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TET1 plays an essential oncogenic role in *MLL*-rearranged leukemia

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The ten-eleven translocation 1 (*TET1*) gene is the founding member of the TET family of enzymes (TET1/2/3) that convert 5-methylcytosine to 5-hydroxymethylcytosine. Although *TET1* was first identified as a fusion partner of the mixed lineage leukemia (*MLL*) gene in acute myeloid leukemia carrying t(10,11), its definitive role in leukemia is unclear. In contrast to the frequent down-regulation (or loss-of-function mutations) and critical tumor-suppressor roles of the three *TET* genes observed in various types of cancers, here we show that *TET1* is a direct target of MLL-fusion proteins and is significantly up-regulated in *MLL*-rearranged leukemia, leading to a global increase of 5-hydroxymethylcytosine level. Furthermore, our both in vitro and in vivo functional studies demonstrate that Tet1 plays an indispensable oncogenic role in the development of *MLL*-rearranged leukemia, through coordination with MLL-fusion proteins in regulating their critical cotargets, including homeobox A9 (*Hoxa9*)/myeloid ecotropic viral integration 1 (*Meis1*)/pre-B-cell leukemia homeobox 3 (*Pbx3*) genes. Collectively, our data delineate an MLL-fusion/Tet1/Hoxa9/Meis1/Pbx3 signaling axis in *MLL*-rearranged leukemia and highlight *TET1* as a potential therapeutic target in treating this presently therapy-resistant disease.

Recently, the ten-eleven translocation (Tet) proteins (including Tet1/2/3) have been shown to be able to convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), resulting in active or passive DNA demethylation (1–4). In contrast to homozygous mutation of *Tet3* that results in neonatal lethality (5), mice carrying either $Tet1^{-/-}$ or $Tet2^{-/-}$ largely appear to develop normally (6–10). Although high abundance of Tet1 and Tet2 has been observed in mouse embryonic stem cells (mESCs) and Tet proteins have been implicated in hydroxymethylation and epigenetic regulation of stem cells (11–16), evidence is emerging that Tet1 and Tet2 are likely not required for the pluripotency and maintenance of mESCs (6, 8–11, 15). *TET1* is the founding member of the family, first identified as a fusion partner of the mixed lineage leukemia (*MLL*) gene in patients with acute myeloid leukemia (AML) carrying t(10,11)(q22;q23) (17, 18), but its definitive function in leukemia is unknown. Loss-of-function mutations of *TET2* (but not *TET1* or *TET3*) have been frequently observed in myeloid cancers, in which *TET2* functions as a critical tumor suppressor (7–9, 19–21). Notably, substantial down-regulation of all three *TET* genes has been reported in various types of solid tumors (3, 22–25), and *TET1* has been shown to be an essential tumor suppressor in prostate and breast cancers (26, 27). Thus, one may expect that all three *TET* genes are tumor suppressors in various cancers.

The *MLL* gene, located at human chromosome 11 band q23 (11q23), is frequently involved in chromosome translocations occurring in ~10% of total leukemia, including ~80% of infant acute leukemia, usually associated with poor prognosis (28–30). The critical feature of *MLL* rearrangements is the generation of a chimeric transcript consisting of 5′ *MLL* and 3′ sequences of a partner gene [80% involving ALL 1-fused gene from chromosome 9 (*AF9*), *AF6*, *AF10*, elongation factor RNA polymerase II (*ELL*), or eleven-nineteen leukemia (*ENL*) in AML] (29, 30). Although *TET1* was first identified as a fusion partner of the *MLL* gene in AML carrying t(10,11)(q22;q23), this fact does not give any clear clue about its pathological role in leukemia because fusion of MLL to even an artificial inducible dimerization domain caused activation of its transforming potential (31–33).

In the present study, through a large-scale, genome-wide gene expression profiling of 100 AML patient samples and nine normal bone marrow (BM) control samples, we show that *TET1* is aberrantly overexpressed in *MLL*-rearranged AML. We then performed a series of in vitro and in vivo functional and mechanistic studies. Our studies indicate that *Tet1* is a direct target gene of MLL-fusion proteins and thus is aberrantly overexpressed in *MLL*-rearranged leukemia, in which it plays a critical oncogenic role through cooperating MLL-fusion proteins in regulating a set of important oncogenic cotargets including homeobox A9 (*Hoxa9*), myeloid ecotropic viral integration 1 (*Meis1*), and pre-B-cell leukemia homeobox 3 (*Pbx3*).

Results

***TET1* Is Aberrantly Overexpressed in *MLL*-Rearranged AML.** We performed a large-scale, global gene expression profiling assay of 100 human AMLs with common chromosomal translocations (12 with *MLL* rearrangements and 88 without; see Table S1) and nine normal BM control [three each for $CD34^+$ hematopoietic stem/progenitor cell, $CD33^+$ myeloid progenitor cell, and mononuclear cell (MNC)) samples by use of microarrays]. We found that *TET1* was expressed at a significantly higher level

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The authors declare no conflict of interest.

Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE34184 and GSE30285).

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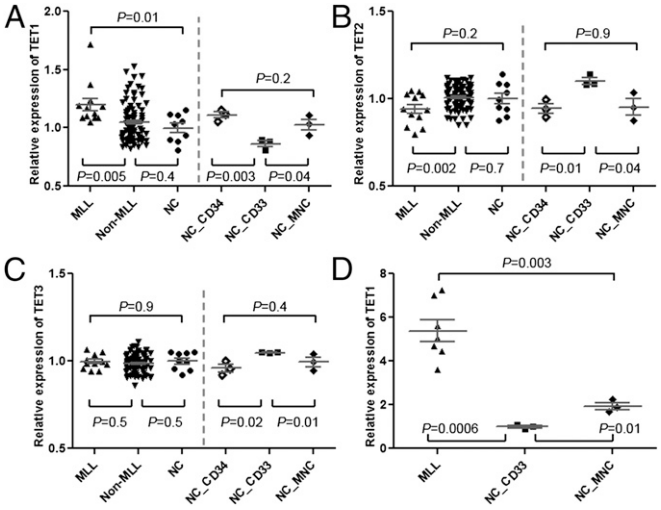

Fig. 1. *TET1* is aberrantly overexpressed in *MLL*-rearranged AML and is likely a direct target of MLL and particularly MLL-fusions. (*A*–*C*) Relative expression levels of *TET1* (*A*), *TET2* (*B*), and *TET3* (*C*) in 12 *MLL*-rearranged AML, 88 non–*MLL*-rearranged AML, and nine normal control samples as detected by microarrays. The average expression level of each *TET* gene in normal controls was set as 1. Note that the gene expression values used in our analyses were log-transformed, not the original absolute values (*SI Materials and Methods*). (*D*) Relative expression levels of *TET1* as detected by qPCR. The average level of *TET1* expression in CD33+ cell samples was set as 1. MLL, *MLL*-rearranged; NC, normal control; MNC, mononuclear cells.

in *MLL*-rearranged AML than in normal controls ($P = 0.01$) (Fig. 1*A*); in contrast, neither *TET2* (Fig. 1*B*) nor *TET3* (Fig. 1*C*) is significantly dysregulated in *MLL*-rearranged AML relative to normal controls. Our cytometry analysis (Fig. S1) and previous studies (34, 35) showed that the *MLL*-rearranged leukemic cells usually are $CD33^+/CD34^{-/low}$. Thus, normal $CD33^+$ cells would be better controls than normal $CD34^+$ cells for *MLL*-rearranged leukemic cells. If compared with normal $CD33^+$ cells, *TET1* was significantly up-regulated ($P = 0.01$) whereas both *TET2* ($P = 0.01$) and *TET3* ($P = 0.05$) were significantly down-regulated in *MLL*-rearranged AML samples (Figs. 1 *A*–*C*). As leukemic MNC cells were used for all human patient samples in the microarray assay, we also compared the expression levels of *TET* genes between *MLL*-rearranged patient samples (i.e., leukemic MNC cells) and normal MNC cell samples; we found that only *TET1* is significantly up-regulated ($P = 0.04$) in *MLL*-rearranged AML whereas *TET2* and *TET3* did not show a significant change. The overexpression of *TET1* in human *MLL*-rearranged AML cell samples compared with normal $CD33^+$ or MNC cells was confirmed by quantitative PCR (qPCR) assay of additional samples (Fig. 1*D*).

Among normal hematopoietic cells, compared with $CD34^+$ hematopoietic stem/progenitor cells, committed $CD33^+$ myeloid progenitor cells (i.e., relatively more mature cells) exhibit significantly lower *TET1* expression but a higher level of *TET2* or *TET3* expression (Figs. 1 *A*–*C*). MNC cells, on the other hand, are a mixed population containing both primitive progenitors and committed cells, in which the *TET* genes are expressed at a level relatively similar to that seen in $CD34^+$ cells, but significantly higher (*TET1*) or lower (*TET2/3*) than that seen in $CD33^+$ cells (Figs. 1 *A*–*C*). Thus, our data suggest that *TET1* is likely down-regulated whereas *TET2* and *TET3* are up-regulated during normal hematopoiesis.

***TET1* Is a Direct Target Gene of MLL and Particularly MLL-Fusion Proteins.** It is well known that MLL-fusion proteins bind to the promoters of a group of critical target genes, such as *HOXA9* and *MEIS1*, and promote their expression through recruiting DOT1 like (DOT1L)-mediated methylation of histone H3 lysine 79 (36-38). To elucidate the mechanism underlying the up-regulation of *TET1* in *MLL*-rearranged leukemia, we performed chromatin immunoprecipitation (ChIP) assays. As shown in Fig. 2*A*, MLL (see MLL-C binding) and particularly MLL-fusion proteins (see the portion of MLL-N binding exceeding that of MLL-C) are significantly enriched at the CpG area (sites 2 and 3), but not the distal upstream site (site 1), of *TET1* in human MONOMAC-6 cells (an *MLL-AF9* leukemia line), associated with a significant enrichment of histone H3 lysine (K) 79 (H3K79) dimethylation (H3K79me2) to the sites. A similar pattern was observed in two other *MLL*-rearranged AML cell lines including THP-1/t(9,11) and KOCL-48/t(4,11) cells. In contrast, no such enrichment was observed in K562, a negative control cell line with no *MLL* rearrangements (Fig. 2*A*). Notably, there is no significant difference between MLL-N binding and MLL-C binding in K562, indicating that the affinity of the antibody against MLL N-terminal is similar to that of the antibody against MLL C-terminal. Thus, the substantially enhanced enrichment of MLL-N binding compared with MLL-C binding in MONOMAC-6 cells is not due to the affinity difference of the antibodies; instead, the enhancement is due to the fact that the antibody against MLL N-terminal can bind to both the wild-type and *MLL*-rearranged alleles whereas the antibody against MLL C-terminal can only bind to the single wild-type allele in MONOMAC-6 cells. Consistent with the direct binding of MLL-fusion proteins to the promoter region of *TET1* observed in *MLL*-rearranged leukemic cells (Fig. 2*A*), forced expression of MLL fusion genes could significantly up-regulate *Tet1* endogenous expression in both mouse (Fig. 2*B*) and human (Fig. 2*C*) hematopoietic progenitor cells. Conversely, *Tet1* expression was significantly ($P < 0.01$) down-regulated in MLL-ENL-estrogen receptor inducible (ERtm) mouse myeloid cells carrying tamoxifen-inducible *MLL-ENL* (39, 40) when expression of *MLL-ENL* was depleted after withdrawal of 4-Hydroxy-tamoxifen (4-OHT). The opposite was true for *Fas*, a control gene that is

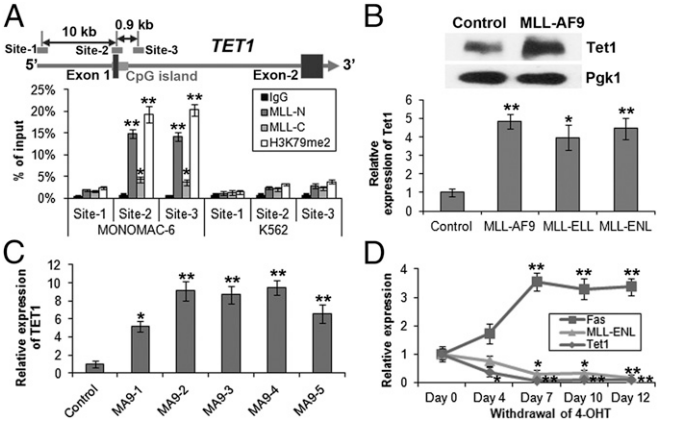

Fig. 2. *TET1* is a direct target of MLL and particularly MLL-fusions. (*A*) ChIP-qPCR assays of the enrichment of MLL-N (i.e., MLL N-terminal, representing both wild-type MLL and MLL-fusion proteins), MLL-C (i.e., MLL C-terminal, representing wild-type MLL only), and H3K79me2 at the promoter region of *TET1* (sites 2 and 3) and a distal upstream region (site 1) in MONOMAC-6 and K562 cells. IgG was used as a negative control. (*B*) qPCR (*Lower*) and Western blotting (*Upper*) analyses of Tet1 expression in mouse normal BM progenitor (Lin^-) cells that were transduced with MSCV-*MLL-AF9*, *-MLL-ELL*, *-MLL-ENL*, or empty vector (Control). Transduced cells were cultured in methylcellulose for 7 d. (*C*) qPCR analysis of *TET1* expression in human cord blood $CD34^+$ cells that were transduced with MSCV-*MLL-AF9* or empty vector and cultured for a period to select transduction-positive cells (MA9-1 to -5, represent different lines of immortalized cells) (35). (*D*) qPCR analysis of *MLL-ENL*, *Tet1*, or *Fas* expression in mouse MLL-ENL-ERtm cells after withdrawal of 4-OHT. MA9, *MLL-AF9*. $*P < 0.05$; $**P < 0.01$, two-tailed *t* test. Pgk1, phosphoglycerate kinase 1.

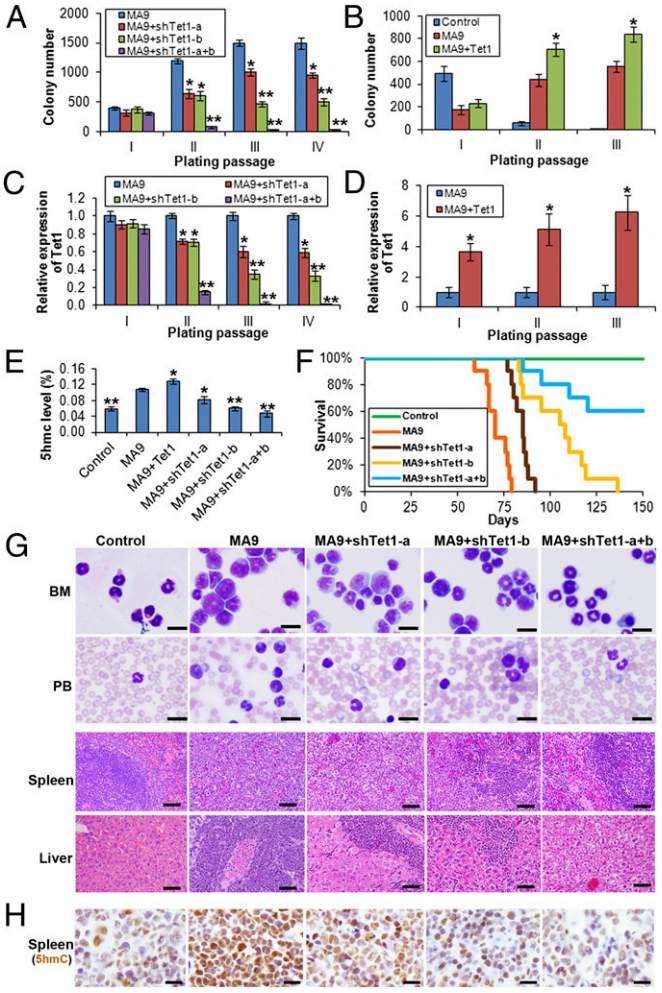

Fig. 3. Effects of *Tet1* in *MLL-AF9* (*MA9*)-mediated cell transformation and leukemogenesis. (*A* and *B*) Effects of depletion (*A*) or forced expression (*B*) of *Tet1* on *MA9*-mediated cell transformation. (*C* and *D*) qPCR analysis of *Tet1* expression in different passages of colony cells shown in *A* and *B*. (*E*) Dot blot assay of 5hmC level in genomic DNA of colony cells (passage II) shown in *A* and *B*. A similar pattern was observed in liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) assays. (*F*) Kaplan–Meier survival analysis of the BM transplantation recipient mice. The median survival of MA9, MA9+shTet1-a, MA9+shTet1-b, and MA9+shTet1-a+b mice ($n = 10$ for each group) is 70, 85, 108, and over 150 d, respectively; MA9+shTet1-a vs. MA9, $P = 0.003$; MA9+shTet1-b vs. MA9, $P = 0.0003$; MA9+shTet1-a+b vs. MA9, $P < 0.00001$; log-rank test. (*G*) Wright–Giemsa-stained BM cell cytospin and peripheral blood (PB) smear, and hematoxylin/eosin (H&E)-stained spleen and liver paraffin sections of transplantation recipient mice are shown. (Scale bars: 10 μm in BM/ PB and 100 μm in spleen/liver.) (*H*) 5hmC antibody-stained spleen. (Scale bars: 20 μm.) $*P < 0.05$; $**P < 0.01$, two-tailed *t* test; in *A–E*, MA9 samples were used as controls for the statistical comparisons. Note: "MA9" represents "MSCVneo-MA9+pGFP-V-RS-scrambled shRNA" in all of the plots except for *B* and *D* where it represents "MSCVneo-MA9+MSCVpuro"; "Control" represents "MSCVneo+MSCVpuro" or "MSCVneo+pGFP-V-RS" (see *SI Materials and Methods* for more details).

down-regulated in *MLL*-rearranged leukemia (41) (Fig.2*D*). Thus, our data suggest that *TET1* is a direct target gene of MLL and, particularly, MLL-fusion proteins.

***Tet1* Plays an Essential Oncogenic Role in *MLL*-Rearranged Leukemia.** To assess the functional importance of *Tet1* expression in *MLL*-rearranged leukemia, we conducted both loss- and gain-of-function studies. First, we synthesized three *Tet1* small hairpin RNAs (shRNAs), including shTet1-a (i.e., the mTet1-shRNA-A used in ref. 14), shTet1-b (i.e., the mTet1-shRNA-5 used in ref. 15), and shTet1-a+b (i.e., a combination to achieve a stronger knock-down effect), along with a scrambled shRNA (as a negative control for Tet1 shRNAs). We then cloned each shRNA into a retroviral vector, namely pGFP-V-RS (OrigGene). Meanwhile, we synthesized a Flag-tagged mouse *Tet1* (amino acids 1367–2039) (12) that has been shown to be able to exhibit a comparable regulatory function as full-length *Tet1* (4, 12) and cloned it into an murine stem cell virus puromycin (MSCVpuro) vector. In colony-forming/replating assays, we showed that depletion of endogenous *Tet1* expression by shTet1-a, shTet1-b, and particularly shTet1-a+b, significantly ($P < 0.05$, *t* test) inhibited *MLL-AF9*-mediated immortalization of mouse BM progenitor cells (Fig. 3*A*) whereas forced expression of *Tet1* led to the opposite effect (Fig. 3*B*). The degree of the above inhibition or enhancement effect on cell immortalization was parallel with the magnitude of the decrease or increase of *Tet1* expression (Fig. 3 *C* and *D*) and that of 5hmC level (Fig. 3*E*), indicating that Tet1 functions in a dosage-dependent manner.

In addition, we transfected *TET1* small interfering RNA (siRNA) oligos into human *MLL*-rearranged leukemic cells. Knockdown of *TET1* by siRNAs resulted in a significant ($P < 0.05$, *t* test) increase in apoptosis (Fig. S2*A*) and decrease in cell viability (Fig. S2*B*) of human MONOMAC-6 and THP-1 cells, both carrying the t(9,11) abnormality. The effects of si*TET1* could be reversed by cotransfected mouse *Tet1*, suggesting that the observed effects of si*TET1* are not due to off-targeting. A similar pattern was observed in analysis of the effects on cell growth/proliferation (Fig. S2*C*). The changes in *TET1* expression were associated with 5hmC level changes (Fig. S2*D*).

Furthermore, we also conducted in vivo mouse BM transplantation (BMT) assays. Knocking down expression of *Tet1* by shTet1-a, shTet1-b, and particularly shTet1-a+b significantly ($P < 0.005$; log-rank test) delayed *MLL-AF9*–mediated leukemogenesis in recipient mice (Fig. 3*F*). All of the leukemic mice died from AML (Fig. S3), and, notably, depletion of *Tet1* expression significantly reduced spleen size and decreased white blood cell counts (Table S2). Depletion of Tet1 expression by shTet1-a, shTet1-b, and particularly shTet1-a+b substantially reduced the proportion of immature blast cells in both BM and peripheral blood (PB), associated with a reduction of leukemia cell infiltration and disruption of organ architecture in spleen and liver (Fig. 3*G*), as well as a decrease of 5hmC in spleen (Fig. 3*H*).

Critical Cotargets of TET1 and MLL Fusions. *HOXA9*, *MEIS1*, and *PBX3* are the three most well-studied critical oncogenic targets of MLL fusions (36, 39, 42–48). Interestingly, previous genome-wide ChIP-seq or ChIP-chip assays of Tet1 in mESCs suggest that *Hoxa9*, *Meis1*, and *Pbx3* are potential direct target genes of Tet1 in mESCs (14–16) (Fig. S4). To determine whether these three MLL-fusion targets are also direct targets of TET1 in *MLL*-rearranged leukemic cells, we conducted conventional ChIP assays using MONOMAC-6 cells as a model. We found that TET1, similar to MLL fusion proteins, could bind to promoter regions of the three genes, associated with significantly increased levels of H3K79me2 (Figs. 4 *A–C*). We next assessed the effect of *TET1* on their expression. We found that knockdown of *TET1* expression by siRNA oligos and ectopic expression of mouse *Tet1* in MONOMAC-6 cells resulted in a significant down-regulation (Fig. 4*D*) and up-regulation (Fig. 4*E*) of the three genes, respectively. Similar effects of knockdown of *Tet1* expression by shRNA constructs on expression of the three targets in mouse BM progenitor cells transduced with *MLL-AF9* were also observed in vitro (Fig. 4*F*) and in vivo (Fig. 4*G*). We next showed that forced expression of *HOXA9*, *MEIS1*, or *PBX3* can largely reverse the effects of *TET1* depletion by siTET1 on cell apoptosis, viability, and growth/proliferation of both MONOMAC-6 and KOCL-48 (the latter carrying *MLL-AF4*) cells (Fig. 4 *H* and *I*).

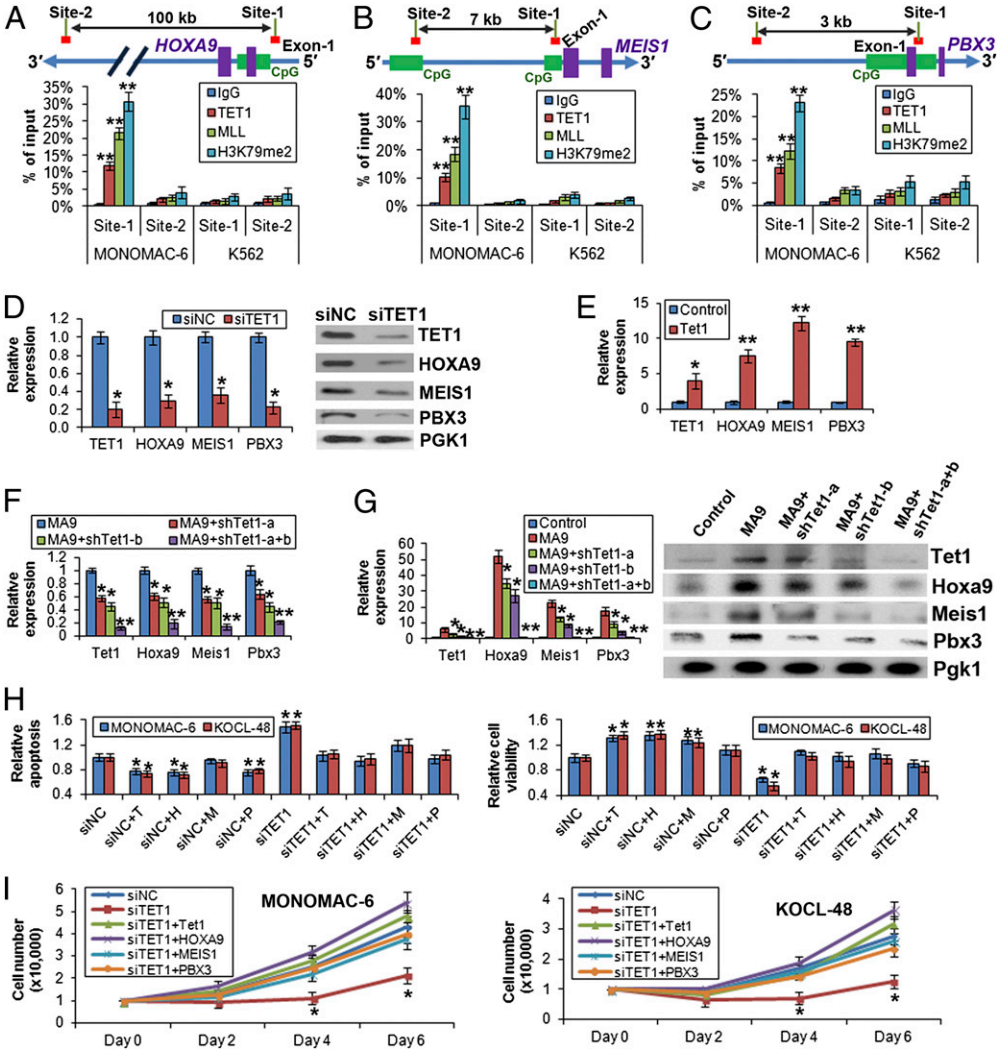

Fig. 4. *HOXA9*, *MEIS1*, and *PBX3* are important targets of TET1. (*A*–*C*) ChIP-qPCR assay of the binding of TET1, as well as MLL-fusion proteins, to the loci of *HOXA9* (*A*), *MEIS1* (*B*), and *PBX3* (*C*). Green bars represent CpG islands. Brown-purple bars represent exons of target genes. (*D* and *E*) The effects of knockdown of *TET1* by siRNA oligos (*D*, *Left*, qPCR; *Right*, Western blot) and of ectopic expression of mouse *Tet1* (*E*) on expression of *TET1*, *HOXA9*, *MEIS1*, and *PBX3* in human MONOMAC-6 leukemic cells. (*F* and *G*) Effects of knockdown of *Tet1* by different shRNAs on expression of the four genes in colony cells (*F*) or in BM cells of transplanted mice (*G*, *Left*, qPCR; *Right*, Western blot); see Fig. 3 for more details about the samples. (*H* and *I*) Effects of knockdown *TET1* by siRNAs with or without cotransfection of *HOXA9*, *MEIS1*, or *PBX3* on apoptosis (*H*, *Left*), cell viability (*H*, *Right*), and cell growth/proliferation (*I*) of *MLL*-rearranged leukemic cells. siNC, scrambled siRNA oligos (as negative control of siTet1); +H (+M, +P, or +T) or + HOXA9 (+MEIS1, +PBX3, or +Tet1), cotransfected with MSCVpuro-HOXA9 (-MEIS1, -PBX3, or -Tet1). *PGK1/Pgk1* was used as endogenous control for qPCR. $*P < 0.05$; $**P < 0.01$, *t* test; in *A*–*C*, K562 was used as a control for MONOMAC-6 for statistics analysis of each item. In *G*, MA9 group was used as the control.

Validation in a Tet1 Knockout Model. After completion of the above studies, we have very recently obtained a Tet1$^{-/-}$ mouse strain (6). As shown in Fig. 5*A*, the expression levels of all above validated target genes including *Hoxa9*, *Meis1*, and *Pbx3* are significantly down-regulated in Tet1$^{-/-}$ BM progenitor [lineage-negative (Lin$^-$)] cells compared with their wild-type counterpart. The Tet1 knockout results in a significant inhibition on cell transformation mediated by MLL-AF9 (Fig. 5*B*), similar to that caused by shRNA-Tet1-a+b (Fig. 3*A*). Such inhibition can be largely reversed by forced expression of *Hoxa9* (Fig. 5*B*). As expected, the expression levels of all three target genes are significantly down-regulated in *MLL-AF9*–transduced Tet1$^{-/-}$ colony cells compared with *MLL-AF9*-transduced wild-type colony cells (Fig. 5*C*). As Hoxa9 has been shown to be able to regulate expression of *Meis1* and *Pbx3* (49–51), it is not surprising that forced expression of *HOXA9* can largely restore the overexpression of those genes in *MLL-AF9*–transduced Tet1$^{-/-}$ cells (Fig. 5*C*). More importantly, knockout of Tet1 significantly inhibited MLL-AF9–mediated leukemogenesis (Fig. 5 *D* and *E*), in a manner similar to that caused by shRNA-Tet1-a+b (Fig. 3 *F* and *G*). As expected, leukemic BM cells from MLL-AF9/Tet1$^{-/-}$ (i.e., Tet1-KO_MA9) mice exhibited a significant decrease of 5hmC than those from MLL-AF9/Tet1-wild-type (i.e., Tet1-WT_MA9) mice (Fig. S5). Again, forced expression of *HOXA9* could partially reverse the delay of leukemogenesis caused by *Tet1* knockout (Fig. 5 *D* and *E*), further indicating that *Hoxa9* is an important target of Tet1. Taken together, the results from the Tet1$^{-/-}$ model are consistent with those that we observed from the *Tet1*-shRNA models, highlighting the important oncogenic role of *Tet1* in the pathogenesis of *MLL*-rearranged leukemia.

Discussion

In contrast to the frequent down-regulation of the *TET* genes in various types of solid tumors (3, 22–27), we found that *TET1* is significantly up-regulated in *MLL*-rearranged leukemia, in which this gene was first identified. Our data indicate that *TET1* is a direct target gene of MLL-fusion proteins, and MLL fusions bind to the promoter region of *TET1* and promote its expression directly in both human and mouse hematopoietic stem/progenitor cells, cumulating in a global increase of 5hmC. More importantly, distinct from the tumor-suppressor roles of both *TET1* and *TET2* reported in various types of cancers (7–9, 24–27), here we provide compelling evidence to show that *TET1* plays an essential oncogenic role in *MLL*-rearranged leukemia. Such a finding has two layers of significance: (*i*) it highlights the critical influence of cell/tissue context on the function of a given gene in tumorigenesis, such as *TET1*, which functions as a tumor-suppressor gene in solid tumors, but as an oncogene in leukemogenesis mediated by MLL fusions; and (*ii*) despite their similar catalytic activities in oxidization of 5mC, TET1 likely plays a distinct pathological role in leukemogenesis compared with TET2.

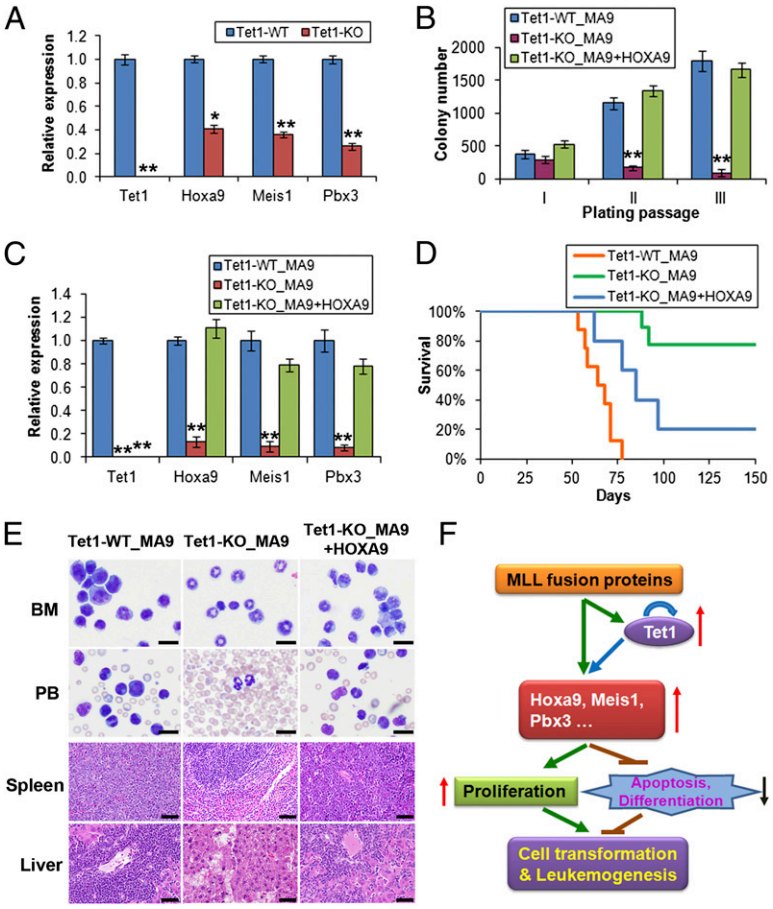

Fig. 5. Effects of *Tet1*-knockout and the signaling-pathway model. (*A*) Expression changes of three target genes in *Tet1* knockout BM progenitor (i.e., Lin^-) cells relative to wild-type controls. (*B* and *C*) Effect of *Tet1* knockout on MA9-mediated cell transformation and the corresponding expression changes of the targets. (*D*) Kaplan–Meier survival analysis of the BM transplantation recipient mice. The median survival of Tet1-WT_MA9 (MSCVneo-MA9+MSCVpuro cotransduced into wild-type mouse BM progenitor cells; $n = 8$), Tet1-KO_MA9 (MSCVneo-MA9+MSCVpuro cotransduced into $Tet1^{-/-}$ mouse BM progenitor cells; $n = 9$), and Tet1-KO_MA9+HOXA9 (MSCVneo-MA9+MSCVpuro-HOXA9 cotransduced into $Tet1^{-/-}$ mouse BM progenitor cells; $n = 5$) is 66, >150, and 85 d, respectively; Tet1-WT_MA9 vs. Tet1-KO_MA9, $P < 0.00001$; Tet1-WT_MA9 vs. Tet1-KO_MA9+HOXA9, $P = 0.018$; Tet1-KO_MA9 vs. Tet1-KO_MA9+HOXA9, $P = 0.019$; log-rank test. (*E*) Wright–Giemsa-stained BM cell cytospin and PB smear, and hematoxylin/eosin (H&E)-stained spleen and liver paraffin sections of transplantation recipient mice are shown. (Scales bars: 10 μm in BM/PB and 100 μm in spleen/liver.) (*F*) The model of the MLL-fusion/Tet1/Hoxa9/Meis1/Pbx3 signaling axis in *MLL*-rearranged leukemia. $*P < 0.05$; $**P < 0.01$, *t* test.

Remarkably, through analysis of potential direct targets of Tet1 detected from mESCs (14–16), we found that a set of critical target genes of MLL-fusion proteins such as *Hoxa9*, *Meis1*, and *Pbx3*, are also likely targeted directly by Tet1 in mESCs. Through conventional ChIP-qPCR assays, we confirmed that these three genes are genuine cotarget genes of MLL-fusion proteins and TET1 in *MLL*-rearranged leukemic cells. As expected, their expression levels are significantly down-regulated when *Tet1* expression is depleted in hematopoetic cells, particularly in those transduced with *MLL* fusions.

It is probably not a coincidence that *TET1* was discovered as a partner gene of *MLL* in a translocation in AML (17, 18). *MLL* has around 100 different partners, and the functions of some of them have been identified (30, 52). MLL itself is a large multifunctional protein (homolog of *Drosophila* trithorax) with many functions related to chromatin structure; many of MLL's partners are members of chromatin-modifying complexes including ENL, AF9, AF4, and AF10, which interact with DOT1L, and ELL, AF4/FMR2 family member 1 (AFF1), and AFF4, which interact with positive transcription elongation factor b (P-TEFb) (37, 53–56). Thus, a fusion protein consisting of MLL and one of the members of the complexes would be much more effective in promoting regulation of critical targets and enhancing cell proliferation than the two proteins produced independently in the cell and meeting at a critical location by chance. It is important, in the future, to systematically investigate how TET1 cooperates with MLL fusions in regulating their cotargets. It is possible that they have critical but transient interactions, or they belong to two distinct functional complexes that exert synergistic functions in regulating transcription of cotargets.

The effects of the aforementioned cotargets of TET1 and MLL fusions on cell viability/growth and apoptosis of *MLL*-rearranged leukemia cells have been well demonstrated (42–44, 48, 50). Thus, it is not a surprise that knockdown of *TET1* induced apoptosis and inhibited cell viability/growth of *MLL*-rearranged leukemic cells. We showed that forced expression of *HOXA9* could only partially rescue the inhibitory effects of Tet1 knockout on *MLL-AF9*-induced leukemogenesis in vivo (Fig. 5 *D* and *E*), which might be owing to the possibility that Tet1 also regulates some other important target genes that are not in the Hoxa9/Meis1/Pbx3 signaling axis.

Taken together, our data delineate an MLL-fusion/Tet1/Hoxa9/Meis1/Pbx3 signaling axis in *MLL*-rearranged leukemia (Fig. 5*F*). Briefly, MLL-fusion proteins bind directly to the *Tet1* locus and promote its expression, and the increased expression of Tet1 (and the corresponding global increase of 5hmC) cooperates with MLL fusions in orchestrating the transcriptional activation of their cotargets, particularly the Hoxa9/Meis1/Pbx3 signaling cascade, which in turn promotes cell proliferation and inhibits apoptosis/cell differentiation, thereby leading to cell transformation and leukemogenesis (Fig. 5*F*). Interestingly, because the knockout of Tet1 expression shows only very minor effects on normal development, including hematopoiesis (6), and given its indispensable oncogenic function in *MLL*-rearranged leukemia, our data also highlight TET1 as a potential target for future therapeutic intervention of this presently therapy-resistant cancer.

Materials and Methods

Microarray Profiling of 109 Human Samples. The samples were analyzed by use of Affymetrix GeneChip Human Exon 1.0 ST arrays (Affymetirx). The data have been deposited in the Gene expression Ominibus (GEO) repository with the accession numbers GSE34184 and GSE30285.

Chromatin Immunoprecipitation and in Vitro or in Vivo Functional Studies. Those were performed as described previously (41, 47, 48, 50) with some modifications. See Table S3 for primer sequences for the chromatin immunoprecipitation (ChIP) assay.

5hmC Labeling Reaction, Dot-Blot, and LC-MS/MS Assays. These assays were conducted as described previously (57–59) with some modifications.

Supplemental Information. A detailed description of all of the materials and methods used appears in *SI Materials and Methods*.

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