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The catalytic activity of TET2 is essential for its myeloid malignancy-suppressive function in hematopoietic stem/progenitor cells

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Mutations/deletions of the *TET2* gene frequently occur in multiple spectra of myeloid malignancies, including myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN), acute myeloid leukemia (AML), and chronic myelomonocytic leukemia (CMML).¹⁻⁴ *TET2* mutations/deletions are often heterozygous and ancestral in these myeloid malignancies.¹⁻⁴ *TET2* plays an important role in hematopoietic stem cell (HSC) biology, and *Tet2*-loss leads to an increased HSC self-renewal and skewed differentiation favoring granulocytic/monocytic lineage.⁵⁻⁸ Furthermore, *Tet2*-deletion (*Tet2*^{-/-}) or -

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haploinsufficiency (*Tet2*^{+/-}) leads to myeloid malignancies in mice.⁵⁻⁷ TET2, therefore, acts as a tumor suppressor in hematopoiesis.

The TET family of proteins shares the conserved Cys-rich domain and double-stranded beta helix domain, which are the catalytic center of Fe²⁺- and 2-oxoglutarate-dependent dioxygenases.⁹ TETs exhibit unique enzymatic function to facilitate the DNA demethylation process, oxidizing 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine and 5-carboxylcytosine in a stepwise manner.¹⁰⁻¹²

Giving the prominent role of TET2 in the pathogenesis of myeloid malignancies, TET2 and its pathways represent potential therapeutic targets for intervention. Therefore, it is important to clarify whether the catalytic activity of TET2 is essential for its myeloid malignancy-suppressive function. In addition, although myeloid malignancies are clonal disorders of hematopoietic stem/progenitor cells (HSC/HPCs), it remains to be established at which HSC developmental stages, *TET2*-loss is capable of inducing myeloid malignancies. In this study, using mice with lineage specific *Tet2*-inactivation and transplantation assays, we reveal that *Tet2* loss in HSC/HPCs, but not more differentiated cells, is capable of inducing myeloid malignancies. By examining the ability of wild-type (WT) and catalytic inactive mutant TET2 to rescue the *in vitro* hyper-replating potential and *in vivo* myeloid malignancy-initiating capacity of *Tet2*^{-/-} HSC/HPCs, we demonstrate that the TET2 catalytic activity is essential and critical for its myeloid malignancy-suppressive function in HSC/HPCs.

We first examined the presence of *TET2* mutations in BM CD34⁺, CD33⁺, CD19⁺ and CD3⁺ cell populations purified from myeloid malignancy patients with known *TET2* mutations. *TET2* mutations were present in each of the CD34⁺, CD33⁺ and CD19⁺, but not CD3⁺, cell populations from all three patients examined (Figs. 1A and S1A), indicating the occurrence of *TET2* mutations at the common myeloid-B-progenitor level. Such primitive progenitor cells have been described.¹³ Deletion of *Tet2* leads to myeloid malignancies in mice.⁵⁻⁷ We next investigated at which HSC differentiation stages *TET2* loss is capable of inducing myeloid malignancies. We first generated *Tet2*^{fl/f}; *LysMCre* mice,¹⁴ in which *Tet2* is inactivated in monocytes/granulocytes/macrophages but not LSK, LK and lymphoid cells (Fig. S1B,C), and then determined if *Tet2*^{fl/f}; *LysMCre* mice develop myeloid malignancy as they age. *Tet2*^{fl/f}; *Vav1Cre* mice were used as positive control as animals with *Tet2*-inactivation in pan-hematopoietic cells (including HSC/HPCs) have been shown to develop myeloid malignancies.^{6, 7} Consistently, aged *Tet2*^{fl/f}; *Vav1Cre* mice (12-14 month of age) had increased WBC counts (mainly caused by neutrophilia and monocytosis), splenomegaly, and dramatically increased proportions of Gr1⁺/Mac1⁺ cells in their BM, spleen and PB as compared to age-matched *Tet2*^{fl/f} mice (Figs. 1B-D and S1D). Histological examination of H&E-stained BM, spleen and liver sections of *Tet2*^{fl/f}; *Vav1Cre* mice revealed extensive infiltration of mature myeloid cells, which disrupted their splenic and hepatic architecture (Fig. 1E). By contrast, no difference was observed in any of these hematological parameters between *Tet2*^{fl/f}; *LysMCre* and *Tet2*^{fl/f} mice (Figs. 1B-E and S1D). In addition, ~36% of the *Tet2*^{fl/f}; *Vav1Cre* mice, but none of the *Tet2*^{fl/f}; *LysMCre* and WT mice, died within 400 days of their life (Fig. 1F). Therefore, aged *Tet2*^{fl/f}; *Vav1Cre*, but not *Tet2*^{fl/f}; *LysMCre* mice

phenotypically show characteristics of CMML or MPN, demonstrating that deletion of *Tet2* in differentiated myeloid cells is not sufficient to cause myeloid malignancy in mice.

Transplantation of *Tet2*^{-/-} bone marrow (BM) cells causes CMML-like phenotype in WT recipient mice.⁵ We next transplanted BM Lin⁻Sca-1⁺c-Kit⁺ (LSK), Lin⁻Sca-1⁻c-Kit⁺ (LK) or Lin⁺c-Kit⁻ cells sorted from 2 independent 6-week old *Tet2*^{-/-} mice into lethally-irradiated WT recipients (3 mice/cell population/donor) (Fig. S2A). The recipient mice were sacrificed 32 weeks after transplantation and their hematological phenotype were analyzed. Five of the 6 mice receiving *Tet2*^{-/-} LSK cells and 3 of the 6 mice receiving *Tet2*^{-/-} LK cells, but none of the mice receiving *Tet2*^{-/-} Lin⁺c-Kit⁻ cells, exhibited monocytosis/neutrophilia, splenomegaly and infiltration of granulocytic/monocytic cells in their BM, spleen and liver (Figs. 1G and S2B-F), reminiscent of the CMML (with over 10% of Mac1⁺F4/80⁺ monocytes in BM cells, Fig. S2E) or MPN (with less than 10% of Mac1⁺F4/80⁺ monocytes in BM cells, Fig. S2E) phenotype displayed in *Tet2*^{-/-} mice. These data indicate that *Tet2*-loss in HSC/HPCs, but not differentiated hematopoietic cells, is capable of inducing myeloid malignancies *in vivo*.

TET2 is a methylcytosine dioxygenase regulating cytosine hydroxymethylation in the genome.¹⁰⁻¹² Loss of *Tet2* in HSC/HPCs increases their replating potential and repopulating capacity.^{5, 14} We next examined the role of the TET2 catalytic activity in *Tet2* loss-mediated hyper-replating potential *in vitro* and myeloid malignancy-initiating capacity *in vivo* by *Tet2*^{-/-} HSC/HPCs. We created lentiviral constructs using synthesized mammalian codon-optimized mouse *Tet2* cDNA sequences (Tet2opt) and catalytic domain-inactive mutant *Tet2* cDNA sequences (Tet2mu, H1295Y×D1297A) to successfully overexpress FLAG-tagged Tet2opt and Tet2mu along with GFP in both MEL and *Tet2*^{-/-} Lin⁻c-Kit⁺ cells (Figs. 2A,B and S3A). MEL cells are a mouse erythroleukemia cell line, arrested at the proerythroblast stage of development. Dot blot assays revealed a dramatically higher genomic 5-hmC level in MEL cells overexpressing Tet2opt, while comparable 5-hmC levels in MEL cells overexpressing Tet2mu as compared to cells expressing GFP alone (Fig. 2C). When replating assays were performed with GFP⁺ *Tet2*^{-/-} Lin⁻c-Kit⁺ cells expressing GFP, Tet2opt/GFP or Tet2mu/GFP, significantly higher colony numbers were documented from *Tet2*^{-/-} Lin⁻c-Kit⁺ cells expressing GFP or Tet2mu in round 2-5 of replating as compared to WT Lin⁻c-Kit⁺ cells (Fig. 2D). By contrast, re-introducing Tet2opt into *Tet2*^{-/-} Lin⁻c-Kit⁺ cells fully rescued their hyper-replating potential (Fig. 2D). Furthermore, the myeloid malignancy-initiating capacity of GFP⁺ *Tet2*^{-/-} Lin⁻c-Kit⁺ cells expressing GFP alone, Tet2opt/GFP or Tet2mu/GFP were determined by transplanting them into WT recipients (3 mice/cell population) (Fig. S3A,B). Interestingly, 32 weeks after transplantation, 66.7% of the mice receiving *Tet2*^{-/-} Lin⁻c-Kit⁺ cells expressing GFP alone or Tet2mu/GFP displayed monocytosis/neutrophilia, splenomegaly, and increased proportion of Gr1⁺/Mac1⁺ granulocytic/monocytic cells and Mac1⁺F4/80⁺ monocytes in their BM, resembling CMML (Fig. 2E-H). By contrast, none of the mice receiving *Tet2*^{-/-} Lin⁻c-Kit⁺ cells expressing Tet2opt/GFP exhibited any sign of abnormal hematopoiesis (Fig. 2E-H). These data indicate that re-introducing WT Tet2, but not the catalytic inactive mutant, rescued the myeloid malignancy-initiating capacity by *Tet2*^{-/-} Lin⁻c-Kit⁺ cells. Thus, the catalytic activity of TET2 is essential and critical for its myeloid malignancy-suppressive function in HSC/HPCs.

In summary, TET2 requires its catalytic activity in HSC/HPCs to exert its myeloid malignancy-suppressive function. The *TET2* mutations/deletions in myeloid malignancy patients are mostly heterozygous (haploinsufficient in TET2),¹⁻⁴ and heterozygous *TET2* mutations also occur in >5% of normal elderly individuals.¹⁵ We have shown that *Tet2*-haploinsufficiency (*Tet2*^{+/-}) is sufficient to cause a CMML-like myeloid malignancy in mice.⁵ Therefore, increasing the remaining WT TET2 enzymatic activity to compensate/overcome the TET2 haploinsufficiency in HSC/HPCs could represent an effective preventive and therapeutic strategy for individuals (including myeloid malignancy patients) with heterozygous *TET2* mutations. Future work is warranted to identify agents that are capable of increasing the TET2 catalytic activity *in vivo* and to test the chemoprevention and therapeutic potential of these agents in animal models.

Supplementary Material

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Figure 1. *Tet2* loss in hematopoietic stem/progenitor cells, but not more differentiated hematopoietic cells results in myeloid malignancies *in vivo*

(A) *TET2* mutations are present in BM CD34⁺, CD33⁺ and CD19⁺, but not CD3⁺ cell populations in CMML and MPN patients with *TET2* mutations. (B) WBC and monocyte counts were performed with cohorts of 12-14 month-old *Tet2*^{fl/fl}, *Tet2*^{fl/fl}; *Vav1Cre* and *Tet2*^{fl/fl}; *LysMCre* mice (n=7-9). (C) The gross morphologies of spleen from representative diseased *Tet2*^{fl/fl}; *Vav1Cre* as well as age-matched *Tet2*^{fl/fl} and *Tet2*^{fl/fl}; *LysMCre* mice. (D) Quantitation of the percent monocytic/granulocytic cells (Gr1/Mac1) in BM, spleen and PB

of diseased *Tet2^{fl/f};Vav1Cre* and age-matched *Tet2^{fl/f}* and *Tet2^{fl/f};LysMCre* mice (mean $\pm$ SD, 4-5 mice/genotype). (E) H&E stained histological sections of femurs, spleen and liver from representative diseased *Tet2^{fl/f};Vav1Cre* as well as age-matched *Tet2^{fl/f}* and *Tet2^{fl/f};LysMCre* mice. (F) Kaplan-Meier survival curve of *Tet2^{fl/f}* (n=25), *Tet2^{fl/f};Vav1Cre* (n=14) and *Tet2^{fl/f};LysMCre* (n=22) mice up to 400 days. *** $P<0.001$. (G) Pie charts show the disease spectra in recipient mice 32 weeks after transplantation of Lin⁻Sca-1⁺c-Kit⁺ (LSK), Lin⁻Sca-1⁻c-Kit⁺ (LK) or Lin⁺c-Kit⁻ cells isolated from 2 independent 6-week old *Tet2^{-/-}* mice (3 mice/cell population/donor). MPN: myeloproliferative neoplasm; CMML: chronic myelomonocytic leukemia.

Figure 2. WT Tet2, but not catalytic domain inactive mutant, is capable of rescuing the hyper-replating and myeloid malignancy-initiating potential by *Tet2*^{-/-} Lin⁻c-Kit⁺ cells

(A) Schematic representation of the lentiviral vectors containing the mammalian codon-optimized cDNA sequences of mouse WT *Flag-Tet2* (Tet2opt) or catalytic domain inactive mutant *Flag-Tet2* (Tet2mu, H1295Y×D1297A). Both vectors contain a Flag-tag at their N-terminal. (B) MEL cells were transduced with lentivirus encoding GFP, Tet2opt/GFP or Tet2mu/GFP. Western blot was performed using whole cell lysates prepared from GFP⁺ MEL cells of each transduction with anti-Flag and anti-Actin antibodies. (C) Genomic DNA

was extracted from GFP⁺ MEL cells of each transduction and blotted onto nitrocellulose membrane after 2-fold serial dilution. 5hmC levels were detected with an anti-5hmC antibody. Methylene blue staining was performed to ensure equal spotting of total DNA on the membranes. Quantification of the signal density was shown in the left panel (mean $\pm$ SD of the 0.5, 1 and 2 μ g DNA dot blots; relative to Tet2opt). **(D)** BM Lin⁻c-Kit⁺ cells were isolated from 6-week old WT or *Tet2*^{-/-} mice. *Tet2*^{-/-} Lin⁻c-Kit⁺ cells were then transduced with lentivirus encoding GFP, Tet2opt/GFP or Tet2mu/GFP for 48 hours. Colony-formation assays were then performed with WT Lin⁻c-Kit⁺ cells and FACS-sorted GFP⁺ *Tet2*^{-/-} Lin⁻c-Kit⁺ cells of each transduction in methylcellulose medium (500 cells/plate). Colonies were passaged every 7 days for 4 sequential plating (P1-5). Data are shown as mean $\pm$ SD, where each plating was performed in triplicate (representative of 3 independent experiments). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ **(E-H)** FACS-sorted GFP⁺ *Tet2*^{-/-} Lin⁻c-Kit⁺ cells (CD45.2⁺) of each transduction were transplanted into irradiated CD45.1⁺ B6.SJL recipients (Fig. S3B). The recipients were sacrificed 32 weeks after transplantation for the following analyses: PB counts, May-Giemsa stained PB smears (E), spleen size (F), and the GFP⁺ cell chimerism (*top*) and the frequency of granulocytic/monocytic cells (Gr1/Mac1, *middle*) and monocytes (Mac1/F4/80, *bottom*) in the BM (G). Pie charts show the disease spectra in recipient mice transplanted with GFP⁺ *Tet2*^{-/-} Lin⁻c-Kit⁺ cells expressing GFP alone, Tet2opt/GFP or Tet2mu/GFP (H). Analyses on representative recipients receiving *Tet2*^{-/-} Lin⁻c-Kit⁺ cells of each transduction are shown (E-G).