Combined Loss of Tet1 and Tet2 Promotes B Cell, but Not Myeloid Malignancies, in Mice

Highlights

- TET1 and TET2 are often concomitantly downregulated in acute B-lymphocytic leukemia
- Tet1 is required for Tet2-deletion-mediated HSC dysregulation and myeloid malignancy
- Deletion of both Tet1 and Tet2 in mice leads to lethal B cell malignancies
- Tet2−/− and DKO HSC/HPCs display distinct DNA 5hmC/5mC signatures

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In Brief
TET1 and TET2 are methylcytosine dioxygenases that are implicated in hematological malignancies. Using Tet1 and Tet2 double-knockout mice, Zhao et al. separate the roles of each enzyme and examine how Tet1 and Tet2 contribute to hematopoiesis and hematological malignancies.

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Combined Loss of Tet1 and Tet2 Promotes B Cell, but Not Myeloid Malignancies, in Mice

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SUMMARY

TET1/2/3 are methylcytosine dioxygenases that regulate cytosine hydroxymethylation. Tet1/2 are abundantly expressed in HSC/HPCs and are implicated in hematological malignancies. Tet2 deletion in mice causes myeloid malignancies, while Tet1-null mice develop B cell lymphoma after an extended period of latency. Interestingly, TET1/2 are often concomitantly downregulated in acute B-lymphocytic leukemia. Here, we investigated the overlapping and non-redundant functions of Tett1/2 using Tet1/2 double-knockout (DKO) mice. DKO and Tet2−/− HSC/HPCs show overlapping and unique 5hmC and 5mC profiles. DKO mice exhibit strikingly decreased incidence and delayed onset of myeloid malignancies in comparison to Tet2−/− mice and in contrast develop lethal B cell malignancies. Transcriptome analysis of DKO tumors reveals expression changes in many genes dysregulated in human B cell malignancies, including LMO2, BCL6, and MYC. These results highlight the critical roles of TET1/2 individually and together in the pathogenesis of hematological malignancies.

INTRODUCTION

The ten-eleven translocation (TET) family of proteins is composed of three members, Tet1, Tet2, and Tet3 (Iyer et al., 2009; Tahiliani et al., 2009). They share a conserved Cys-rich domain and double-stranded beta helix domain (Iyer et al., 2009). TETs exhibit their unique enzymatic function and facilitate DNA demethylation, oxidizing 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) in a stepwise manner (He et al., 2011; Ito et al., 2011; Tahiliani et al., 2009). TETs can thus act as epigenetic regulators and modulate gene transcription and cellular functions (Pastor et al., 2013).

Mutations and/or deletions of the Tet2 gene have been reported to frequently occur in multiple myeloid malignancies, including approximately 30% of myelodysplastic syndromes (MDS), 20% of myeloproliferative neoplasms (MPN), 17% of de novo acute myeloid leukemias (AML), 30% of secondary AMLs, and 50%–60% of chronic myelomonocytic leukemias (CMMLs) (Delhommeau et al., 2009; Jankowska et al., 2009; Langemeijer et al., 2009). Tet2 mutations have also been found in approximately 2% of Hodgkin’s lymphoma cases and 11.9% of T cell lymphoma patients (Quivoron et al., 2011). In addition, Musialik et al. recently showed that Tet2 expression is significantly lower in acute B-lymphocytic leukemia (B-ALL) patients when compared to control CD19+ samples. Tet1 was first identified as a fusion partner of the mixed lineage leukemia (MLL) gene in AML patients carrying t(10;11)(q22;q23) (Ono et al., 2002). Recently, Tet1 was shown to be transcriptionally downregulated in human B cell non-Hodgkin lymphomas (B-NHL), including diffuse large B cell lymphoma (DLBCL) and follicular lymphoma (FL) (Cimmino et al., 2015). Huang et al. reported that Tet1 is significantly upregulated and plays an oncogenic role in MLL-rearranged leukemia, rendering TET1 as a potential target for treating this form of hematopoietic malignancy (Huang et al., 2013). However, Cimmino et al. recently showed that deletion of Tet1 promoted the development of B cell lymphoma in
Acute B-Lymphocytic Leukemia Patients

Tet2 decreased frequency and delayed onset of myeloid malignancies in peripheral blood (PB) mononuclear cells from B-ALL patients (n = 27) and PB CD19+ cells such as B cells and myeloid cells (Huang et al., 2013; Li et al., 2011; Moran-Crusio et al., 2011). Tet2 plays a prominent and oncogenic role in the pathogenesis of hematological malignancies. TET1 and TET2 in the pathogenesis of hematological malignancies.

RESULTS

TET1 and TET2 Are Often Concomitantly Downregulated in Human Acute B-Lymphocytic Leukemia Patients

TET2 is frequently mutated in both myeloid and lymphoid malignancies, whereas TET1 mutations are rare. We first examined by quantitative real-time PCR the mRNA expression of TET1 and TET2 in peripheral blood (PB) mononuclear cells from a cohort of B-ALL patients, and compared it with that of PB CD19+ B cells from healthy controls. Downregulation of TET1 mRNA expression was observed in 21 of the 27 B-ALL patients as compared to healthy controls (Figure 1A), while downregulation of TET2 was noted in nine of the 27 B-ALL patients (Figure 1B). Interestingly, all of the nine B-ALL patients with TET2 downregulation had concomitant TET1 downregulation (Figures 1A and 1B). These data suggest that combined TET1 and TET2 loss may play important roles in the pathogenesis of B cell malignancies and prompted us to examine the hematological phenotype displayed in Tet1/2 DKO mice.

Tet1/2 DKO Mice Develop Lethal B Cell Malignancies

Analysis of hematological parameters on young wild-type (WT), Tet1−/−, Tet2−/−, and DKO mice showed that up to 4 months of age DKO mice do not exhibit signs of CMML development that Tet2−/− mice display at this age (Figure S1) (Li et al., 2011). To determine whether DKO mice develop hematological malignancies later in life, we aged a cohort of WT, Tet1−/−, Tet2−/−, and DKO mice. Analysis of total blood counts and blood smears of 12- to 15-month-old Tet2−/− mice revealed features that are consistent with development of myeloid malignancies. Most of the aged Tet2−/− mice developed significantly higher WBC counts (mainly caused by neutrophilia and monocytes), compared to age-matched WT mice (Figures 2A and 2B). The blood cell counts of aged Tet1−/− mice were comparable to those of WT mice (Figure 2A). In contrast, 12 of the 19 aged DKO mice exhibited increased WBC counts, with 11 caused by marked lymphocytosis but normal monocyte and neutrophil counts (Figures 2A and 2B). Only one of these DKO mice (G3-21) displayed prominent neutrophilia and monocytosis but normal lymphocyte count. Two of the 19 DKO (10.6%) and three of the 14 Tet2−/− (21.4%) mice were anemic, evidenced by reduced RBC counts. PB smears from most of the DKO mice showed absolute lymphocytosis but normal monocyte and neutrophil counts (Figures 2A and 2B). Interestingly, 68.4% of the DKO mice either became moribund or died within 20 months of their life, whereas none of the WT, 7.6% of Tet1−/−, and 90% of Tet2−/− mice died at the end of our evaluation (Figure 2C; Table S1). Necropsy of the moribund/deceased DKO mice revealed pronounced...
hepatosplenomegaly in all and enlarged lymph nodes in most of the mice (Figure 2D; Table S1). Flow cytometric analyses on PB, spleen, and BM cell preparations from the moribund/deceased DKO mice revealed predominant proportions of B220+IgM+/low CD43+/CD19+ B-lymphocytes with high forward scatter (FSC) in nine of the ten DKO mice, demonstrating a neoplastic monomorpic expansion of B cell origin (Figures 2E, S2A, and S2B; Table S1). Furthermore, the neoplastic B-lymphocytes expressed CD71 in all and CD5 in two-thirds of the DKO mice (Figure S2B; Table S1). In each of the DKO mice with predominant neoplastic B cells, the proportion of myeloid and T cells in the PB, BM, and spleen were either decreased or comparable to WT mice (Figure S2C), which is likely related to the predomi-
nance of B cell populations rather than a primary defect. Note that in one moribund DKO animal (G3-21) with monocytosis/ neutrophilia, its BM and spleen cells showed predominant proportions of CD11b+F4/80+Gr-1low monocytic cell populations (Table S1), indicating CMML.

Examination of H&E-stained BM, spleen, liver, and lymph node sections and May-Giemsa-stained cytospin preparations of BM and spleen cells from the moribund/deceased DKO mice revealed extensive infiltration with intermediate to large-sized immature-appearing lymphocytes with large nuclei and small amount of cytoplasm, slightly irregular nuclei and dispersed nuclear chromatin (Figures 2F and S2D). BM showed diffuse neoplastic lymphoid infiltration with decreased normal tri-
lineage hematopoiesis (Figure 2F). The normal architecture of spleen and lymph nodes was effaced and replaced by diffuse atypical lymphoid infiltrates (Figure 2F). Both red and white pulp of the spleen were involved. The liver displayed nodular and sinusoidal lymphoid infiltration, which was also confirmed by immunostaining with anti-B220 and anti-CD43 antibodies (Figures 2F and 2G). Furthermore, to determine whether these monotypic expansions/infiltrations were clonal, PCR analysis of immunoglobulin H (IgH) D-J rearrangements was performed. Clonal gene rearrangements were detected for IgD-J junctions in five of six mice tested. Splenic B cells from three DKO mice showed monoclonal Ig rearrangements. Two mice dem-
onstrated bclonal Ig rearrangements. These Ig gene rearrange-
ment assays revealed that the splenic B220+ cells from each of these DKO mice with monomorphic B cell expansions/infiltrations were clonal, while B220+ cells from the DKO case with CMML or WT displayed oligoclonal patterns (Figure 2H). Collectively, the majority of these aged DKO mice developed B cell malignancy, with features most closely resembling human B-ALL (Table S1).

**B Cell Malignancy in DKO Mice Is Transplantable to Secondary Recipients**

To evaluate the malignant nature of the abnormally infiltrated B-lymphocytes in DKO mice, spleen cells from a moribund DKO (G2-53) and a WT mice were transplanted into sublethally irradiated WT recipients (Figure S3A). None of the recipients receiving WT spleen cells developed pathology or gross evi-
dence of disease within 4 months of transplantation (Figures 3A–3D). In contrast, all of the mice receiving DKO spleen cells developed diseases with similar characteristics as those observed in the primary mouse including elevated WBC counts, lymphocytosis, splenomegaly, enlarged lymph nodes, and death (Figures 3A, 3B, S3B, and S3C). Flow cytometric analysis and histological and cytospin examination of PB, spleen, BM, liver, lymph node, lung, and kidney cells of the recipients revealed predominant infiltration of a uniform B cell population (high FSC, B220+CD19+IgM+/low CD43+/CD5+CD71+) similar to that observed in the primary DKO mice (Figures 3C, 3D, S3D, and S3E; data not shown). These data demonstrate that the Tet1/2-deletion-induced B cell malignancy is transplantable, suggesting a malignant and neoplastic nature of the infiltrated B cells in DKO mice.

In addition, long-term observation of a cohort of Tet1+/--; Tet2−/− mice (n = 15) showed that Tet1+/--; Tet2−/− mice have an improved survival rate compared to Tet2+/− mice, but a compar-
ible survival rate compared to DKO mice (Figure S2E; Table S2). Interestingly, analysis of nine moribund/deceased Tet1+/--; Tet2−/− mice demonstrate that seven of nine developed B cell malignancies (B-ALL) and two of nine developed myeloid malignancies (Figure S2F; Table S2). Our long-term observation of 196 Tet2−/− mice demonstrate that ~93% of Tet2−/− mice developed myeloid malignancies and only ~4% developed B cell malignancies (Figure S2F; our unpublished data). These data indicate that Tet1 loss modulates Tet2-deletion-mediated disease phenotype in mice, not only decreasing the incidence and delaying the onset of myeloid malignancies, but also promo-
ning the pathogenesis of B cell malignancies.

**Deletion of Tet1/2 Increases CLP/BLP Compartment and Affects B Cell Development in Mice**

To explore the cellular mechanisms by which deletion of Tet1/2 causes high frequency of B cell malignancies but low frequency of myeloid malignancies, we analyzed HSC, myeloid, and lymphoid progenitors and various maturation stages of B cell popula-
tions in the BM of 5- to 7-week-old DKO mice prior to their development of hematological malignancies. The proportion of LSK cells and each of the myeloid progenitors (CMP, GMP, and MEP) was comparable to WT, Tet1−/−, and DKO mice, whereas LSK and GMP cell populations were significantly increased in Tet2−/− mice (Figures 4A and 4B; data not shown). Similar results were obtained with conditional MxCre-mediated Tet1 and/or Tet2 deletion in adult mice (Figures S4 and S5). Tet2fl/f;MxCre mice have increased LSK and GMP cell populations compared to that of Tet1fl/fl; Tet2fl/fl;MxCre, Tet1fl/fl;MxCre and Tet1fl/fl; Tet2fl/fl mice 5–6 weeks after p/pc injection (Figures S5A–S5D).

We also assessed the replating potential of purified LSK cells from 5- to 7-week-old WT, Tet1−/−, Tet2−/−, and DKO mice by in vitro myeloid colony formation assays. A significant in-
crease in colony formation was observed for Tet2−/− LSK cell cultures in each round of replating, while Tet1−/− and DKO LSK cell cultures exhibited a moderate increase in the number of colonies at P2, but not P3 and P4 (Figure 4C). Furthermore, re-introduction of WT Tet1, but not catalytic domain inactive mutant Tet1, into DKO LSK cells significantly increased their replating potential at P2–P4 (Figures S5E and S5F). These data suggest that Tet1 loss abrogated the Tet2-deletion-caused LSK pool increment in vivo and their hyper-replating potential in vitro. In addition, the catalytic activity of Tet1 is likely required for Tet2 loss-induced hyper-replating potential in HSC/HPCs.

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Figure 2. Most of the DKO Mice Developed Lethal B-Lymphoid Malignancies
(A) Most of the aged DKO mice exhibited elevated WBC and lymphocyte counts. WBC, neutrophil, monocyte, lymphocyte, and RBC counts were performed with 12- to 15-month-old DKO mice (n = 19) and age-matched WT (n = 13), Tet1−/− (n = 12), and Tet2−/− (n = 14) mice. *p < 0.05, **p < 0.01, ***p < 0.001.
(B) May–Giemsa-stained PB smears prepared from a representative moribund DKO mouse and age-matched WT, Tet1−/−, and Tet2−/− mice. Bar, 20 µm.
(C) Kaplan–Meier survival curve of WT (n = 25), Tet1−/− (n = 13), Tet2−/− (n = 20), and DKO (n = 19) mice up to 650 days.
(D) The gross morphologies of spleen, liver, and lymph nodes from a representative moribund DKO and an age-matched WT mouse.
(E) Flow cytometric analysis of B cell lineage (B220/IgM) in PB, BM, and spleen of representative moribund DKO (#G3-53 and #G2-53) and age-matched WT mice. The numbers indicate the percentages of cells in each cell population.
(legend continued on next page)
Interestingly, a significantly increased frequency of common lymphoid progenitors (CLPs) and B-lymphocyte progenitors (BLPs) is observed in DKO mice (5–7 weeks old), whereas comparable CLP and BLP frequencies are seen in Tet1−/−, Tet2−/−, and WT mice (Figures 4G–4H). In addition, when BM cells of young WT, Tet1−/−, Tet2−/−, and DKO mice are examined for their Pro-, Pre-, Immature-, and Mature-B cell populations, DKO mice show significantly higher frequencies of Pro-, Pre-, and Immature-B cells, but not mature-B cells, whereas, Tet1−/− mice show higher frequencies of Immature-B cells in comparison to WT control mice (Figures 4G and 4H). Frequencies for each B cell population is comparable or slightly decreased in DKO compared to WT control mice (Figures 4G and 4H). Consistently, B cell colony formation assay shows significantly higher frequencies of Pro-, Pre-, Immature-, and Mature-B cell populations, DKO mice (5–7 weeks old), whereas comparable CLP and BLP frequencies are seen in Tet1−/−, Tet2−/−, and WT mice (Figures 4G–4H). In addition, when BM cells of young WT, Tet1−/−, Tet2−/−, and DKO mice are examined for their Pro-, Pre-, Immature-, and Mature-B cell populations, DKO mice show significantly higher frequencies of Pro-, Pre-, and Immature-B cells, but not mature-B cells, whereas, Tet1−/− mice show higher frequencies of Immature-B cells in comparison to WT control mice (Figures 4G and 4H). Frequencies for each B cell population is comparable or slightly decreased in Tet2−/− mice in comparison to WT mice (Figures 4G and 4H), which might be related to an increased Gr1/Mac1 cell population in their BM. Consistently, B cell colony formation assay shows significantly increased B cell colonies in the BM of DKO mice compared to Tet1−/−, Tet2−/−, and WT mice (Figure 4I). A moderate increase in B cell colony formation is also seen in Tet1−/− and Tet2−/− mice compared to WT controls (Figure 4I). These results indicate that deletion of both Tet1/2 results in increased CLP, BLP, and Pro-/Pre-/Immature-B cell populations in vivo manner in vivo, we performed a competitive BM reconstitution assay by transplanting total CD45.2+ nucleated BM cells (1 × 10^6) from 6- to 7-week-old Tet1f/f, Tet2f/f, Tet2f/f, MxCre, or Tet1f/f, Tet2f/f, MxCre mice along with an equal number of WT CD45.1+ BM cells, into lethally irradiated F1 recipient mice (Figure 5A). Four weeks after transplantation, the recipients were assessed for CD45.2/CD45.1 chimeras in their PB by FACS analysis and then were induced for Tet1 and/or Tet2 deletion by pIpC injections. The contribution of CD45.1+ versus CD45.2+ cells in the PB was monitored every 4 weeks for 28 weeks after pIpC injections. CD45.2+ Tet1f/f, Tet2f/f cell chimeras remained around 50%, whereas there was a steady increase in the CD45.2+ Tet2f/f, MxCre cell chimeras that reached over 70% at 28 weeks. Interestingly, Tet1f/f, Tet2f/f, MxCre cell chimeras increased to ~60% during the first 16 weeks and then slightly declined with no significant differences compared to Tet1f/f; Tet2f/f CD45.2+ cell chimeras at 24 and 28 weeks. The Tet1f/f; Tet2f/f, MxCre cell chimeras were significantly smaller than that of Tet2f/f, MxCre cells after 20 weeks (Figures 5B and 5C). Furthermore, Tet2f/f, MxCre, but not Tet1f/f; Tet2f/f, MxCre BM cells contributed to a greater proportion of Gr1/Mac1 cell malignancy developed a disease similar to their donor mouse. (Figure 4J), which might predispose and/or facilitate the development of B cell malignancies.

Tet1/2-Deficient HSCs Exhibit an Increased Short-Term, but Not Long-Term, Hematopoietic Repopulating Capacity

To determine whether deletion of Tet1/2 affects HSC activity in a cell intrinsic...
Figure 4. Frequencies of HSC, Myeloid, and Lymphoid Progenitor Compartments, as well as Various B Cell Sub-populations in DKO Mice

(A) Flow cytometric analysis of Sca-1+c-Kit+ and Sca-1-c-Kit+ cell populations in the total BM cells of representative WT, Tet1−/−, Tet2−/−, and DKO mice (7 weeks old).

(B) Quantitation of the percentage of Sca-1+c-Kit+ cells in the BM of each mouse genotype (mean ± SD, four to five mice/genotype).

(C) Colony-formation assay of LSK cells sorted from each genotype of BM cells in methylcellulose medium (500 cells/plate). Colonies were passaged every 7 days for three sequential platings (P1-4). Data are representative of three experiments.

(D) Flow cytometric analysis of common lymphoid progenitors (CLP, Lin IL-7R+CD135+ Sca-1low) and B-lymphocyte progenitors (BLP, Lin IL-7R+CD135+Ly6D+ Sca-1low) in the BM of representative WT and DKO mice.

(E and F) Quantitation of the percentage of CLP (E) and BLP (F) in the total BM cells of each mouse genotype (mean ± SD, five to six mice/genotype).

(G) Flow cytometric analysis of Pro-B (B220+IgM+CD19+CD43+), Pre-B (B220+IgM+CD19+CD43−), Immature-B (B220IgM+), and Mature-B (B220IgMlow) cell populations in the BM cells of representative WT, Tet1−/−, Tet2−/−, and DKO mice (7 weeks old).

(H) Quantitation of the percentage of Pro-B, Pre-B, Immature-B, and Mature-B cells in the total BM cells of each genotype of mice (mean ± SD, four to seven mice/genotype).

(legend continued on next page)
granulocytic/monocytic cells and a smaller proportion of B220+ B cells in the recipient BM compared to controls (Figure 5D).

Consistent with PB, at 24 weeks after pl:pC injections, CD45.2 chimeras in the BM was also significantly higher in Tet2fl/fl;

MxCre BM transplants than Tet1fl/fl;

MxCre or Tet1fl/fl;

Tet2fl/fl BM transplants (Figures 5E and 5F). Furthermore, within the CD45.2 BM cell populations, the frequencies of LSK and GMP, but not CLP were increased in Tet2fl/fl;

MxCre BM transplants, whereas the frequency of CLP, but not LSK and GMP, was increased in Tet1fl/fl;

Tet2fl/fl BM transplants as compared to the control Tet1fl/fl;

Tet2fl/fl BM transplants (Figures 5G–5I). These data suggest that Tet1/2 deletion in HSC/HPCs moderately increases their short-term, but not long-term repopulating capacity while promoting the CLP expansion. The data also confirm previous observations that Tet2-deficient HSCs exhibit an enhanced HSC activity and skewed differentiation toward granulocytic/monocytic lineage.

In addition, three of the five mice receiving Tet2fl/fl;

MxCre BM cells had moderate monocytosis and splenomegaly 24 weeks after pl:pC injections (Figure 5J; data not shown), reminiscent of the CMMML phenotype displayed in Tet2−/− mice. Moreover, two of the five mice receiving Tet1fl/fl;

Tet2fl/fl;

MxCre BM cells had mild lymphocytosis and splenomegaly (Figure 5I; data not shown), reminiscent of the B cell malignancy phenotype displayed in DKO mice. None of the mice receiving Tet1fl/fl;

Tet2fl/fl BM cells developed any signs of hematological malignancies. These data suggest that the Tet1/2-deletion-induced phenotype is likely cell autonomous.

**Tet2−/− and DKO LK Cells Display Distinct DNA Methylation/Hydroxymethylation Signatures**

Given the role of Tet proteins in 5mC oxidation, we employed a previously established chemical labeling and affinity purification method coupled with high-throughput sequencing (hMe-Seal) to profile the genome-wide distribution of 5hmC. We also used methylated DNA immunoprecipitation (MeDIP) coupled with high-throughput sequencing (MeDIP-seq) to profile 5mC using BM LK cells purified from young WT, Tet2−/−, and DKO mice (5–8 weeks old). The loss of Tet2 or Tet1/2 leads to genome-wide alterations of both 5mC and 5hmC (Figure 6A). We observed significant overlap in differential hydroxymethylated regions (DhMRs) or differential methylated regions (DMRs) between two genotypes of LK cells (Figures 6B and 6A). Intriguingly, the overlap between DhMRs and DMRs within each genotype of LK cells was minimal, indicating that DhMRs and DMRs might represent distinct loci with altered epigenetic modifications under these conditions (Figures 6C and 6B).

To further explore molecular mechanisms underlying phenotypes associated with these two mutant lines of mice, we applied RNA sequencing (RNA-seq) to the LK cells from young Tet2−/− and DKO mice that we used for genepicomic analyses. We primarily focused on 654 genes that are known to be involved in regulating hematopoietic cell development and/or to promote leukemogenesis, based on previously published studies (Abdel-Wahab et al., 2013; Chambers et al., 2007; Shlush et al., 2014). By overlapping with the DhMRs or DMRs identified above, we observed a significant number of these genes with altered 5hmC or 5mC modifications (Figures 6D and S6C–S6E). However, we did not observe significant expression changes in these genes in either Tet2−/− or DKO LK cells, and there was no clear correlation between gene expression changes and the 5hmC/5mC alteration (Figures 6E and 6F). This is consistent with previous observations that no direct correlation is observed between gene expression alteration and 5hmC changes in mouse ES cells (Ficz et al., 2011; Pastor et al., 2011; Williams et al., 2011). These observations suggest the roles of distinct cytokine modifications (particularly 5hmC) could play in marking specific genes, which may enable cells to respond to additional stimuli to subsequently alter gene expression.

**Altered Expression of Genes Implicated in Human B Cell Malignancies in the B-ALL DKO B220+ Cells**

Next, we performed RNA-seq to identify the dysregulated genes in preleukemic and B-ALL DKO B220+ cells. B220+ cells were isolated from the spleen cells of B-ALL DKO mice, age-matched WT, and pre-leukemic DKO mice. RNA-seq analysis of preleukemic DKO B220+ cells identified a pool of dysregulated genes, compared to WT B220+ cells, which are implicated in B cell development such as Sox4, Ets2, and Irf4 (Mallampati et al., 2014; Simonetti et al., 2013; Yoshimatsu et al., 2011), and consistent with the observation that impaired B cell development occurs in these DKO mice (Figure 7A). Moreover, analysis of the RNA-seq data identified a panel of differentially expressed genes in B-ALL DKO B220+ cells compared to WT or pre-leukemic DKO B220+ cells. When compared to pre-leukemic DKO B220+ cells, 294 genes were upregulated and 205 genes were downregulated in B-ALL DKO B220+ cells (false discovery rate [FDR] <0.05). The significantly altered genes in B-ALL DKO B220+ cells included many dysregulated genes in human B cell malignancy such as Lmo2, Bcl6, Myc, Pten, and Blk (Natkanem et al., 2007; Ott et al., 2013; Pfeifer et al., 2013), further validating B cell malignancy occurring in these B-ALL DKO mice (Figure 7B).

We then integrated the previously published TET1 and TET2 chromatin immunoprecipitation sequencing (Chip-seq) data with our RNA-seq data to search for known TET1 target genes (Williams et al., 2011) and TET2 target genes (Deplus et al., 2013) within the significantly dysregulated genes in B-ALL DKO B220+ cells. Interestingly, nearly 40% of these dysregulated genes were direct targets of TET1 (such as Jun, Myc, and Lmo2), and ~10% were direct targets of TET2 (such as Pbx3, Rras2, and Flt3) (Figure 7C). These data suggest that Tet1 and/or Tet2 are capable of regulating the expression of these genes in the transformed B-ALL DKO B220+ cells, and Tet1 seems more capable of controlling its target gene expression.

(I) Pre-B colony-formation assays of BM cells from each mouse genotype. Colonies were scored after 12–14 days of culture. Data are representative of three experiments.

(J) Schematic overview of the effects of Tet1 and/or Tet2 deletion on B cell development/maturation.

*p < 0.05, **p < 0.01, ***p < 0.001.
Figure 5. Hematopoietic Repopulating Capacity of DKO HSCs

(A) Schematic depiction of the competitive transplantation scheme. CD45.2+ BM cells from Tet1f/f;Tet2f/f, Tet2f/f;MxCre, and Tet1f/f;Tet2f/f;MxCre mice were mixed with CD45.1+ competitor cells at a ratio of 1:1 (1 x 10^6 cells each) and transplanted into lethally irradiated F1 recipients. Recipients were injected with pI:pC 4 weeks after transplantation to induce MxCre-mediated Tet1/2 deletion in the CD45.2+ donor cells.

(B) The kinetics of the CD45.2+ cell chimerism in the PB of mice receiving each genotype of BM cells. Data are shown as mean ± SD from four to seven animals per genotype.

(C) Flow cytometric analysis of PB cells from representative recipients of each group at 0 or 24 weeks after pI:pC injection.

(D) The lineage distribution within the PB CD45.2+ cells of each group of recipients 24 weeks after pI:pC injection (mean ± SD from four to seven animals/genotype).

(E and F) The CD45.2+ cell chimerism in the BM of mice receiving each genotype of BM cells 24 weeks after pI:pC injection (mean ± SD, three to five animals/genotype).

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compared to Tet2. These data also suggest that altered genes in B-ALL DKO B220+ cells are largely a direct result of combined Tet1/2 loss.

**DISCUSSION**

TET1/2/3 promote DNA demethylation by catalyzing conversion of 5mC primarily to 5hmC as well as 5-fC and 5-caC (He et al., 2011; Ito et al., 2011; Tahiliani et al., 2009). Previous work has shown that loss-of-function mutations of **TET2** frequently occur in myeloid malignancies (Delhommeau et al., 2009; Jankowska et al., 2009; Langemeijer et al., 2009), in which **TET2** functions as a critical tumor suppressor (Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011). Somatic **TET2** mutations also occur in T cell lymphoma (such as angioimmunoblastic T lymphomas, 33%) and B-NHL (such as DLBCL, 12%; and mantle cell lymphoma, 4%). Besides loss-of-function mutations and deletions of **TET2**, **TET2** inactivation or haploinsufficiency
could result by other means such as gene downregulation. Indeed, TET2 mRNA expression level is significantly decreased in B-ALL compared to normal controls (Musialik et al., 2014). Our data show that TET1 and TET2 are often concomitantly downregulated in B-ALL patients. Recently, TET1 was shown to be transcriptionally downregulated in B-NHL (DLBCL and FL) (Cimmino et al., 2015). Given the frequency of TET2 mutations in these patients, TET1 and TET2 are likely simultaneously inactivated in some cases of B-NHL. Although the definitive function of TET1 in leukemogenesis remains to be determined, a recent study has implicated loss of Tet1 in onset of B cell lymphoma late in life (Cimmino et al., 2015). Nonetheless, Tet1 has also been implicated in an essential oncogenic role in MLL-rearranged leukemia (Huang et al., 2013). The seemingly opposite function of TET1 and TET2 in leukemogenesis and their combined inactivation in B cell malignancy pose a challenge to
the investigation of their overlapping and non-redundant roles in hematopoietic regulation and pathogenesis of hematological malignancies.

The generation of both germline and conditional Tet1/2 DKO mice has allowed us to gain valuable information toward addressing this important question and to determining the in vivo consequences of both Tet1/2 loss in hematopoiesis and hematological malignancies. Our findings can be summarized as follows: (1) unlike Tet2 deletion, deficiency of Tet1/2 does not cause an increased HSC pool in vivo and Tet1/2-deficient HSCs do not possess an increased long-term repopulating capability and skewed monocytic differentiation; (2) Tet1 loss dramatically decreases the incidence, and markedly delays the onset of Tet2-deletion-related myeloid malignancies; (3) deletion of both Tet1/2 but not either in mice leads to increased CLP/BLP/Pro-B/Pre-B/Immature-B cell populations and results in the development of predominantly B-ALL; (4) Tet2-/- and DKO LK cells display distinct DNA methylation and 5hmC methylation signatures; however, no correlation between gene expression and DhMRs or DMRs is observed in both Tet2-/- and DKO HSC/ HPCs; (5) transcriptome analyses of B-ALL DKO B220+ cells identify alteration of genes that are dysregulated in human B cell malignancies such as Lmo2, Bcl6, Myc, Pten, and Btk.

Although TET2 is frequently mutated in T cell malignancies like angioimmunoblastic T lymphomas and peripheral T cell lymphoma (Sakata-Yanagimoto et al., 2014), and a fraction of Tet2-/- mice develop T cell malignancies (our unpublished data), none of the DKO mice we analyzed developed T cell malignancy, suggesting a low incidence of T cell malignancy in these mice. Future studies are required to determine the frequency of T cell malignancy in DKO mice.

Recent genome-wide profiling of 5mC distribution in murine HSC/HPCs highlights the significance of dynamic changes in DNA methylation (Bock et al., 2012; Sun et al., 2014), while little is known about 5hmC distribution in HSC/HPCs. Understanding the biological significance of 5mC and 5hmC dynamics and their relevance to TET1 and TET2 enzymes in HSC/HPCs is important. In this study, we demonstrate that the loss of Tet2 or Tet1/2 leads to genome-wide alterations of both 5mC and 5hmC. The significant amount of overlap of DhMRs or DMRs in LK cells between Tet2-/- and DKO mice represents the impact of Tet2 loss on the genome of these cells. The small amount of overlap between DhMRs and DMRs within each genotype of LK cells is surprising, but these results suggest that decreased-5hmC does not necessarily result in increased-5mC in a specific locus of genome. It will be very informative to map in these cells, 5fC and 5caC genomic profiles simultaneously to determine their overlap with 5hmC or 5mC changes in the genome. As well, we observed no correlation between gene expression and DhMRs or DMRs in these LK cells that were isolated from pre-leukemic Tet2-/- or DKO mice. Similar observations have been reported in mouse ES cells (Ficz et al., 2011; Pastor et al., 2011; Williams et al., 2011). These observations suggest that distinct cytosine modifications (particularly 5hmC) mark specific genes in Tet2-/- or DKO LK cells without altering their expression. Such could work together with other genomic insults to alter gene expression. Indeed, the global transcriptome changed dramatically in transformed B-ALL DKO tumor cells, and most of these dysregulated genes are direct targets of Tet1 or Tet2. A recent study has shown that Tet2 loss cooperates with AML1-ETO oncoprotein to promote DNA hypermethylation on enhancers (leading to the loss of enhancer activity), resulting in lowered gene expression (Rasmussen et al., 2015), while Tet2 loss alone is likely unable to achieve this effect. Another recent study has shown that combined Tet2 loss and Flt3 mutation alter DNA methylation and gene expression in LSK cells, but not with either mutation alone, and similar results are seen in human AML patients with combined TET2 and FLT3 mutations (Shih et al., 2015).

These findings have several layers of implications: (1) they highlight the critical roles of each of the enzymes and the possible lines of communication between Tet1 and Tet2 in homeostasis of hematopoiesis and pathogenesis of hematological malignancies; (2) despite their similar catalytic activities in oxidation of 5mC, Tet1 is required for the 5hmC dysregulation, myeloid skewing, and myeloid malignancy development; TET1 therefore, likely plays a distinct role in myeloid leukemogenesis in the context of TET2 loss; (3) Tet1 and Tet2 seem to have overlapping roles in B cell development/tumorigenesis, where loss of Tet1 alone causes B cell malignancies and loss of both enzymes accelerates pathogenesis of B cell malignancies. This study expands upon our previous work that implicated Tet2 in myeloid malignancies (Li et al., 2011) and Tet1 in lymphoid malignancies (Cimmino et al., 2015). Our present studies suggest a novel role for Tet1 in Tet2-loss-mediated leukemogenesis as such making it a potential target for therapeutic intervention in myeloid malignancy patients. Moreover, it reveals that combined loss of Tet1/2 in mice induces B cell malignancies with a long latency. However, early changes in DNA 5hmC/5mC, CLP/BLP pool, and B cell development precede the development of disease. Whether Tet1/2 loss promotes changes in chromatin accessibility that facilitates acquisition of additional mutations requires further investigation. In summary, the studies presented here identify overlapping, non-redundant, and even opposite functions for Tet1 and Tet2 in hematopoietic regulation and leukemogenesis. Therefore, these studies provide a framework on pathology for further elucidating molecular mechanisms of critical lines of communication between TET1 and TET2 in the pathogenesis of hematological malignancies.

**EXPERIMENTAL PROCEDURES**

**Generation of Tet1 and Tet2 Conditional Knockout Mice**

Tet2:lox allele mice were generated as follows: the targeting vector was constructed in a plasmid containing a Neo cassette flanked by two FRT sites. A 0.8-kb genomic fragment containing the 11th exon of Tet2 (coding part of the catalytic domain) was inserted between twoloxP sites (cassette map: loxP-flx-Neo-FLP-Neo-FLP). For gene targeting, 4.4-kb 5’ and 3.6-kb 3’ arm genomic fragments were subcloned into the vector. The targeting vector was electroporated into 129/sv ES cells and subsequently screened by Southern blot (Figure S4B). Two positive clones were selected for the blastocyst injection. Male chimeric mice were crossed to C57BL/6 females to screen for germline transmission of Tet2:lox allele (Tet2lox). Tet2lox/+ mice were crossed to Flippase deleter mice to remove the Neo cassette (Figure S4B) (Li et al., 2011; Wang et al., 2014). Tet1:lox allele mice were generated from the previously reported Tet1loxlox mice (Dawlaty et al., 2011) as outlined in Figures S4C and S4D. Tet2loxMxCre, Tet1loxMxCre and Tet1loxMxCre mice were produced for studies. MxCre expression was induced...
by intraperitoneal injection of three doses of 300 μg of polyinosine-polycytosine (p(10)C010) (Figure S4E). Animal care was in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee (IACUC), University of Miami Miller School of Medicine and Department of Comparative Medicine, and Massachusetts Institute of Technology.

**Bioinformatics and Statistical Analyses**

hME-Seal was performed as described previously (Song et al., 2011). MeDIP was performed according to the manufacturer’s protocol. Bioinformatics analysis for 5hmC-seq and MeDIP-seq were described previously (Szulwach et al., 2011; Yao et al., 2014). Differences between experimental groups were determined by the Student’s t test or ANOVA followed by Newman-Keuls multiple comparison tests as appropriate. p values <0.05 were considered significant.

**ACCESSION NUMBERS**

Sequence data have been deposited to the NCBI GEO and are available under accession number GEO: GSE73611.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.10.037.

**AUTHOR CONTRIBUTIONS**

Z.Z., M.M.D., F.P., J.W., and S.C. performed the experiments involving animal models; Z.Z., Y.Z., Z.C., H.S., and W.Y. performed experiments involving human specimens; H.N. reviewed the blood smears and histopathologic sections; L.C., L.L., Z.Q., and P.J. analyzed the RNA-seq/5mC/5hmC data. M.M.D., O.W., S.D.N., F.-C.Y., and R.J. participated in designing the study and revised the manuscript; P.J. and M.X. designed and supervised the studies, performed the experiments, analyzed data, wrote the manuscript, and are responsible for its final draft.

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