H4K16ac in these cells (Figure 3). Interestingly, the observations that inhibition of HDAC1/HDAC2 has no significant effect on some important genes in DNA repair, including *RAD50* and *BRCA1* (25), and HDAC1/HDAC2 does not have occupancy on the promoter of *RAD50*, *BRCA1*, *RAD51* and *TP53BP1* in ChIP-seq data from mESCs (Supplementary Table S2), imply that TET1/SIN3A may not form a complex with HDAC1/HDAC2 to repress these DNA repair genes. Additionally, there was no alteration in the methylation level of the promoters of these DNA repair genes in Tet1 knockout mESCs by analyzing the published data

from Gary *et al.* (26) (Supplementary Figure S9). This result partly exclude the possibility that down-regulation of these genes is epigenetically regulated via its DNA methylation.

H4K16ac is a critical epigenetic modification involved in chromatin organization, transcriptional regulation, DNA repair, cellular life span, and X-chromosome dosage compensation in *Drosophila* (19,27,28). Previous studies have determined that hMOF, a HAT that specifically modifies H4K16ac, is indispensable for facilitating both HRR and NHEJ pathways through I-SceI induced HRR and NHEJ reporter assays (17) and frequently downregulated in various types of cancers (14,15). In parallel, there is deficiency of DNA repair in TET1-deficient non-Hodgkin B cell lymphoma (B-NHL) (8) and expression of TET1 is decreased in several cancers, such as breast cancer, hepatocellular carcinoma, colon cancer, and gastric cancer (29–32). In this study, we also demonstrated that an accumulation of DNA damage in Tet1^{-/-} MEF cells occurs and a phenotype of DNA repair deficiency upon X-ray exposure in Tet1^{+/-} knockout mice (Figure 6), suggesting that Tet1 plays an important role in facilitating DNA repair and maintaining genomic stability. In addition, our findings in which TET1 interacts with and positively regulates enzymatic activity of hMOF against H4K16ac residue (Fig-

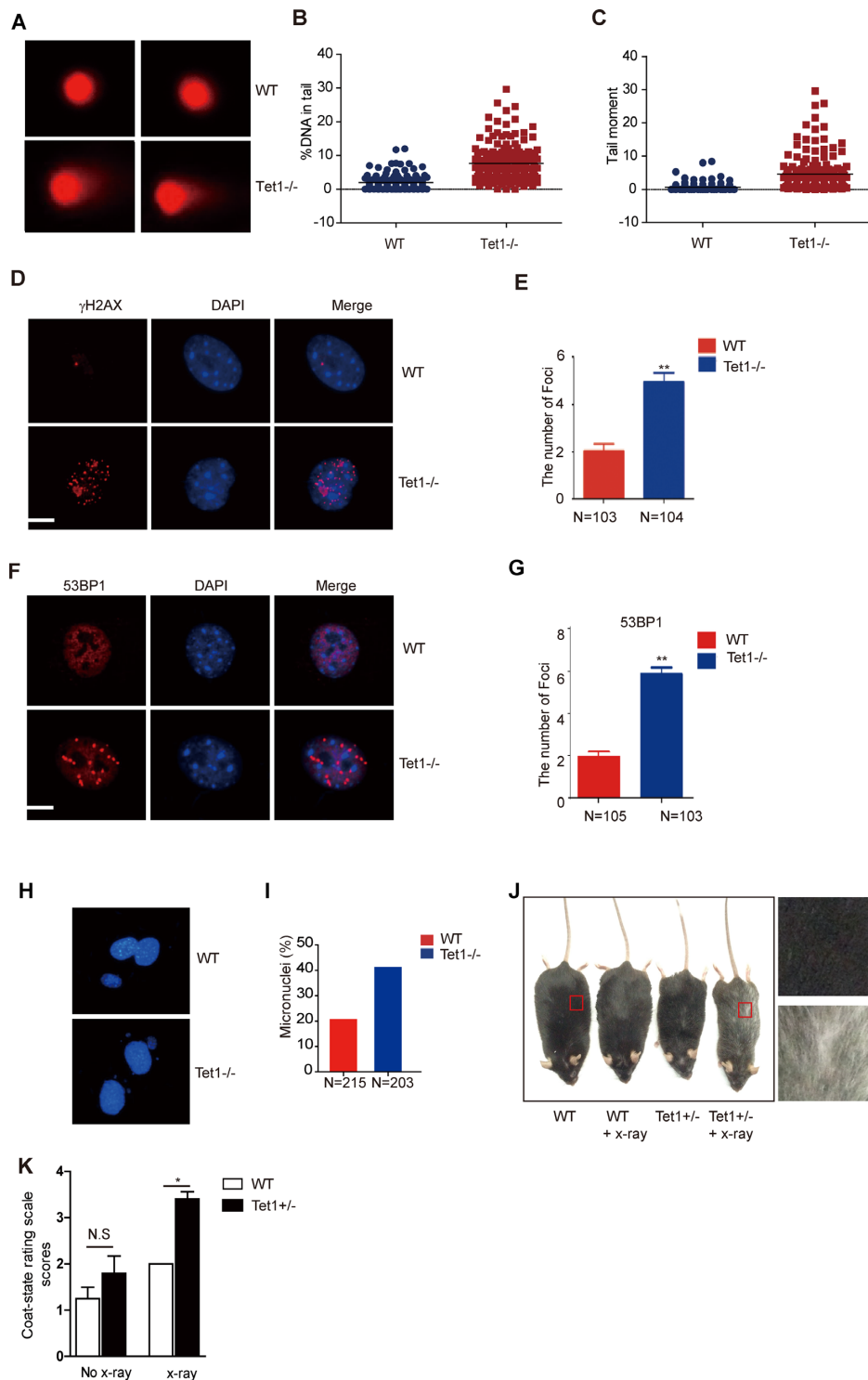


Figure 6. TET1-depletion causes an accumulation of DNA damage and genomic instability. (A) DNA damage in WT and Tet1^{-/-} MEF cells, as measured by neutral comet assay. (B, C) Quantification of comet experiments (shown in a) the percentage of DNA in the comet tail or the tail moment was measured and statistical analysis was performed using GraphPad software. (D) DNA damage marker γ H2AX was stained in WT and Tet1^{-/-} MEF cells. Nuclei were stained with DAPI. (E) Statistical analysis of γ H2AX foci pictured in (A). More than 10 number of foci was calculated as ten (** $P < 0.01$, $N =$ the number of the cells). (F) Immunofluorescence staining of 53BP1 foci formation in Tet1^{-/-} MEF cells. Nuclei were stained with DAPI. (G) Statistical analysis of 53BP1 foci in Tet1^{-/-} MEF cells and WT MEF cells. (H) DAPI staining and microscope analysis of micronuclei in Tet1^{-/-} MEF cells. (I) Statistical analysis of micronuclei in WT and Tet1^{-/-} MEF cells (** $P < 0.01$, $N =$ the number of the cells). (J) The phenotype of WT mice and Tet1^{+/-} mice after 3 Gy X-ray irradiation. Mice were irradiated with a single whole-body dose of 3 Gy X-rays at 60 days of age. Concurrent sham-irradiated control groups were also examined from the same litter where possible to minimize genetic bias. (K) Statistical analysis of the coat-state condition of mice after 3 Gy X-ray radiation, according to the method published by Nasca *et al.* (no X-ray: WT $n = 14$, Tet1^{+/-} $n = 15$; X-ray: WT, $n = 13$, Tet1^{+/-}, $n = 10$; * $P < 0.05$, N.S. $P > 0.05$).

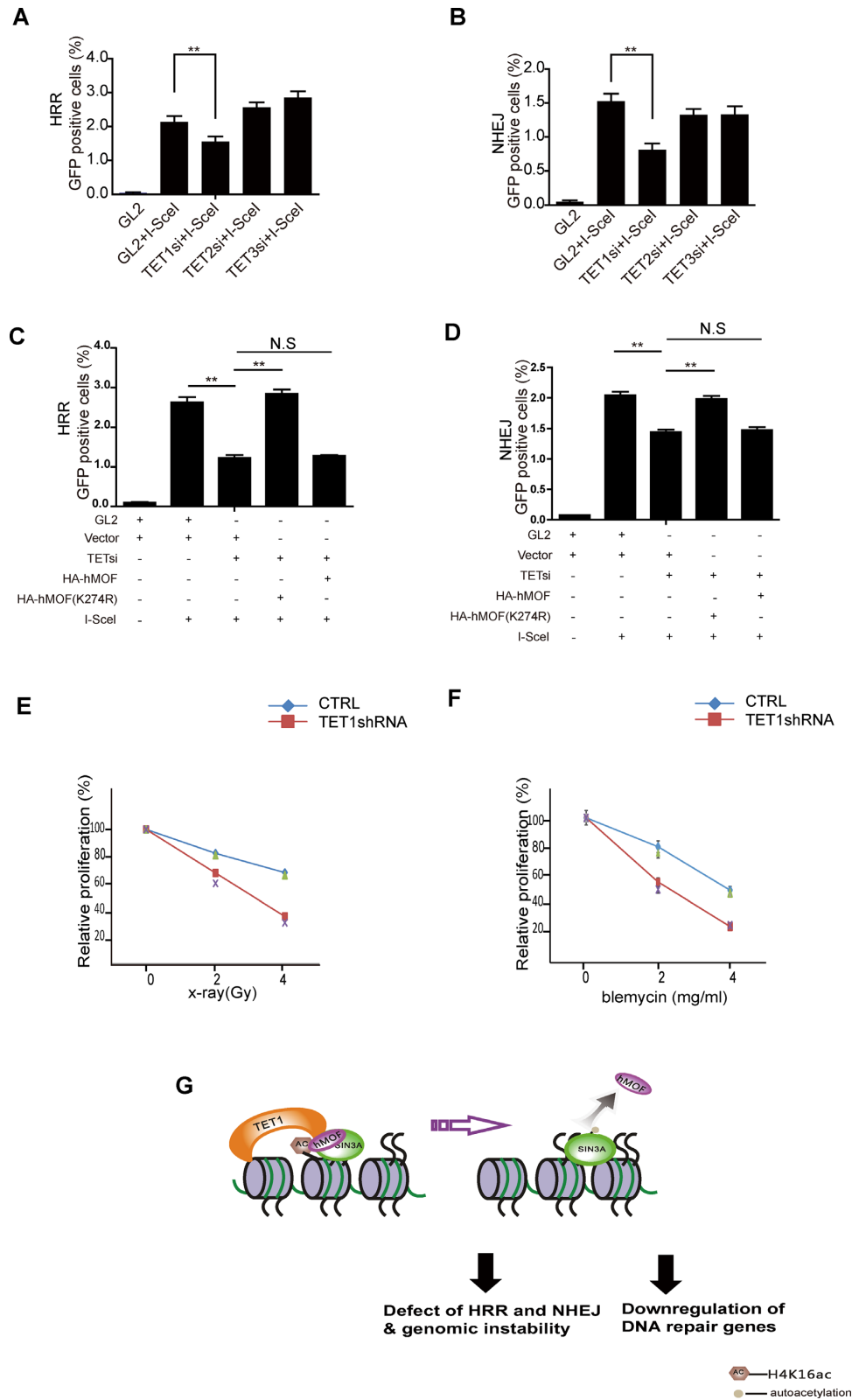


Figure 7. TET1 facilitates HRR and NHEJ via modulating auto-acetylation of hMOF. (A) Frequency of HRR after TET1, TET2 or TET3 depletion (** $P < 0.01$). (B) Frequency of NHEJ after TaET1, TET2 or TET3 depletion (** $P < 0.01$). (C) Frequency of HRR in TET1-knockdown cells, with or without overexpressing hMOF or hMOF (K274R) respectively (** $P < 0.01$). (D) Frequency of NHEJ in TET1-knockdown cells, with or without overexpressing hMOF or hMOF (K274R), respectively (** $P < 0.01$). (E, F) TET1 stable knockdown cells were transfected with shRNA. Transfectants were treated with x-ray or bleomycin with increasing concentrations as indicated for 72h. Relative cell proliferation was determined by MTT assay. P value was calculated by unpaired Student's t test. (G) The left graph depicts that TET1 forms a chromatin complex with SIN3A and hMOF. SIN3A may function as a scaffold on chromatin. The right graph depicted the process that TET1 depletion promotes auto-acetylation of hMOF to dissociate from chromatin and subsequently induces hypo-acetylation of H4K16, ultimately leading to down-regulation of DNA repair genes and defect of DNA repair.

ure 2), indicates that TET1 maybe epigenetically modulate H4K16ac to involve in DNA repair via complexing with hMOF. Mechanistically, our findings further revealed that TET1 elevates chromatin affinity and enzymatic activity of hMOF against H4K16ac through preventing hMOF's auto-acetylation (Figure 4). This observation was supported by previous result in which deacetylation of hMOF is correlated with the increased recruitment of hMOF to chromatin by protein deacetylases SIRT1 (33), providing additional evidence that hMOF's autoacetylation is reversely correlated to control H4K16ac. Therefore, it is speculated that TET1 may, through forming the complex with hMOF, act as a regulator to interfere with the auto-acetylation of hMOF so as to help the targeting of hMOF to H4K16ac, thereby facilitating DNA repair and maintaining genomic stability. Recently, a study showed that phosphorylated hMOF is required for the recruitment of different DNA repair proteins to involve in the appropriate DNA DSB repair pathways (34), thus the possibility in which hMOF functions in DNA repair via modification of its phosphorylation cannot be excluded. It is well-known that H4K16ac functions in keeping a more open conformation of the chromatin serving as a platform for DNA repair at the site of the damage. Thus, the question whether TET1-dependent H4K16ac directly affects on DNA repair genes or just serve as a platform need to be further investigated.

In summary, the roles of TET family proteins in regulating chromatin architecture and transcriptional expression independent of their DNA methylation have been gradually uncovered. Here, our findings reveal a novel molecular mechanism that TET1 specially modulates level of H4K16ac through forming TET1/hMOF complex to prevent auto-acetylation of hMOF and thereby increase its chromatin affinity to involve in regulation of gene expression, DNA repair and maintain genomic stability. These findings deepen our understanding for the role of TET1 in tumorigenesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We are grateful to Dr. Guoliang Xu for the kind gift of Tet1-knockout MEF cells, Dr. Jie Du for critically evaluating the manuscript and Dr. Xingda Ju and the other members in our lab for their invaluable assistance in experiments.

FUNDING

National High Technology Research and Development Program of China [2012AA02A202]; Natural Science Foundation of China (NSFC)-Canadian Institutes of Health Research Collaborative Research Project [81161120541]; NSFC [81301778]; Zhejiang Provincial Natural Science Foundation (ZNSF) [LY13C060002]; Zhejiang Provincial Department of Education Research Project [Y201223513]; NSFC [31271266]; Startup Fund for Scholars of Wenzhou Medical University [QTJ12001]; Innovative Center of China, AstraZeneca. Funding for

open access charge: National High Technology Research and Development Program of China [2012AA02A202]; NSFC-Canadian Institutes of Health Research Collaborative Research Project [81161120541]; NSFC [81301778]; ZNSF [LY13C060002]; Zhejiang Provincial Department of Education Research Project [Y201223513]; NSFC [31271266]; Startup Fund for Scholars of Wenzhou Medical University [QTJ12001]; Innovative Center of China, AstraZeneca.

Conflict of interest statement. None declared.

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