Tet1 is not required for myeloid leukemogenesis by MLL-ENL in novel mouse models

Ryoichi Ono1,*, Masahiro Masuya2, Naokazu Inoue3, Makoto Shinmei1, Yuri Maegawa1,2, Bishnu Devi Maharjan1, Naoyuki Katayama2, Tetsuya Nosaka1*

1 Department of Microbiology and Molecular Genetics, Mie University Graduate School of Medicine, Tsu, Mie, Japan, 2 Department of Hematology and Oncology, Mie University Graduate School of Medicine, Tsu, Mie, Japan, 3 Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

* Current address: Department of Cell Science, Institute of Biomedical Sciences, School of Medicine, Fukushima Medical University, Fukushima, Japan

*onor-ty@umin.net (RO); nosaka@doc.medic.mie-u.ac.jp (TN)

Abstract

The Ten Eleven Translocation 1 (TET1) gene encodes an epigenetic modifying molecule that is involved in demethylation of 5-methylcytosine. In hematological malignancies, loss-of-function mutations of TET2, which is one of the TET family genes including TET1, are frequently found, while the mutations of TET1 are not. However, clinical studies have revealed that TET1 is highly expressed in some cases of the hematological malignancies including acute myeloid leukemia. Indeed, studies by mouse models using conventional Tet1 knockout mice demonstrated that Tet1 is involved in myeloid leukemogenesis by Mixed Lineage Leukemia (MLL) fusion gene or TET2 mutant. Meanwhile, the other study showed that Tet1 is highly expressed in hematopoietic stem cells (HSCs), and that deletion of Tet1 in HSCs enhances potential self-renewal capacity, which is potentially associated with myeloid leukemogenesis. To examine the role of Tet1 in myeloid leukemogenesis more precisely, we generated novel conditional Tet1-knockout mice, which were used to generate the compound mutant mice by crossing with the inducible MLL-ENL transgenic mice that we developed previously. The leukemic immortalization in vitro was not critically affected by conditional ablation of Tet1 in HSCs with the induced expression of MLL-ENL or in hematopoietic progenitor cells retrovirally transduced with MLL-ENL. In addition, the leukemic phenotypes caused by the induced expression of MLL-ENL in vivo was not also critically affected in the compound mutant mouse model by conditional ablation of Tet1, although we found that the expression of Evi1, which is one of critical target genes of MLL fusion gene, in tumor cells was remarkably low under Tet1-ablated condition. These results revealed that Tet1 was dispensable for the myeloid leukemogenesis by MLL-ENL, suggesting that the therapeutic application of Tet1 inhibition may need careful assessment.
Introduction

Emerging evidence has demonstrated critical roles of epigenetic modifying molecules in oncogenesis [1]. Several findings over these decades suggest that cancer stemness is associated with aberrant epigenetic pathways caused by oncogenic driver mutations, including mutation of epigenetic modifier genes [2,3], similarly to the finding that stemness of embryonic stem cells (ESCs) is closely associated with epigenetic pathways [4].

The Ten Eleven Translocation 1 (TET1) gene, which was originally identified as a fusion partner of Mixed Lineage Leukemia (MLL) in acute myeloid leukemia (AML) [5,6], encodes an epigenetic modifying molecule [7]. Tet1 has a catalytic dioxygenase domain consisting of a cysteine (Cys)-rich region and a double-stranded β-helix (DSBH) at the C-terminal part [8]. The dioxygenase is capable of converting 5-methylcytosine (5-mC) to 5-hydroxymethyl-C (5-hmC) and catalyzing further stepwise oxidation of the derivatives [9]. Tet1 belongs to the Tet family, including Tet2 and Tet3, which is characteristic of conserved Cys-rich and DSBH motifs [8,9].

Previous studies revealed that loss-of-function mutations of TET2 are frequently found in hematological malignancies, while the mutations of TET1 are not [10]. Tet2 deficiency in mice recapitulated myeloproliferative disease (MPD) and lymphoma in humans [11]. In contrast, Tet1 deficiency did not lead to MPD, but developed lymphoma with a long latency [12,13]. Meanwhile, the conventional knockout/knockdown of Tet1 suppressed leukemic transformation by MLL-AF9 through the downregulation of critical downstream molecules [14]. In addition, Tet1 deficiency suppressed a Tet2-loss-driven myeloid malignancy, but promoted the lymphoid malignancy [15]. In line with the results, high TET1 expression is found to be associated with a poor prognosis in cytogenetically normal AML [16]. These results suggested that TET1 is a tumor-suppressor gene in lymphoid malignancies, but an oncogene in myeloid malignancies.

Several studies have demonstrated that, at least in part, normal hematopoietic stem cells (HSCs) share molecular mechanism with leukemic stem/initiating cells and ESCs [17]. Interestingly, Tet1 is relatively highly expressed in HSCs and ESCs in comparison with their progenitor cells [7,13], suggesting the possible essential role of Tet1 in these stem cells. Also, a report suggested an important role of Tet1 in reprogramming [18]. However, Tet1 deficiency in ESCs did not abrogate pluripotency [12], and on the contrary, the deletion in HSCs enhances potential self-renewal capacity [13], reminiscent of the deletion of Tet2. HSCs are considered to be one of major target cells of driver mutation(s) that initiate AML [19]. Thus, a concept of Tet1 as an oncogene in myeloid malignancies might be contradictory in the leukemogenesis arising in HSCs, but this hypothesis remains to be investigated.

To clarify the role of Tet1 in myeloid leukemogenesis more closely, we have established a new mouse model with conditional ablation of Tet1 and induction of MLL-ENL in bone marrow (BM) cells. Using this model, we demonstrate that leukemic transformation by MLL-ENL in vivo and in vitro was not critically affected by the Tet1-ablation, although the expression of Evi1, which is one of critical target genes of MLL fusion gene, in tumor cells was remarkably low under Tet1-ablated condition. Also, leukemic immortalization by retroviral transduction of MLL-ENL in vitro was not inhibited by the conditional Tet1-ablation. Our results suggest that the therapeutic application of Tet1 inhibition in AML may need careful assessment.

Materials and methods

Reagents

4-hydroxytamoxifen (4-OHT) was dissolved in ethanol, and used at a final concentration of 0.1 μM. Tamoxifen (Sigma-Aldrich, St. Louis, MO) was dissolved in ethanol (100 mg/ml), and
diluted in corn oil to 10 mg/ml. Starting at 8–12 weeks of age, or 5 weeks after bone marrow transplantation (BMT), tamoxifen was injected intraperitoneally at a dose of 100 mg/kg for 3 consecutive days. The time course started with the date of the third treatment as day 0. For drug selection in retroviral transduction, G418 (Thermo Fisher Scientific Inc., Waltham, MA) was used at a final concentration of 1 mg/ml.

**Mice**

C57BL/6N (B6) mice harboring a floxed allele (Tet1^fl/+^) where loxP sites flank exons 8 and 9 of *Tet1* were produced with standard techniques of gene targeting of B6 ES cells and flippase (Flp)-mediated recombination, as described, with some modifications [20]. Briefly, to generate Tet1^fl/+^ mice, a targeting vector to introduce the 5' and 3' loxP sites, upstream of exon 8 and downstream of exon 9, respectively, was constructed on the pNT1.1 backbone vector (kindly provided by Dr. M. Okabe, Fig 1A). A 1.7-kb genomic fragment upstream of exon 8, a 1.1-kb genomic fragment covering exon 8, intron 8, and exon 9, and a 6.1-kb genomic fragment downstream of exon 9 were generated by polymerase chain reaction (PCR) of DNA from RP24-174M19 BAC clone (BACPAC Resources Center, Oakland, CA) using Phusion High-Fidelity DNA Polymerase (NEB, Ipswich, MA) according to the manufacturer’s protocol. These three fragments were inserted upstream of the proximal loxP site ligated with the neo cassette flanked by Flp recognition target (FRT) sites, between the neo cassette and the distal loxP, and between the distal loxP and the thymidine kinase (tk) cassette, respectively, in the pNT1.1. The primer sequences were as follows: 5'-GAAACTGCAGCCTATCATGCTGTGCTGACCCTTTTGGGCT-3' and 5'-GAAAGACTCGAGGATTAAAGGCGTGCCAC-3', 5'-GAAAGAATTCCTAGCACACGGAAAGCCAGA-3' and 5'-GTGTTGTACCGGTACAGAAGAAAGATTTGATCATACATGCCTTAGTGTG-3', and 5'-CACGTCAGAGATGGTGTTGCTGACAAACATGGTCACCTTGTC-3' and 5'-GAAACATCTAGAGAGCCCTTTTAGACCCACAGTTG7AGGGTTCCCAGCAAC-3', respectively (underlined nucleotides are added for subcloning). Each subcloned plasmid was validated by sequencing.

The targeting vector was linearized by Not I digestion just upstream of the insertion site of the 1.7-kb fragment, and electroporated into B6 EGR-101 male ES cells, followed by positive and negative selection with G418 and ganciclovir, respectively, as previously described [20]. To detect the correct homologous recombination, PCR screening of genomic DNA from the engineered ES cells was performed using LA Taq (Takara Bio Inc., Otsu, Japan) according to the manufacturer’s protocol. The primer sequences were as follows: 5'-GGCCTATCATGCTGTGCTGACCCTTTTGGGCT-3' and 5'-GCTCCCGATTCGCAGCGCATCGCCTTCTAT-3' for the first screening, and 5'-CTGCTAAAGCGCATGCTCCAGACTGCCTTGGGAAAAG-3' (first PCR) or 5'-GAAAGCCTCCTCCCCCTAAGCGGTTGAATTTGACCT-3' (second PCR), and 5'-CCCCTACAACCATATTAGCAAAAGGTTATACATGAGAGGAGGCTCTAT-3' for the second screening. To validate the screening, the karyotypes of PCR-positive clones were analyzed, and Southern blot analysis of genomic DNA from the clones with the correct karyotype was performed.

Chimeric male mice (F0), which were generated by the blastocyst injection of properly targeted ES cell clones, were bred with B6 wild-type female mice. F1 mice that were heterozygous for the targeted allele on a pure B6 background were bred with Flpe deleter mice (kindly provided by Dr. A. F. Stewart) to remove the FRT-flanked neo cassette. F2 mice that were heterozygous for the modified allele harboring the proximal loxP-FRT and the distal loxP sequences, as well as Flpe, were bred with B6 wild-type mice, to produce Tet1^fl/+^ mice that were heterozygous for the floxed allele of Tet1 alone, and Tet1^fl/fl^ mice were generated by intercrossing of the Tet1^fl/+^ mice.

C57BL/6-Gt(Rosa)26Sortm9(Cre/ESR1)Arte (CreER) mice were purchased from Taconic Biosciences (Rensselaer, NY), and inducible MLL-ENL transgenic, Tg-ME (ME), mice harboring...
**Fig 1. Generation of Tet1^fl/fl^ mice.** (A) Schematic illustration of the targeting vector and the wild-type (wt) and genetically engineered Tet1 alleles. The targeting vector was constructed by inserting 3 fragments (indicated by horizontal bold lines) corresponding to the wt regions (as shown), before the proximal loxP, between both loxP, and after distal loxP sequences, into the backbone vector. To generate the floxed (fl) allele, the neomycin resistance gene (neo) cassette flanked by the flippase (Flp) recognition target (FRT) sequences in the targeted allele was removed by mating with Flpe mice. The floxed allele can be recombinated between loxP sequences by Cre activity, resulting in the deleted (Δ) allele lacking exons 8 and 9. The restriction sites of BamHI (B), and the 5′ and 3′ probes, used for Southern blot analysis, are indicated with vertical dashed lines and short horizontal lines, respectively. Coding and non-coding exons are indicated by closed and open boxes, respectively. The exons coding a cysteine-rich (C) and a double-stranded β-helix (D) regions are shown. tk, thymidine kinase gene. (B) Southern blot analysis of ES clones. Each genomic DNA extracted from 6 candidate ES clones and wt ES cells was digested with BamHI and hybridized with the 5′ probe, resulting in the wt 15-kb and targeted 6.4-kb bands. The blot image was cropped from S1 Raw images.

https://doi.org/10.1371/journal.pone.0248425.g001
the cytomegalovirus early enhancer/chicken β actin (CAG) promoter-driven loxP-enhanced green fluorescent protein (EGFP)-poly A-loxP-MLL-ENL-poly A-loxP cassette were described [21].

The genotyping of mice or cells harboring a floxed/recombinated allele of Tet1 and a CreER allele was performed by PCR of genomic DNA using LA Taq with the following individual 3 primer sets: dFRT-S2 (5’-GGTGGTGTTGGTAAGTTGAGGC-3’), L-FS2 (5’-GGTGGTGTTGGTAAGTTGAGGC-3’), and L-dP-AS2 (5’-GGTGGTGTTGGTAAGTTGAGGC-3’) for Tet1, and R26-S (5’-GGTGGTGTTGGTAAGTTGAGGC-3’), R26-AS1 (5’-GGTGGTGTTGGTAAGTTGAGGC-3’), and Cre-1AS (5’-GGTGGTGTTGGTAAGTTGAGGC-3’) for CreER. Genotyping of each offspring of Tg-ME mice expressing EGFP were performed by a fluorescence activated cell sorting (FACS) analysis of peripheral blood (PB) samples, and the recombinated allele was detected by PCR as previously described [21].

All animal studies were approved by the Animal Care Committees of Mie University (No. 24–34). Mice were kept in the specific pathogen-free facility under standard conditions (temperature 22–24°C, 12-h light/12-h dark cycle, and free access to food and water). In BMT experiments, recipient mice were housed in sterilized cages with filter-cap, and were administered neomycin (1 mg/ml (Merck Millipore, Burlington, MA)) in drinking water, for 4 weeks after BMT. Both male and female mice were subjected to experiments, except for that female mice were used for recipients in BMT. Animal health was monitored carefully and regularly in all experiments by trained staff. Hematological analyses of conditionally Tet1-ablated mice were performed, compared with the corresponding littermate controls, as previously described [22]. The mice were euthanized by carbon dioxide (CO₂) inhalation, to analyze or collect BM cells. The total number of mice used in this study was 108.

**Purification of mouse hematopoietic stem and/or progenitor cells**

Mouse c-Kit(+), c-Kit<sup>hi</sup>Sca-1<sup>hi</sup>Lineage<sup>–</sup> (KSL), CD34(-/+) KSL, c-Kit<sup>hi</sup>Sca-1<sup>–</sup>Lineage<sup>–</sup> (hereafter designated as MP) cells were purified from BM cells using FACS Aria (BD Biosciences, Franklin Lakes, NJ) as described, with some modifications [21]. In brief, BM mononuclear cells (BMMNCs) were prepared from 10- to 15-week-old B6 mice (n = 1–2 per each experiment). In myeloid immortalization assays and BMT assays, non-littermate mice with the indicated genotypes were used, since it was difficult to obtain a pair of littermates with four types of genotypes (described later). Using a MACS cell separation system (Miltenyi Biotec, Auburn, CA), c-Kit(+)-BM cells were enriched from BMMNCs labeled with biotinylated anti-c-Kit antibody (2B8), or lineage-depleted cells were isolated from BMMNCs labeled with Lineage Cell Detection Cocktail-Biotin (Miltenyi Biotec). The lineage-depleted cells were stained with streptavidin (SAV)-peridinin chlorophyll protein (PerCP)-Cy5.5, and Alexa Fluor 647-conjugated anti-CD34 (RAM34), phycoerythrin (PE)-conjugated anti-c-Kit (2B8), and PE-Cy7-conjugated anti-Sca-1 (D7) monoclonal antibodies. KSL, CD34(-/+) KSL, and MP cells were purified from the stained cells using a FACS Aria II (BD Biosciences). All monoclonal antibodies were purchased from Biolegend (San Diego, CA), with the exception of the anti-CD34 antibody (Thermo Fisher Scientific Inc.).

**Retroviral transduction**

The pMYs retroviral vector harboring MLL-ENL-internal ribosomal entry site (IRES)-neo was previously described [21]. Retroviral transduction was performed using RetroNectin (Takara Bio Inc.) with retroviral supernatants, which were harvested 48 hours after transfection from Plat E cells transfected with a retroviral construct, as previously described [23,24].
Myeloid immortalization assay

Myeloid immortalization assays using serial replating were performed as described [21]. Briefly, the purified CD34(-) or CD34(+) KSL (150) and MP (1500) cells were directly sorted into 1.5 ml of methylcellulose medium prepared from M3234 (StemCell Technologies, Vancouver, Canada) according to the manufacturer’s protocol, supplemented with 25 ng/ml SCF, 10 ng/ml each of IL-6, mouse IL-3, mouse GM-CSF (Miltenyi Biotec), and 0.1 μM of 4-OHT. The sorted cells in 1 ml of the mixture were immediately plated in 35 mm dishes. Alternatively, retrovirally transduced KSL, CD34(+) KSL, and MP cells were placed in the same M3234-based medium supplemented with G418 in addition to the cytokines. After culturing for 5 (for MP cells), 6 (for CD34(+) KSL cells), and 10 days (for retrovirally transduced cells and CD34(-) KSL cells), colonies were enumerated, and single-cell suspensions (0.3–1x10⁴ cells) of colonies were subsequently replated in α-MEM-based medium containing 1% methylcellulose (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) supplemented with only the same cytokines. Every 5–7 days, replating of cells collected from colonies was repeated in the same way using the α-MEM-based methylcellulose medium. The immortalized cells were harvested from colonies in the third plating, and were cultured in α-MEM supplemented with 20% FBS and the same cytokines.

BMT and leukemogenesis assay

Hematopoietic reconstitution of BM and leukemogenesis assays using BMT using B6 mice aged 10–15 weeks were performed as previously described [21], with some modifications. Briefly, for BM chimera mice, 1x10⁶ BM cells derived from the tested genotype of mice that were positive for CD45.2 were transplanted into lethally irradiated (7.5 Gy) wild-type recipient B6 mice that were positive for CD45.1. The recipient mice were periodically monitored, and analyzed morphologically and immunophenotypically at the indicated intervals as previously described [24].

For the mixed-BM chimera mice for use in leukemogenesis assays, a mixture of 1x10⁶ BM cells each derived from wild-type CD45.1-positive mice and the tested genotype of CD45.2-positive mice were also transplanted into wild-type CD45.1- (BMT #1 (#1)), or CD45.1/CD45.2- (BMT #2 (#2)) positive recipient female B6 mice that were lethally irradiated in the same way. Mixed-BM chimera mice were treated with tamoxifen after engraftment. For secondary transplantation, 3x10⁴ MPD cells harvested from BM were transplanted into sublethally irradiated (5.25 Gy) recipient B6 mice. To assess the mixed-BM chimera mice hematologically, PB samples collected from tail vein were measured 5 weeks after BMT to confirm the engraftment of transplanted cells, and also every 4 weeks after the treatment of tamoxifen. Mouse health was monitored for signs of illness such as less activity, scruffiness, weight loss, and hind-limb paralysis every day. The mice showing these signs were considered moribund, and were immediately euthanized by CO₂ inhalation with all efforts to minimize animal suffering (n = 11 (#1), 13 (#2), and 7 (secondary BMT)), however, some mice unexpectedly died without showing suspicious signs of illness (n = 10 (#1), 6 (#2), and 6 (secondary BMT)). The control mice in #1 were euthanized in the same way at the end of monitoring (n = 9). The monitoring was ended after all the mice transplanted with Tg-ME cells met the criteria of humane endpoints described above. The analyses of BM and spleens from the tested mice were performed as previously described [24], showing the development of lethal MPD in the mice transplanted with Tg-ME cells.

Immunophenotyping by FACS analysis

Immunophenotypic analyses were performed using a FACS Calibur or a FACS Canto II (BD Biosciences) as previously described [21]. Briefly, red blood cells in PB, BM, and spleen
samples were lysed with Ammonium-Chloride-Potassium lysis buffer beforehand. For the assessment of conditional Tet1-knockout mice, PB cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD11b (M1/70), PE-conjugated anti-CD3ε (145-2C11), PerCP-Cy5.5-conjugated anti-Gr-1 (RB6-8C5), and APC-conjugated anti-B220 (RA3-6B2) monoclonal antibodies. BM cells were labeled with Lineage Cell Detection Cocktail-Biotin, followed by staining with SAV-PerCP-Cy5.5, FITC-conjugated anti-CD34 (RAM34 (BD Biosciences)), PE-conjugated Sca-1 (D7), and APC-conjugated anti-c-Kit antibodies (2B8; for CD34+/− KSL), or SAV-FITC, PE-conjugated anti-IL-7 receptor α (A7R34), PE-Cy7-conjugated anti-Sca-1, and APC-conjugated anti-c-Kit antibodies (for CLP). For MEP, GMP, and CMP, BM cells were labeled with Lineage Cell Detection Cocktail-Biotin and biotinylated anti-Sca-1 antibody (E13-161.7), followed by staining with SAV-PerCP-Cy5.5, FITC-conjugated anti-CD34, PE-conjugated anti-CD16/32 (93), and APC-conjugated anti-c-Kit antibodies. For the assessment of colony-forming cells, the cells were stained with FITC-conjugated anti-CD45.2 (104) and PE-conjugated anti-c-Kit antibodies. For the assessment of cells from BMT recipient mice, chimerism in PB and BM cells was analyzed by staining with FITC-conjugated anti-CD45.2 (104) and PerCP-Cy5.5-conjugated anti-CD45.1 (A20) antibodies. The BM cells were stained with FITC-conjugated anti-CD11b, PerCP-Cy5.5-conjugated anti-Gr-1, and APC-conjugated anti-B220 antibodies, or PE-conjugated Sca-1, PerCP-Cy5.5-conjugated anti-Gr-1, and APC-conjugated anti-c-Kit antibodies. With the exception of the anti-CD34 antibodies, all monoclonal antibodies were purchased from BioLegend. Data were analyzed with FlowJo version 7.2.5 (BD Biosciences).

Dot blot assay
Dot blot assay was performed as previously described [25], with some modifications. Briefly, Genomic DNA was manually spotted onto Hybond-N+ membranes (Cytiva, Marlborough, MA). After ultraviolet crosslinking, the membranes were probed with anti-5-hmC rabbit polyclonal (Active Motif, Carlsbad, CA), and anti-5-mC mouse monoclonal (33D3, Active Motif) antibodies, followed by probe with horseradish peroxidase (HRP)-conjugated anti-rabbit/anti-mouse immunoglobulin G (IgG) polyclonal antibodies (MBL, Nagoya, Japan), respectively. Detection was performed using Western Blotting Luminol Reagent (SANTA CRUZ BIOTECHNOLOGY, INC., Dallas, TX). The dot blot intensity was quantified by ImageJ software (National Institute of Health, version 1.53e, http://rsb.info.nih.gov/ij/download.html).

Southern blot analysis
BamHI I-digested genomic DNA was analyzed as previously described [20], with some modifications. Briefly, spotted membranes were probed with DIG-labeled DNA probes, and visualized with CDP-star, using DIG-High Prime DNA Labeling and Detection Starter Kit I (Sigma-Aldrich), according to the manufacturer’s protocol. The template DNA for 5’ probe or 3’ probe was generated by PCR of genomic DNA from wild-type ES cells with LA Taq using the following primers: 5'-GTCTGGCTCCTGATGTATAAAGCTTG-3' and 5'-GTTCCTGCTACTCAGGCTAGT-3', and 5'-GTCTTCTGAAGTCACCTCGTCAATTAACT-3' and 5'-GGCAGAACACTGGAAACAAAGTAATC-3'.

RNA extraction and reverse transcription (RT)
Total RNA was extracted from cells using TRI Reagent LS (Molecular Research Center Inc., Cincinnati, OH), followed by treatment with RQ1 RNase-free DNase I (Promega, Madison,
Tet1 is not required for myeloid leukemogenesis by MLL-ENL
RT was performed using SuperScript II (Thermo Fisher Scientific Inc.) with random hexamers, as previously described [24].

Northern blot analysis

Poly-A+ RNA (0.5 μg) purified from total RNA using an Oligotex™-dT30 mRNA Purification Kit (Takara Bio Inc.) was analyzed as previously described [5], with some modifications. Briefly, blotted membranes were probed with DIG-labeled RNA probes synthesized using T3 (for Actb) or T7 (for Tet1) RNA polymerase and DIG RNA Labeling Mix (Sigma-Aldrich) supplemented with RNAse inhibitor (recombinant RNasin (Promega)), in DIG Easy Hyb solution (Sigma-Aldrich), according to the manufacturer’s protocol. Detection was performed using CDP-star in the same way as done in Southern blot analysis. The corresponding sequences of probes were as follows: Actb, 112–909; Tet1 exons 8–9, 4518–4764; and Tet1 exons 10–13, 4883–6120.

PCR

To analyze the exon structure of Tet1 transcripts around the region flanked by lox P sequences by RT-PCR, PCR of cDNA was performed using LA Taq with primers spanning exons 7–11 (5’-TTGAGGTCCTACAGCGGACAT-3’ and 5’-ACGCCCCTCTTCATTTCCA-3’), as previously described [24]. RT-quantitative PCR (qPCR) was performed using PowerSYBR® Green PCR Master Mix on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA), as described [21]. After quantifying the expression levels of the samples using the 2^ΔΔCT method and normalization relative to B2m, the relative expression levels were calculated. The sequences of the primers used in qPCR, with the exception of those for B2m, MLL-ENL, Hoxa9, Meis1a, Evi1, and Dnm3b [21], were as follows: 5’-CATCCCAACAGCGGAGATGTC-3’ and 5’-TTTGGGTCGATGCGCTTGAC-3’ for Tet1, 5’-TTGGGCAAGTGTGGCATATG-3’ and 5’-TCTCACCCTGACAACAACAGTTTC-3’ for Tet2, 5’-ACAGCTGCTGAGTTTCTTCT-3’ and 5’-ACGACCGCATTGACTCTCTTCT-3’ for Tet3, 5’-AGAGATGCCATCACCCAAAAA-3’ and 5’-AAGCCTCGATGCTCACCTTCTGATAGTAAG-3’ for Dnm1, and 5’-GGCATCCACTGTGAATGATAAGGC-3’ and 5’-GTGGTAATGGTCCTCACTTTG-3’ for Dnm3a.

Statistical analysis

Unpaired Student’s t-tests, and a one-way analysis of variance (ANOVA) followed by Tukey-Kramer test as a post hoc test, were used to compare two groups, and more than two groups, respectively. A two-way repeated measures ANOVA followed by Shaffer’s modified sequentially rejective Bonferroni procedure as post hoc analyses was used to compare several groups at different timepoints. Data from RT-qPCR were log2-transformed before the statistical analysis. To compare the gene expression from CD34(-) KSL-derived cells, after a one-way ANOVA was used to compare four genotypes of samples at the first round of plating and a
Fig 3. Hematological assessment of conditionally Tet1-ablated mice. (A) Measurement of the white blood cell (WBC) counts and hemoglobin (Hb) concentration in peripheral blood just before (0), 4, 8, and 12 weeks after tamoxifen treatment as shown in Fig 2A (n = 5 per each (total 10)). (B, C, and D) A FACS analysis of hematopoietic stem and progenitor cells (B and C), the expression levels of Tet1, Tet2, and Tet3 in bone marrow (BM) 12 weeks after tamoxifen treatment, determined by RT-qPCR (D). Representative FACS data
two-way ANOVA was used to compare two pairs of two genotypes samples at the first and third rounds of plating were performed, the obtained p values were adjusted using the Holm-Bonferroni correction. The probability of overall survival of the mice was estimated using the Kaplan-Meier method and compared by the log-rank test. All statistical tests were performed using the R software program (version 3.5.2, https://www.r-project.org/), except that two-way repeated measures ANOVA tests were performed using the ANOVAKUN software (version 4.8.4, http://riseki.php.xdomain.jp/index.php?ANOVA%E5%90%9B), which was executed on the R software program.

Results

Generation of conditional Tet1-knockout mice

To conditionally ablate Tet1 in vivo, we designed a strategy to induce Cre/loxP-mediated excision of the exons 8 and 9 of Tet1, which led to a truncated product lacking the catalytic domain by a frameshift mutation (Fig 1A). The targeting vector was constructed, and the targeted ES clones (Fig 1B) were used to generate chimeric founder mice, from which two lines (57 and 71) of F1 mice were obtained. After removing the neomycin' cassette by Flpe deleter mice, Tet1

Validation of Tet1-ablation in hematopoiesis

First, to validate the genetic effect focused on hematopoiesis in C/T1

of 4 independent experiments (with 1 pair per each (total 8)) are shown in (B). Bar graphs show the mean and SD of data combined from two (A) and four independent experiments (C and D), respectively. **p<0.005; n.s., not significant. (determined by two-way repeated measures ANOVA followed by Shaffer’s modified sequentially rejective Bonferroni procedure (A), and two-tailed unpaired t-tests (D), respectively).

https://doi.org/10.1371/journal.pone.0248425.g003

Tet1 is not required for myeloid leukemogenesis by MLL-ENL

https://doi.org/10.1371/journal.pone.0248425.t003

Hematological assessment of conditionally Tet1-ablated mice

We next assessed the biological effect of conditional Tet1-ablation on hematopoiesis. At the timepoints of 0 (just before the treatment), 4, 8, and 12 weeks after tamoxifen treatment, PB and BM (only 12 weeks) samples were collected from C/T1

https://doi.org/10.1371/journal.pone.0248425.g004

of 4 independen t experime nts (with 1 pair per each (total 8)) are shown in (B). Bar graphs show the mean and SD of data combined from two (A) and four independen t experime nts (C and D), respectively. **p<0.005; n.s., not significant. (determined by two-way repeated measures ANOVA followed by Shaffer’s modified sequentially rejective Bonferroni procedure (A), and two-tailed unpaired t-tests (D), respectively).
Fig 4. The conditional ablation of Tet1 in cells retrovirally immortalized by MLL-ENL. (A) The experimental strategy for myeloid immortalization assays of the retrovirally transduced cells using colony replating, and conditional ablation of Tet1 in the immortalized CreER;Tet1<sup>fl/fl</sup> cells. Wild-type (Wt) KSL and MP cells (from one mouse per each (total 3)) were retrovirally transduced with MLL-ENL or an empty vector, respectively. CreER;Tet1<sup>fl/fl</sup> CD34(+) KSL and MP cells were immortalized with the retroviral transduction of MLL-ENL after serial replating, and treated with 4-hydroxytamoxifen (4-OHT). (B) The expression level of Tet1 in the wt MLL-ENL-transduced KSL and MP cells (from 2 mice) at the end of the first round of colony plating, as assessed by RT-qPCR. (C and D) The expression levels of Tet1, Tet2, Tet3, MLL-ENL, Hoxa9, Meis1, and Evi1, assessed by RT-qPCR (C) and clonogenicities (D) in the CreER;Tet1<sup>fl/fl</sup> CD34(+) KSL/MP-derived cells retrovirally immortalized by MLL-ENL with treatment of 4-OHT or vehicle control (ethanol, EtOH). CFU, colony forming unit. Bar graphs show the mean and SD of three independent experiments in (B), (C), and (D). *p<0.05; **p<0.005; n.s., not significant. (determined by two-tailed unpaired t-tests).
Tet1 is not required for myeloid leukemogenesis by MLL-ENL
Fig 5. Leukemic immortalization by the induction of Tg-ME-derived MLL-ENL under simultaneous Tet1-ablation. (A) The experimental strategy for the myeloid immortalization assays of cells purified by sorting. CD34(-), CD34(+), and MP cells were purified from Tet1+/fl, CreER; Tet1+/fl; CreER; Tet1+/fl; Tg-ME, and CreER; Tet1+/fl; Tg-ME bone marrow cells (from one mouse per each genotype per each experiment (total 12 mice)), and were directly plated in the presence of 4-hidroxyl tamoxifen (4-OHT). (B) Genomic PCR of Lin-depleted (Lin(-)) cells prepared for sorting (pre-sorting), and the cells harvested at the end of the first round (1R) of plating. Bands derived from wild-type (wt), floxed (fl), and deleted (Δ) alleles regarding Tg-ME (right panel) are shown. (C) Expression levels of Tet1 and MLL-ENL in the cells harvested at the end of the first (1R) and the third (3R, only in CD34(-) KSL-derived cells) rounds of plating, assessed by RT-qPCR. A red circle indicates recombinated (R) alleles regarding Tg-ME (right panel) are shown. (D) Colony numbers along serial replating in the myeloid immortalization assays. Red circles indicate colony forming units (CFUs) in the CD34(+) KSL sample with an exceptionally high two-tailed unpaired two-way repeated measures ANOVA followed by Shaffer’s modified sequentially rejective Bonferroni procedure (CD34(-) KSL), and the end of the first (1R) and the third (3R, only in CD34(-) KSL-derived cells) rounds of plating, assessed by RT-qPCR. A red circle indicates recombinated (R) alleles regarding Tg-ME (right panel) are shown. (E) The typical morphology of the cells constituting the colonies derived from CreER; Tet1+/fl; Tg-ME and CreER; Tet1+/fl; Tg-ME and CreER; Tet1+/fl; Tg-ME and CreER; Tet1+/fl; Tg-ME CD34(-) KSL cells at the end of the third round of plating. Cells were stained with Wright-Giemsa. Magnification, 200×; scale bars, 50 μm. Bar graphs show the mean and SD of three independent experiments in (C) and (D). **p<0.0005; *p<0.005; †p<0.05; n.s., not significant; †, not significant in comparison of the other combinations. With the exception of MLL-ENL, the p-values for the expression levels of the genes tested were determined by one-way ANOVA followed by Tukey-Kramer tests (CD34(+)) and MP), or a combination of one-way ANOVA followed by Tukey-Kramer tests (1R) and two-way repeated measures ANOVA followed by Shaffer’s modified sequentially rejective Bonferroni procedure (CreER; Tet1+/fl; ΔTet1; Tg-ME and CreER; Tet1+/fl; Tg-ME) adjusted using the Holm-Bonferroni correction (CD34(-) KSL). The gel images were cropped from S2 Raw images.

https://doi.org/10.1371/journal.pone.0248425.g005

Tet2 or Tet3 expression (Fig 3D). In addition, we generated BM chimera mice, in which the BM cells of recipient mice were replaced with C/Tet1+/fl or T1+/fl BM cells using BMT (S2A Fig), and treated the chimera mice with tamoxifen after confirmation of engraftment. In this experimental system, it was assumed that the donor-derived HSCs were subjected to proliferative stress, which was partly associated with aging, in the process of reconstitution of hematopoiesis before the conditional ablation of Tet1. However, no significant differences concerning WBCs in PB were found within an observation period of 34 weeks (S2B Fig). Furthermore, no lethal disease in the tamoxifen-treated mice receiving C/Tet1+/fl or T1+/fl BM cells. These results suggested that Tet1 was dispensable in the maintenance of proper hematopoiesis, at least during the period of 34 weeks.

A role of Tet1 in leukemogenesis by MLL-ENL in HSPCs in vitro

Based on the finding that Tet1 is upregulated by MLL-AF9 [14], we analyzed the expression levels of Tet1 in retrovirally MLL-ENL-transduced KSL and MP cells (Fig 4A), and found similar increase of Tet1 expression, which was significant only in MP cells (Fig 4B). Next, to examine whether Tet1 might be required for the maintenance of leukemic immortalization, C/Tet1+/fl CD34(-) KSL and MP cells were immortalized by MLL-ENL (Fig 4A). CD34(-) KSL cells were not analyzed in these immortalization assays, due to difficulty in retroviral transduction of MLL-ENL into CD34(-) KSL cells, as previously described [21]. The 4-OHT treatment of the immortalized C/Tet1+/fl cells led to an approximately 80% reduction in Tet1 expression (Fig 4C), but clonogenicities in the treated cells were not significantly different from those in the control cells (Fig 4D). We also found no significant changes in the expression levels of the critical genes downstream of MLL-ENL or the epigenetic regulator genes associated with DNA methylation, Tet2, Tet3, and Dnmt genes, between the treated and the control cells (Figs 4G and S3). These results suggested that Tet1 was upregulated by MLL-ENL in HSPCs, but was not required for the maintenance of MLL-ENL-mediated immortalization in CD34(+) KSL and MP cells.

A role of Tet1 in leukemogenesis by MLL-ENL in LT-HSCs in vitro

In ME mice we established [21], induction of MLL-ENL led to leukemic transformation of CD34(-) KSL cells enriched for long-term HSCs (LT-HSCs), but not to the transformation of CD34(+) KSL and MP cells. To examine the role of Tet1 in MLL-fusion-mediated...
Tet1 is not required for myeloid leukemogenesis by MLL-ENL
Fig 6. Characterization of hematopoietic stem/progenitor cells after the induction of Tg-ME-derived MLL-ENL under simultaneous Tet1-ablation. (A) The typical immunophenotype of the cells constituting the colonies derived from CreER; Tet1+/+, Tg-ME and CreER; Tet1f/+; Tg-ME CD34(-) KSL cells at the end of the third round of plating in Fig 5. (B) The expression levels of Tet2, Tet3, Dnmt3b, Hoxa9, Meis1a, and Evil in the cells harvested at the end of the first (1R) and the third (3R, only in CD34(-) KSL-derived cells) rounds of plating in Fig 5, as assessed by RT-qPCR. Bar graphs show the mean and SD of three independent experiments in (B). ***p<0.0005; **p<0.005; *p<0.05; n.s., not significant; †, not significant in comparison of the other combinations. The p-values for the expression levels of the genes tested were determined by one-way ANOVA followed by Tukey-Kramer tests (CD34(+) and MP), or a combination of one-way ANOVA followed by Tukey-Kramer tests (1R) and two-way repeated measures ANOVA followed by Shaffer’s modified sequentially rejective Bonferroni procedure (CreER; Tet1f/+; Tg-ME and CreER; Tet1f/+; Tg-ME) adjusted using the Holm-Bonferroni correction (CD34(-) KSL).

https://doi.org/10.1371/journal.pone.0248425.g006

leukemogenesis in LT-HSCs, we generated CreER;Tet1f/+;Tg-ME (C/ME) and CreER;Tet1f/+; Tg-ME (C/T1f/+)/ME) mice by mating the individual genetically engineered mice.

We first performed myeloid immortalization assays of CD34(-) KSL, CD34(+) KSL, and MP cells from T1f/+; C/T1f/+; C/T1f/+;ME, and C/ME mice, respectively (Fig 5A). In the first plating, efficient recombination between loxP sites in Tet1f and/or Tg-ME alleles was confirmed (Fig 5B), accompanied by the similar reduction of Tet1 and/or induction of MLL-ENL among the cells harboring CreER, and Tet1f/+ and/or Tg-ME (Fig 5C). Also, the four genotypes of CD34(-) KSL, CD34(+) KSL, and MP cells formed similar numbers of colonies, respectively. In the third plating, the induced MLL-ENL enabled C/T1f/+;ME and C/ME CD34(-) KSL-derived cells to form numerous highly proliferative colonies, leading to immortalization, without significant differences in the number of the colonies between the two genotypes (Fig 5D). Furthermore, both genotypes of the cells constituting the colonies exhibited similar morphological and immunophenotypic features of myeloid cells at various differentiation stages (Figs 5E and 6A). However, it should be noted that, in one of C/T1f/+;ME CD34(+) KSL samples, a relatively higher MLL-ENL expression enhanced clonogenicity temporally, but failed to immortalize after additional replating (Fig 5C and 5D).

Next, to examine whether the conditional Tet1-ablation may affect the molecular mechanism of leukemic immortalization by the induced MLL-ENL, we analyzed the critical genes downstream of MLL-ENL and the epigenetic regulator genes associated with DNA methylation, in the first and third (only for CD34(-) KSL) plating (Figs 6B and 5A). In the first plating, the expression levels of these genes tested were not significantly changed among T1f/+, C/T1f/+, C/T1f/+;ME, and C/ME-derived cells in each subpopulation, except for a small reduction of Dnmt3a in C/T1f/+, and C/T1f/+;ME-derived cells in CD34(-) KSL, respectively. In comparison of C/T1f/+;ME and C/ME CD34(-) KSL-derived cells in the third plating with those in the first plating, the Tet1 expression was non-significantly enhanced in the third plating of C/ME-derived cells, while the Tet1 expression did not increase with escape from the conditional ablation along serial plating in C/T1f/+;ME-derived cells. The MLL-ENL expression in the third plating was significantly enhanced in C/T1f/+;ME-derived cells, but was still almost comparable to that in C/ME-derived cells (Fig 5C). The expression levels of Hoxa9, Meis1, and Evil in the third plating were significantly elevated in both genotypes, but were not significantly different between the two genotypes. Among the epigenetic genes tested, expression of Dnmt3b and the Dnmt1 was significantly more strongly enhanced in the C/T1f/+;ME- and C/ME-derived cells, respectively, in comparison with each counterpart cells. Regarding Dnmt3b, our results were compatible with the finding in ES cells [26].

Taken together, these results indicated that the Tet1-ablation did not affect the leukemic immortalization by induced MLL-ENL in CD34(-) KSL, CD34(+) KSL, or MP cells.

The phenotype of MPD by the induction of MLL-ENL was not affected by conditional ablation of Tet1 in vivo

To examine whether the conditional Tet1-ablation may affect leukemogenesis by induced MLL-ENL in vitro, C/T1f/+;ME and C/ME mice were preliminarily treated with tamoxifen,
Fig 7. The leukemogenesis by induction of Tg-ME-derived MLL-ENL under simultaneous Tet1 ablation in vivo. (A) The experimental strategy for leukemogenesis assays enabling leukemic initiation in vivo. The two types (BMT #1 and #2) of the mixed-bone marrow (BM) chimera mice (from one mouse per each genotype per each experiment (total 8 (#1) and 4 (#2)) were prepared using BMT into CD45.1-, and CD45.1/45.2-positive recipient mice, respectively. Wt, wild-type; Tm, tamoxifen; ip, intraperitoneal injection; MPD, myeloproliferative disease. (B) Genomic PCR of PB cells from the mixed-BM chimera mice receiving CreER; Tet1fl/fl; Tg-ME (Tet1fl/fl) and CreER; Tet1+/+; Tg-ME (Tet1+/+) BM cells, just before (pre) and 4 weeks after (post) tamoxifen treatment. Bands derived from wild-type (wt), floxed (fl), and deleted (Δ) alleles at Tet1 locus (top panel), and those derived from germline (G) and recombinated (R) alleles regarding Tg-ME (bottom panel) are shown. (C) Chimerism analyses in peripheral blood cells just before (0) and 4 weeks after tamoxifen treatment in the cohort #2. P-values were determined by two-way repeated measures ANOVA followed by Shaffer’s modified sequentially rejective Bonferroni procedure. (D) Survival curves of the mixed-BM chimera mice (cohort #2) receiving CreER; Tet1fl/fl; Tg-ME (n = 11) and CreER; Tet1++; Tg-ME (n = 8) BM cells that were treated with tamoxifen. (E) Weight of the spleens from the moribund mice that developed lethal MPD. Data from two independent experiments were combined in (D). Bar graphs show the mean with SD of data combined from two independent experiments in (C) and (E). *** p<0.0005; n.s., not significant. (determined by two-way repeated measures ANOVA followed by Shaffer’s modified sequentially rejective
leading to lethal MPD. To avoid ubiquitous induction of MLL-ENL, we beforehand prepared two types (#1 and #2) of mixed-BM chimera mice by BMT of mixture of equal ratios of BM cells from T1<sup>fl/fl</sup> (#1), C/T1<sup>fl/fl</sup> (#1), C/T1<sup>fl/fl</sup>/ME (#1, #2), or C/ME (#1, #2) mice (CD45.2(+)), and wild-type mice (CD45.1(+)), into recipient mice (CD45.1(+) (#1) or CD45.1/CD45.2(+) ( #2)) (Fig 7A). In PB cells collected 4 weeks after the treatment of tamoxifen, efficient recombinination between loxP sites in Tet1<sup>fl</sup> and/or Tg-ME alleles was confirmed (Fig 7B), while no significant changes of chimerism in each genotype were found, except for a little increase in the chimera mice (#2) receiving C/T1<sup>fl/fl</sup>/ME cells (Figs 7C and S5A). Over a period of about 200 days of observation after the treatment, the chimera mice receiving C/T1<sup>fl/fl</sup>/ME or C/ME cells died with similar latency in each cohort (#1 and #2), while the mice (#1) receiving T1<sup>fl/fl</sup> or C/T1<sup>fl/fl</sup> cells did not (Figs 7D and S5B). Almost comparable splenomegaly was found in the chimera mice receiving C/T1<sup>fl/fl</sup>/ME or C/ME cells, in each cohort (Fig 7E). Immunophenotyping analyses of BM cells from the moribund mice revealed that the majority of the cells derived from CD45.2-positive donor cells, and expressed myeloid markers and c-Kit, and that these cells also expressed markers of immaturity, c-Kit/Sca-1, in some recipient mice (Fig 8A), similarly in each cohort. These features were also compatible with the morphological findings of BM cells showing MPD where myeloid cells at various stages of differentiation occupied BM (S5C Fig). In addition, in secondary transplantation of BM cells from moribund mice, both genotypes of MPD were aggressively reproduced with similar latencies (Fig 8B).

The Evi1 expression was markedly lower in CreER;Tet1<sup>ΔΔ</sup>;Tg-ME samples

To further examine whether the conditional Tet1-ablation may affect the molecular mechanism of leukemogenesis by induced MLL-ENL in vivo, we analyzed the critical downstream genes and epigenetic genes in MPD cells from the moribund chimera mice (Fig 9A). The Tet1 expression was significantly and remarkably lower in C/T1<sup>ΔΔ</sup>/ME MPD cells. However, the expression levels of other epigenetic genes did not differ between the two genotypes of MPD cells (Figs 9A and S6). Unexpectedly, the MLL-ENL expression was mildly but significantly higher in C/T1<sup>ΔΔ</sup>/ME MPD cells, accompanied with significantly higher expression levels of Meis1 and Hoxa9. Nevertheless, in C/T1<sup>ΔΔ</sup>/ME MPD cells, the Evi1 expression was significantly and markedly lower (median: approximately 3%) in comparison with C/T1<sup>+/+</sup>/ME MPD cells, while it was exceptionally higher in one C/T1<sup>ΔΔ</sup>/ME MPD sample. In comparison of the expression levels in the MPD samples with those of the third plating of CD34(-) KSL-derived cells in myeloid immortalization assays, the lower expression levels of Evi1 in the C/T1<sup>ΔΔ</sup>/ME MPD samples were highlighted, although those of MLL-ENL were higher in the MPD samples (Fig 9B).

Taken together, in the leukemogenesis by induced MLL-ENL in vivo, the conditional Tet1-ablation led to marked reduction of Evi1 expression, but did not critically affect the MPD phenotype, although an unexpected increase of the MLL-ENL expression in C/T1<sup>ΔΔ</sup>/ME MPD cells might cancel out the phenotypic change. Furthermore, Tet1 was not involved in the maintenance of the leukemogenesis.

Discussion

The present study redefines the role of TET1/Tet1 in myeloid leukemogenesis by the MLL-fusion gene more closely. The aberrantly increased and reduced expression of TET1 is found
Fig 8. Characterization of tumor cells induced by Tg-ME-derived MLL-ENL under simultaneous Tet1 ablation. (A) FACS analyses of bone marrow (BM) cells from the moribund mice that developed lethal MPD in Fig 7. (B) Survival curves of the secondarily transplanted mice that received primary MPD cells with CreER; Tet1Δ/Δ; Tg-ME (n = 5) and CreER; Tet1+/+; Tg-ME (n = 8) harvested from the respective BM. Data from two independent experiments were combined in (B). n.s., not significant. (determined by log-rank tests (B)).

https://doi.org/10.1371/journal.pone.0248425.g008
Tet1 is not required for myeloid leukemogenesis by MLL-ENL

to be associated with myeloid and lymphoid leukemogenesis [13–15], respectively, although loss-of-function mutations of TET2/Tet2 develops both leukemogenesis [11]. MLL-fusion protein upregulates the TET1/Tet1 expression in the myeloid leukemogenesis [14], as confirmed in the present study. Interestingly, TET1 is found to be crucial for the proliferation of human T-cell acute lymphoblastic leukemia, suggesting context-dependent roles of TET1/Tet1 in leukemogenesis [27]. Indeed, our results suggested that Tet1 may not be necessarily essential for MLL-fusion-mediated myeloid leukemogenesis.

Our leukemogenesis assays in vivo showed that the conditional Tet1-ablation did not affect the myeloid leukemogenesis phenotype by induced expression of MLL-ENL. In comparison with BMT of retrovirally MLL-fusion-transduced HSPCs [14,17,22], our mixed-BM chimera enabled leukemic initiation to occur without temporary culture in vitro, thereby excluded the
bias of engraftment and some inevitable influence by culture *in vitro*, as in conditional/inducible MLL-fusion-mediated mouse models [28,29]. Meanwhile, compared with conventional Tet1 knockout mice [13–15], the Tet1 protein expression remained at the early phase of the conditional ablation. Thus, partly due to differences in the approaches, our results may differ from the finding [14] that Tet1 plays an essential role for the myeloid leukemogenesis.

Tet1 was dispensable for the leukemic immortalization by MLL-ENL in CD34(-) KSL cells enriched for LT-HSCs, in which the role of Tet1 had not been well-characterized, while, in retrovirally MLL-AF9-transduced Lin- BM cells, Tet1 is critically involved in the myeloid leukemogenesis [14]. Since the retroviral transduction into LT-HSCs is inferred to be very difficult [21], this discrepancy concerning the role of Tet1 may reflect the difference of a subpopulation of cells targeted by MLL-fusion genes.

Unexpectedly, the expression level of induced MLL-ENL in MPD cells was higher under the Tet1-ablated condition. Since the induced MLL-ENL expression at the third plating of CD34 (-) KSL-derived cells under the Tet1-ablated condition was comparable to that under the Tet1+/+ condition *in vitro*, the higher expression of induced MLL-ENL *in vivo* implied that the enrichment of cells that highly expressed MLL-ENL was more enhanced in association with the Tet1-ablation, in the process leading to MPD. The unexpected genetic effect, which may accelerate MPD under the Tet1-ablated condition, might reduce the effect of deceleration by loss of Tet1. However, in contrast to the finding that Hoxa9 and Meis1a were markedly down-regulated under Tet1-deficient conditions [14], our results showed that those genes were upregulated by MLL-ENL under Tet1-ablated conditions in the same way as Tet1+/+ conditions. In addition, irrespective of the genotypes, secondary BMT reproduced the MPD in similar latencies. In retrovirally MLL-ENL-immortalized CD34(+) KSL/MP cells, the conditional Tet1-ablation did not cause any significant changes in the clonogenicity or expression of critical downstream genes, although it should be noted that the ablation was incomplete presumably due to escape from recombination in a part of 4-OHT- treated cells, and/or the remaining expression of Tet1 transcripts after the recombination. These results suggested that Tet1 might not be essential for the MLL-ENL-mediated leukemogenesis, even after taking into consideration of the high expression of induced MLL-ENL in Tet1-ablated MPD cells.

The conditional Tet1-ablation was closely associated with very low Evi1 expression in myeloid leukemogenesis by the induced MLL-ENL *in vivo*, but not in the myeloid immortalization assays *in vitro*. Considering the epigenetic function of Tet1 [8–10] and the recent finding that loss-of-function Tet leads to a localized increase in DNA methylation [30], further analyses, such as comprehensive profiling of the methylation status of regulatory genomic regions of Evi1 [31], may be needed in the future. Since MLL-ENL upregulates Evi1/Evi1 through promoter binding [32] and epigenetic regulation by histone modifications [33], epigenomic analyses regarding Evi1 may be also helpful. Another hypothesis is that Tet1-ablation might lead to leukemic initiation by MLL-ENL more efficiently in relatively mature progenitor cells with low expression of Evi-1, since MLL-fusion genes upregulate Evi1 in KSL cells more strongly than in myeloid progenitor cells [32,34,35]. Thus, further analyses including hematopoietic stem/progenitor-specific ablation of Tet1 will be also needed to test this hypothesis.

In conclusion, to examine the role of Tet1 in the myeloid leukemogenesis more precisely, we generated conditional Tet1-knockout mice, and constructed a mouse model enabling leukemic initiation, in combination with inducible MLL-ENL transgenic mice. The phenotypes of leukemic transformation by MLL-ENL were not critically affected by conditional Tet1-ablation. However, the low Evi1 expression in MPD cells under the Tet1-ablated condition may be associated with a role of Tet1 in leukemic initiation by MLL-ENL. Furthermore, Tet1 was dispensable in leukemic maintenance in secondary BMT, suggesting that the role of Tet1 may not be essential for, at least, MLL-ENL-mediated myeloid leukemogenesis. Also, our results
suggested that the therapeutic application of Tet1 inhibition in AML may need careful assessment.

**Supporting information**

**S1 Checklist. ARRIVE guidelines checklist.**
(PDF)

**S1 Fig.** The proportion of myeloid, B-, and T lymphoid cells in peripheral blood from conditionally Tet1-ablated mice. The blood samples were measured just before (0), 4, 8, and 12 weeks after tamoxifen treatment as shown in Fig 2A (n = 5 per each (the same mice as used in Fig 2A)). Bar graphs show the mean and SD of data combined from two independent experiments. n.s., not significant. (determined by two-way repeated measures ANOVA followed by Shaffer’s modified sequentially rejective Bonferroni procedure).
(TIF)

**S2 Fig.** Assessment of hematopoiesis derived from conditionally Tet1-ablated bone marrow cells. (A) The experimental strategy for the hematological assessment of BM chimera mice where bone marrow (BM) cells were replaced with CreER;Tet1^{fl/fl} or Tet1^{fl/fl} BM cells (from one mouse per each genotype (total 2)) in bone marrow transplantation. Wt, wild-type; Tm, tamoxifen; ip, intraperitoneal injection; PB, peripheral blood. (B) Time course of measurement of the white blood cells (WBCs) counts and proportion of myeloid, B-, and T lymphoid cells in peripheral blood just before (0) and at the indicated number of weeks after tamoxifen treatment (n = 5 per each (total 10)). **p<0.005; n.s., not significant. (determined by two-tailed unpaired t-tests).
(TIF)

**S3 Fig.** Expression of Dnmt genes in the conditionally Tet1-ablated cells that were retrovirally immortalized by MLL-ENL beforehand. The expression levels of Dnmt1, Dnmt3a, and Dnmt3 assessed by RT-qPCR in the CreER;Tet1^{fl/fl} CD34(+) KSL/MP-derived cells retrovirally immortalized by MLL-ENL with treatment of 4-OHT or vehicle control (ethanol, EtOH). Bar graphs show the mean and SD of three independent experiments. n.s., not significant. (determined by two-tailed unpaired t-tests).
(TIF)

**S4 Fig.** Expression of Dnmt1 and Dnmt3a genes in myeloid immortalization assays by the induction of Tg-ME-derived MLL-ENL under simultaneous Tet1-ablation. The expression levels of Dnmt1 and Dnmt3a in the cells harvested at the end of the first (1R) and the third (3R, only in CD34(-) KSL-derived cells) rounds of plating, as assessed by RT-qPCR. Bar graphs show the mean and SD of three independent experiments. *p<0.05; n.s., not significant; †, not significant in comparison of the other combinations. The p-values for the expression levels of the genes tested were determined by one-way ANOVA followed by Tukey-Kramer tests (CD34(+) and MP), or a combination of one-way ANOVA followed by Tukey-Kramer tests (1R) and two-way repeated measures ANOVA followed by Shaffer’s modified sequentially rejective Bonferroni procedure (CreER; Tet1^{fl/fl}; Tg-ME and CreER; Tet1^{+/+}; Tg-ME) adjusted using the Holm-Bonferroni correction (CD34(-) KSL).
(TIF)

**S5 Fig.** The leukemogenesis by induction of Tg-ME-derived MLL-ENL under simultaneous Tet1 ablation, compared with the counterpart controls. (A) Chimerism analyses in peripheral blood cells (shown in Fig 5A) just before (0) and 4 weeks after tamoxifen treatment in the cohort #1. P-values were determined by two-way repeated measures ANOVA followed by
Shaffer’s modified sequentially rejective Bonferroni procedure. (B) Survival curves of the mixed-bone marrow (BM) chimera mice (cohort #1) receiving CreER; Tet1^{fl/fl}; Tg-ME (n = 13), CreER; Tet1^{+/+}; Tg-ME (n = 8), CreER; Tet1^{fl/fl} (n = 4), and Tet1^{fl/fl} (n = 5) BM cells that were treated with tamoxifen. (C) The typical morphology of BM cells from the moribund mice that developed lethal MPD. Cells were stained with Wright-Giemsa. Magnification, 200×; scale bars, 50 μm. Bar graphs show mean with SD of data combined from two independent experiments in (A). n.s., not significant. (determined by two-way repeated measures ANOVA followed by Shaffer’s modified sequentially rejective Bonferroni procedure).

S6 Fig. Expression profiling of Dnmt genes in the leukemogenesis assays. The expression levels of Dnmt1, Dnmt3a, and Dnmt3b in BM cells from the moribund mice developing lethal MPD in the leukemogenesis assays under Tet1-ablated (Tet1^{Δ/Δ}) and Tet1^{+/+}-conditions, as assessed by RT-qPCR. Data are shown in box and whisker plots. n.s., not significant. (determined by two-tailed unpaired t-tests).

Acknowledgments
We thank Dr. M. Okabe for the pNT1.1 vector, Dr. A. F. Stewart for the Flp e deleter mice, and Mr. Brian Quinn for providing language assistance.

Author Contributions
Conceptualization: Ryoichi Ono, Tetsuya Nosaka.
Data curation: Ryoichi Ono, Tetsuya Nosaka.
Formal analysis: Ryoichi Ono, Masahiro Masuya, Makoto Shinmei, Satomi Ishii, Yuri Maegawa, Bishnu Devi Maharjan.
Funding acquisition: Ryoichi Ono, Tetsuya Nosaka.
Investigation: Ryoichi Ono, Tetsuya Nosaka.
Methodology: Ryoichi Ono, Naokazu Inoue, Tetsuya Nosaka.
Project administration: Ryoichi Ono, Tetsuya Nosaka.
Resources: Ryoichi Ono.
Supervision: Naoyuki Katayama, Tetsuya Nosaka.
Validation: Ryoichi Ono, Tetsuya Nosaka.
Visualization: Ryoichi Ono.
Writing – original draft: Ryoichi Ono, Tetsuya Nosaka.
Writing – review & editing: Ryoichi Ono, Tetsuya Nosaka.
References

1. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet. 2003; 33 Suppl: 245–254. https://doi.org/10.1038/ng1089 PMID: 12610534

2. Michalak EM, Burr ML, Barnister AJ, Dawson MA. The roles of DNA, RNA and histone methylation in ageing and cancer. Nat Rev Mol Cell Biol. 2019; 20: 573–589. https://doi.org/10.1038/s41580-019-0143-1 PMID: 31270442

3. Flavahan WA, Gaskell E, Bernstein BE. Epigenetic plasticity and the hallmarks of cancer. Science. 2017; 357: eaal2380. https://doi.org/10.1126/science.aal2380 PMID: 28729483

4. Aitasi Y, Stunnenberg HG. The interplay of epigenetic marks during stem cell differentiation and development. Nat Rev Genet. 2017; 18: 643–658. https://doi.org/10.1038/nrg.2017.57 PMID: 28804139

5. Ono R, Taki T, Taketani T, Taniwaki M, Kobayashi H, Hayashi Y. LCX, leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23). Cancer Res. 2002; 62: 4075–4080. PMID: 12124344

6. Lorsbach RB, Moore J, Mathew S, Raimondi SC, Mukatira ST, Downing JR. TET2 in Normal and Malignant Hematopoiesis. 2017. Cold Spring Harb Perspect Biol. https://doi.org/10.1101/cshperspect.a026518 PMID: 28242787

7. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 2009; 324: 930–935. https://doi.org/10.1126/science.1170116 PMID: 19372391

8. Rasmussen KD, Helin K. Role of TET enzymes in DNA methylation, development, and cancer. Genes Dev. 2016; 30: 733–750. https://doi.org/10.1101/gad.276568.115 PMID: 27036965

9. Wu X, Zhang Y. TET-mediated active DNA demethylation: mechanism, function and beyond. Nat Rev Genet. 2017; 18: 517–534. https://doi.org/10.1038/nrg.2017.33 PMID: 28555658

10. Lio CJ, Yuita H, Rao A. Dysregulation of the TET family of epigenetic regulators in lymphoid and myeloid malignancies. Blood. 2019; 134: 1487–1497. https://doi.org/10.1182/blood.2019791475 PMID: 31467060

11. Bowman RL, Levine RL. TET2 in Normal and Malignant Hematopoiesis. 2017. Cold Spring Harb Perspect Med 7: a026518. https://doi.org/10.1101/cshperspect.a026518 PMID: 28242787

12. Dawlaty MM, Ganz K, Powell BE, Hu YC, Markoulaki S, Cheng AW, et al. Tet1 is dispensable for maintaining pluripotency and its loss is compatible with embryonic and postnatal development. Cell Stem Cell. 2011; 9: 166–175. https://doi.org/10.1016/j.stem.2011.07.010 PMID: 21816367

13. Cimmino L, Dawlaty MM, Ndiaye-Lobry D, Yap YS, Bakogianni S, Yu Y, et al. TET1 is a tumor suppressor of hematopoietic malignancy. Nat Immunol. 2015; 16: 653–662. https://doi.org/10.1038/nl.3148 PMID: 25867473

14. Huang H, Jiang X, Li Z, Li Y, Song CX, He C, et al. TET1 plays an essential oncogenic role in MLL-rearranged leukemia. Proc Natl Acad Sci USA. 2013; 110: 11994–11999. https://doi.org/10.1073/pnas.1310656110 PMID: 23818607

15. Zhao Z, Chen L, Dawlaty MM, Pan F, Weeks O, Zhou Y, et al. Combined Loss of Tett and Tet2 Promotes B Cell, but Not Myeloid Malignancies, in Mice. Cell Rep. 2015; 13: 1692–1704. https://doi.org/10.1016/j.celrep.2015.10.037 PMID: 26586431

16. Wang J, Li F, Ma Z, Yu M, Guo Q, Huang J, et al. High Expression of TET1 Predicts Poor Survival in Cytogenetically Normal Acute Myeloid Leukemia From Two Cohorts. EBioMedicine. 2018; 28: 90–96. https://doi.org/10.1016/j.ebiom.2018.01.031 PMID: 29402726

17. Somerville TC, Matheny CJ, Spencer GJ, Iwasaki M, Rinn JL, Witten DM, et al. Hierarchical maintenance of MLL myeloid leukemia stem cells employs a transcriptional program shared with embryonic rather than adult stem cells. Cell Stem Cell. 2009; 4: 129–140. https://doi.org/10.1016/j.stem.2008.11.015 PMID: 19200802

18. Gao Y, Chen J, Li K, Wu T, Huang B, Liu W, et al. Replacement of Oct4 by Tet1 during iPSC induction reveals an important role of DNA methylation and hydroxymethylation in reprogramming. Cell Stem Cell. 2013; 12: 453–469. https://doi.org/10.1016/j.stem.2013.02.005 PMID: 23499384

19. Kitamura T, Watanabe-Okoichi N, Enomoto Y, Nakahara F, Oki T, Kornemo Y, et al. Novel working hypothesis for pathogenesis of hematological malignancies: combination of mutations-induced cellular phenotypes determines the disease (cMiP-DD). J Biochem. 2016; 159: 17–25. https://doi.org/10.1093/jb/mvw014 PMID: 26990301

20. Ono R, Ihara M, Nakajima H, Ozaki K, Kataoka-Fujisawa Y, Taki T, et al. Disruption of Sept6, a fusion partner gene of MLL, does not affect ontogeny, leukemogenesis induced by MLL-SEPT6, or phenotype induced by the loss of Sept4. Mol Cell Biol. 2008; 25: 10965–10978. https://doi.org/10.1128/MCB.25.24.10965-10978.2005 PMID: 16314519
21. Ono R, Masuya M, Nakajima H, Enomoto Y, Miyata E, Nakamura A, et al. Plzf drives MLL-fusion-mediated leukemogenesis specifically in long-term hematopoietic stem cells. Blood. 2013; 122: 1271–1283. https://doi.org/10.1182/blood-2012-09-456665 PMID: 23838347

22. Ono R, Nakajima H, Ozaki K, Kumagai H, Kawashima T, Taki T, et al. Dimerization of MLL fusion proteins and FLT3 activation synergize to induce multiple-lineage leukemogenesis. J Clin Invest. 2005; 115: 919–929. https://doi.org/10.1172/JCI22725 PMID: 15761502

23. Kitamura T, Koshino Y, Shibata F, Oki T, Nakajima H, Nosaka T, et al. Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. Exp Hematol. 2003; 31: 1007–1014. PMID: 14585362

24. Ono R, Masuya M, Ishii S, Katayama N, Nosaka T. Eya2, a Target Activated by Plzf, Is Critical for PLZF-RAA-Induced Leukemogenesis. Mol Cell Biol. 2017; 37: e00585–16. https://doi.org/10.1128/MCB.00585-16 PMID: 28416638

25. Kunimoto H, Fukuchi Y, Sakurai M, Sadahira K, Ikeda Y, Okamoto S, et al. Tet2 disruption leads to enhanced self-renewal and altered differentiation of fetal liver hematopoietic stem cells. Scientific Reports. 2012; 2: 273. https://doi.org/10.1038/srep00273 PMID: 22355785

26. Freudenberg JM, Ghosh S, Lackford BL, Yellaboina S, Zheng X, Li R, et al. Acute depletion of Tet1-dependent 5-hydroxymethylcytosine levels impairs LIF/Stat3 signaling and results in loss of embryonic stem cell identity. Nucleic Acids Res. 2012; 40: 3364–3377. https://doi.org/10.1093/nar/gkr1253 PMID: 22210859

27. Bamezai S, Demir D, Pulikkottil AJ, Ciccarone F, Fischbein E, Sinha A, et al. TET1 promotes growth of T-cell acute lymphoblastic leukemia and can be antagonized via PARP inhibition. Leukemia. 2020; in press. https://doi.org/10.1038/s41375-020-0664-3 PMID: 32409690

28. Krivtsov AV, Feng Z, Lemieux ME, Faber J, Vempati S, Sinha AU, et al. H3K79 methylation profiles define murine and human MLL-AF4 leukemias. Cancer Cell. 2008; 14: 355–368. https://doi.org/10.1016/j.ccr.2008.10.001 PMID: 18977325

29. Stavropoulou V, Kaspar S, Brault L, Sanders MA, Juge S, Morettini S, et al. MLL-AF9 Expression in Hematopoietic Stem Cells Drives a Highly Invasive AML Expressing EMT-Related Genes Linked to Poor Outcome. Cancer Cell. 2016; 30: 43–58. https://doi.org/10.1016/j.ccell.2016.05.011 PMID: 27344946

30. López-Moyado IF, Tsagaratou A, Yuita H, Seo H, Delatte B, Heinzet S, et al. Paradoxical association of TET loss of function with genome-wide DNA hypomethylation. Proc Natl Acad Sci USA. 2019; 116: 16933–16942. https://doi.org/10.1073/pnas.1903091116 PMID: 31371502

31. Vázquez I, Maicas M, Cervera J, Agirre X, Marin-Béjar O, Marcotegui N, et al. Down-regulation of EVI1 is associated with epigenetic alterations and good prognosis in patients with acute myeloid leukemia. Haematologica. 2011; 96: 1448–1456. https://doi.org/10.3324/haematol.2011.040535 PMID: 21750091

32. Arai S, Yoshimi A, Shimabe M, Ichikawa M, Nakagawa M, Imai Y, et al. Evi-1 is a transcriptional target of mixed-lineage leukemia oncoproteins in hematopoietic stem cells. Blood. 2011; 117: 6304–6314. https://doi.org/10.1182/blood-2009-07-234310 PMID: 21190993

33. Garcia-Cueillar MP, Büttner C, Bartenhagen C, Dugas M, Slany RK. Leukemogenic MLL-ENL Fusions Induce Alternative Chromatin States to Drive a Functionally Dichotomous Group of Target Genes. Cell Rep. 2016; 15: 310–322. https://doi.org/10.1016/j.celrep.2016.03.018 PMID: 27050521

34. Bindels EM, Havermans M, Lugthart S, Erpelinck C, Wocjtowicz E, Krivtsov AV, et al. EVI1 is critical for the pathogenesis of a subset of MLL-AF9-rearranged AMLs. Blood. 2011; 119: 5838–5849. https://doi.org/10.1182/blood-2011-11-393827 PMID: 22553314

35. Krivtsov AV, Figueroa ME, Sinha AU, Stubbs MC, Feng Z, Valk PJM, et al. Cell of origin determines clinically relevant subtypes of MLL-rearranged AML. Leukemia. 2013; 27: 852–860. https://doi.org/10.1038/leu.2012.393 PMID: 23235717