

Published in final edited form as:

Cancer Cell. 2011 July 12; 20(1): 11–24. doi:10.1016/j.ccr.2011.06.001.

Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation

Kelly Moran-Crusio^{1,2,11}, Linsey Reavie^{1,2,11}, Alan Shih^{3,4,11}, Omar Abdel-Wahab^{3,4}, Delphine Ndiaye-Lobry^{1,2}, Camille Lobry^{1,2}, Maria E. Figueroa⁵, Aparna Vasanthakumar⁶, Jay Patel³, Xinyang Zhao⁷, Fabiana Perna⁷, Suveg Pandey³, Jozef Madzo⁶, Chunxiao Song⁸, Qing Dai⁸, Chuan He⁸, Sherif Ibrahim¹, Miloslav Beran⁹, Jiri Zavadil¹⁰, Stephen D. Nimer^{4,7}, Ari Melnick⁵, Lucy A. Godley⁶, Iannis Aifantis^{1,2,12,13}, and Ross L. Levine^{3,4,12,13}

¹Department of Pathology and NYU Cancer Institute, New York University School of Medicine, New York, NY 10016, USA

²Howard Hughes Medical Institute, New York University School of Medicine, New York, NY 10016 USA

³Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York 10016, NY, USA

⁴Leukemia Service, Department of Medicine, Memorial Sloan Kettering Cancer, New York 10016, NY, USA

⁵Division of Hematology/Oncology, Weill Cornell Medical College, New York 10016, NY, USA

⁶Department of Medicine, The University of Chicago, Chicago, Illinois 60637, USA

⁷Molecular Pharmacology and Chemistry Program, Sloan Kettering Institute, New York 10016, NY, USA

⁸Department of Chemistry and Institute for Biophysical Dynamics, The University of Chicago, Chicago, Illinois 60637, USA

⁹Department of Leukemia, M.D. Anderson Medical Center, Houston, TX, USA

¹⁰Department of Pathology, NYU Cancer Institute and Center for Health Informatics and Bioinformatics, NYU Langone Medical Center, New York, New York 10016, USA

SUMMARY

Somatic *loss-of-function* mutations in the ten-eleven-translocation-2 (*TET2*) gene occur in a significant proportion of patients with myeloid malignancies. Although there are extensive genetic data implicating *TET2* mutations in myeloid transformation, the consequences of *Tet2* loss in the hematopoietic compartment have not been delineated. We report here an animal model of conditional *Tet2* loss in the hematopoietic compartment which leads to increased stem cell self-renewal *in vivo* as assessed by competitive transplant assays. *Tet2* loss leads to a progressive

© 2011 Elsevier Inc. All rights reserved.

¹³**Address Correspondence To:** Iannis Aifantis, Ph.D., Howard Hughes Medical Institute, Department of Pathology, NYU School of Medicine, 550 First Avenue, MSB 504, New York, NY, 10016, USA, iannis.aifantis@nyumc.org or to: Ross L. Levine, M.D., Human Oncology and Pathogenesis Program, Leukemia Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, 1275 York Ave, Box 20, New York, NY, 10065, leviner@mskcc.org.

^{11, 12}These authors contributed equally to this study

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

enlargement of the hematopoietic stem cell compartment and eventual myeloproliferation *in vivo* including splenomegaly, monocytosis, and extramedullary hematopoiesis. In addition, *Tet2*^{+/-} mice also displayed increased stem cell self-renewal and extramedullary hematopoiesis, suggesting *Tet2* haploinsufficiency contributes to hematopoietic transformation *in vivo*.

INTRODUCTION

Genetic studies of patients with myeloid malignancies have identified recurrent somatic alterations in the majority of patients with myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). A subset of myeloid disease alleles can be classified into two distinct complementation groups; one class which includes mutations which activate oncogenic signaling pathways, and a second class of mutations that perturb myeloid differentiation (Gilliland and Griffin, 2002). However, recent studies have suggested that this model of myeloid transformation does not accurately represent the biologic and clinical heterogeneity of MPN, MDS and AML, and that not all leukemogenic disease alleles can be classified by their ability to directly affect signaling and/or differentiation. Consonant with this notion, recent studies have identified recurrent mutations of known and putative epigenetic modifiers in patients with myeloid malignancies. These include somatic mutations in chromatin modifying enzymes (Ernst et al., 2010; Nikoloski et al., 2010) and in DNA methyltransferases (Ley et al., 2010; Yamashita et al., 2010). In addition, biologic studies of recurrent chromosomal translocations, including MLL fusions, have shown that leukemogenic fusion proteins alter epigenetic regulation in hematopoietic cells resulting in changes in chromatin state at specific loci (Dorrance et al., 2006; Krivtsov et al., 2008). Taken together, these data suggest that somatic alterations in genes that regulate the epigenetic state of hematopoietic cells are a common pathogenetic event in leukemogenesis (Abdel-Wahab and Levine, 2010). Importantly, recent studies have identified recurrent mutations in epigenetic master regulators in lymphoid malignancies (Morin et al., 2010) and in solid tumors (van Haften et al., 2009; Varela et al., 2011), suggesting mutational dysregulation of the epigenetic machinery is a common theme in oncogenic transformation.

Recent studies have identified a novel class of somatic mutations in myeloid malignancies. Specifically, somatic deletions and *loss-of-function* mutations in the *TET2* gene were identified in 10–20% patients with MDS and MPN (Delhommeau et al., 2009; Langemeijer et al., 2009), subsequent studies identified recurrent *TET2* mutations in patients with chronic myelomonocytic leukemia (CMML) and AML (Abdel-Wahab et al., 2009; Jankowska et al., 2009), and demonstrated that *TET2* mutations were associated with adverse outcome in intermediate-risk AML (Metzeler et al., 2011). Within the TET family of proteins, TET1, TET2, and TET3 have been shown to modify DNA by hydroxylating 5-methylcytosine (5mC) (Ko et al., 2010; Tahiliani et al., 2009) and the *TET2* mutant proteins observed in these patients with myeloid malignancies have been shown to be deficient in this enzymatic function (Ko et al., 2010). In addition, recent work demonstrated that mutations in the metabolic enzymes IDH1 and IDH2 (Mardis et al., 2009; Parsons et al., 2008; Ward et al., 2010; Yan et al., 2009) are mutually exclusive with *TET2* mutations, and that production of 2-hydroxyglutarate by neomorphic IDH1/2 mutant proteins (Dang et al., 2009) inhibits *TET2* catalytic activity (Figuroa et al., 2010; Xu et al., 2011). Taken together, these data indicate that mutations that impair 5-hydroxymethylation represent a novel mechanism of transformation in myeloid malignancies.

Although genetic data has implicated loss of *TET2* function in myeloid transformation, the *in vivo* effects of *Tet2* loss have not been delineated. Recent *in vitro* studies using shRNA-based approaches have suggested a role for *TET2* in regulating myeloid differentiation

(Figuroa et al., 2010; Ko et al., 2010) and in regulating stem/progenitor cell proliferation (Figuroa et al., 2010). In order to elucidate the function of *Tet2* in hematopoiesis *in vivo*, we describe here the characterization of conditional deletion of *Tet2* in the hematopoietic compartment demonstrating a role for *Tet2* in regulating hematopoietic stem cell renewal and differentiation. These findings provide a model for human disease in which loss of *Tet2*, often through loss of a single allele, leads to increased self-renewal and myeloid transformation, and provides insight into how mutations in epigenetic modifiers contribute to malignant transformation.

RESULTS

Tet2 expression silencing leads to increased re-plating capacity

We first investigated the effects of *Tet2* silencing in hematopoiesis by transducing whole mouse bone marrow cells with retroviral vectors encoding validated *Tet2* shRNA vectors (Figuroa et al., 2010), and sorting for GFP-positive cells. Stable shRNA-mediated knock-down led to stable reductions in TET2 expression by 50–70% (Figure 1A). Since TET2 is an enzyme that hydroxylates 5-methylcytosine on DNA, its knock-down should lead to loss of 5-hydroxylation of methylcytosine (5hmC). We used a mass-spectrometry-based approach (Shah et al., 2010) to demonstrate that *Tet2* silencing results in a significant reduction in 5-hmC in GFP⁺ hematopoietic cells stably expressing *Tet2* shRNAs (Figure 1B). GFP⁺ cells were then plated in methylcellulose for CFU *in vitro* assays. We observed no significant differences in the number or the lineage specificity of *Tet2*-expressing and *Tet2*-silenced colonies after the first plating. However, at the second plating there was a two-fold increase in the number of colonies expressing *Tet2*-specific shRNAs (Figure 1C). Strikingly, *Tet2*-silenced cells retained the ability to serially replate and generate colonies, although essentially no wild type colonies were detected after the second plating. In agreement with the putative acquisition of a more immature phenotype, *Tet2* shRNA-expressing colonies upregulated c-Kit, a hematopoietic stem and progenitor cell marker (Figure 1D). These initial studies demonstrated that *Tet2* silencing reduces global 5hmC levels and increases the re-plating capacity of hematopoietic stem and progenitor cells.

Generation of a conditional *Tet2* knock-out allele

Although these studies provide important information on TET2 function, hematopoiesis is a dynamic process that is most accurately studied *in vivo*. We thus decided to conditionally inhibit *Tet2* gene expression in the hematopoietic compartment. We first assessed *Tet2* expression by performing qRT-PCR of sorted hematopoietic stem/progenitor subsets (LSK: Lin⁻ Sca-1⁺ c-Kit⁺; CMP: Lin⁻ Sca-1⁻ c-Kit⁺, FcγR¹⁰ CD34⁺, GMP: Lin⁻ Sca1⁻ c-Kit⁺ FcγR⁺ CD34⁺, MEP: Lin⁻ Sca-1⁻ c-Kit⁺ FcγR⁻ CD34⁻) and in differentiated hematopoietic lineages. Expression studies revealed that *Tet2* expression was ubiquitously expressed in the hematopoietic compartment, including in stem and progenitor subsets and in mature myeloid and lymphoid cells (Figure 2A). Purified human CD34⁺ peripheral blood mobilized cells also demonstrated expression of *TET2* in human hematopoietic stem cells (Figure S1A). Two different *TET2* isoforms are expressed including a shorter isoform b that lacks the C-terminal enzymatic domain. To understand the nature and location of *TET2* mutations in human myeloid malignancies and how this would inform our mouse model, we analyzed *TET2* mutations from 991 patients with a variety of myeloid malignancies (MPN, chronic myelomonocytic leukemia (CMML), and acute myeloid leukemia (AML)). Exon 3 in the murine *Tet2* locus encodes for approximately half of the TET2 protein; analysis from the mutational data identifies the corresponding exon 3 of human *TET2* as the most frequently mutated exon in myeloid malignancies, including a large number of nonsense mutations, insertions and deletion mutations which truncate the protein. Specifically, we found that 41.5% (51/123) of *TET2* mutations occurred in the first coding exon of *TET2* (exon 3) while

29.3% (36/123) of *TET2* mutations occurred in the last coding exon (exon 11) (Abdel-Wahab et al., 2009; Figueroa et al., 2010) (Figure 2B and Figure S1B). Moreover, the largest proportion of mutations which result in a premature stop codon occur in exon 3 (Figure 2B). In addition, exon 3 of *Tet2* is contained within the two described *Tet2* transcripts (*Tet2a* and *Tet2b*), which are both expressed in the hematopoietic compartment (Figure S1A). We therefore decided to target exon 3 using homologous recombination. We utilized ES cell targeting to insert two LoxP sites flanking exon 3, as well as an Frt-flanked neomycin selection cassette in the upstream intron (Figure 2C). The generated mice (*Tet2^{fl/fl}*) were initially crossed to a germline Flp-deleter murine line to eliminate the neomycin cassette and subsequently crossed to IFN α -inducible *Mx1-cre*, the hematopoietic specific *Vav-cre* and the germline *Ella-cre* mice (Kuhn et al., 1995; Lakso et al., 1996; Stadtfeld and Graf, 2005). Figures 2D and 2E demonstrate the efficient targeting of the locus and the generation of a recombined (E) allele upon Cre recombinase expression in hematopoietic cells. Although *Tet2* was recently suggested to play an important role in ES cell differentiation (Ito et al., 2010; Koh et al., 2011) *Ella-cre⁺Tet2^{-/-}* mice were born with the expected Mendelian ratios with normal growth and organ development. These data suggested that other members of the *Tet* family may have redundant functions in embryonic development and in homeostasis of different tissues. *Vav-Cre* mediated deletion led to a complete silencing of *Tet2* expression in the bone marrow, thymus and spleen of *Vav-cre⁺Tet2^{fl/fl}* animals. Interestingly, *Tet2* expression silencing was not accompanied by changes in expression of either *Tet1* or *Tet3*, which were both expressed in hematopoietic cells at similar levels before and after *Tet2* deletion (Figure 2F).

Tet2* deletion leads to an enhanced re-populating capacity of hematopoietic progenitors *in vitro

Given that RNAi-mediated *Tet2* silencing led to increased re-plating potential, we assessed whether deletion of TET2 in different hematopoietic subsets led to similar effects. We performed methylcellulose CFU assays using FACS-sorted Lin⁻c-Kit⁺ progenitors and LSK CD150⁺ HSC from *Vav-cre⁺Tet2^{-/-}* and *Vav-cre⁺Tet2^{+/+}* mice. In both cases, *Tet2* deficient cells demonstrated serial replating capacity (for at least 10 replatings followed by continuous liquid culture for 4 additional months) whereas *Tet2^{+/+}* counterparts differentiated and did not generate colonies after the second plating (Figure 3A). *Tet2^{-/-}* colonies were more homogeneous and less differentiated and contained cells with a promyeloblastic morphology (Figure 3B). Indeed, expression of c-Kit, a classical marker of hematopoietic progenitors and the receptor for stem cell factor, was up-regulated significantly in *Tet2^{-/-}* and was expressed in nearly all *Tet2^{-/-}* cells in the third plating and beyond (Figure 3C). Furthermore, the c-Kit expressing cells also upregulated the myeloid progenitor markers CD34 and Fc γ R (Figure 3C). Interestingly, comparison of gene expression profiles of *Tet2^{-/-}* cells at the fifth plating (CFU5) to different stem and progenitor populations showed that *Tet2^{-/-}* cells share a common gene expression program with common myeloid progenitors (CMP), suggesting that these are indeed c-Kit⁺ myeloid progenitors with the ability to replate indefinitely in culture (Figure S2). *Tet2^{-/-}* myeloid progenitor cells were also characterized by increased expression of the self-renewal regulators *Meis1* and *Evi1*, and by reduced expression of multiple myeloid specific factors (including *Cebpa*, *Cebpd*, *Mpo*, and *Csf1*), more consistent with multipotent LSK cells than committed CMP or GMP (Figure S2 and data not shown). These findings are consistent with the shRNA knock-down experiments shown in Figure 1 and suggest that *Tet2* loss leads to enhanced serial re-plating ability *in vitro*.

***Tet2* deletion leads to progressive defects in hematopoiesis**

We next performed detailed analysis of *in vivo* hematopoiesis in *Tet2^{-/-}* mice. Detailed phenotypic characterization of young (4–6 weeks) *Vav-Cre⁺Tet2^{-/-}* mice failed to reveal

gross alterations in bone marrow stem/progenitor numbers or in the myeloid compartment (Figure 4A, first panel). Likewise, there were no effects on the differentiation of lymphocyte lineages in the bone marrow (data not shown). However, detailed FACS analysis of *Tet2*^{-/-} spleens from young mice identified significant extramedullary hematopoiesis, as defined by a significant increase in the absolute numbers of c-Kit⁺ (data not shown), LSK cells (including CD150⁺ HSC), and myeloid progenitors (Figure 4B, C). Moreover, purification of *Tet2*^{+/+} and *Tet2*^{-/-} bone marrow progenitor populations (LSK, CMP, GMP) and subsequent transcriptome analysis revealed that *Tet2*^{-/-} LSK cells display a significant enrichment of a CMP gene expression signature (Figure S3 and Experimental Procedures), suggesting *Tet2* loss leads to aberrant hematopoiesis *in vivo*.

We next analyzed older (20 week) *Tet2*^{-/-} animals to determine the consequences of *Tet2* loss on long-term hematopoiesis. At 20 weeks after *Tet2* deletion, we noted an increase in the size of the bone marrow LSK compartment (Figure 4A, second panel). This phenotype was not accompanied by expansion of a specific stem or multipotential progenitor compartment as defined by the utilization of CD150/CD48 marker labeling. Further analysis of the progenitor compartment showed a marked increase in the GMP population, which includes progenitors of monocytes and granulocytes (Figure 4A). However, the most striking effects of *Tet2* deletion were noted in the spleen of these animals; *Tet2*^{-/-} but not *Tet2*^{+/+} mice displayed splenomegaly at 20 weeks of age, which was associated with an increase in the proportion of total CD11b⁺ cells (see below). Notably, extramedullary hematopoiesis was prominent, with a significant increase in the absolute number of LSK (Figure 4B, C) as well as CD150⁺ HSC (not shown) cells. These combined studies suggested that *Tet2* deletion leads to progressive defects in blood differentiation and to a significant elevation of extramedullary hematopoiesis.

Tet2* deficient hematopoietic stem cells show increased self-renewal ability *in vivo

As we have previously shown that *Tet2* deletion leads to increased re-plating capacity *in vitro*, we tested the ability of *Tet2*^{-/-} cells to compete directly against wild-type counterparts in an *in vivo* transplantation setting. We mixed identical numbers of either *Mx1-cre*⁺*Tet2*^{wt/wt}CD45.2⁺ or *Mx1-cre*⁺*Tet2*^{fl/fl} CD45.1⁺/CD45.2⁺ BM from littermate mice with wild-type CD45.1⁺ bone marrow and transplanted them in lethally irradiated CD45.1⁺ recipients (Figure 5A). Four weeks after transplantation, equal engraftment (approximately 50:50) rates were detected in peripheral blood and subsequently *Tet2* deletion was induced by polyI-polyC injections. The presence of the *Tet2* recombined allele was verified using genomic PCR of peripheral blood mononuclear cells. Chimerism was followed by FACS assessment of peripheral blood staining for CD45.1 vs CD45.2 (or CD45.1⁺45.2⁺). As evident from Figure 5B, upon *Tet2* deletion, *Tet2*^{-/-} chimerism increased rapidly as these cells out-competed their wild type counterparts. Indeed at 23 weeks post polyI-polyC deletion more than 95% of the peripheral blood cells (both myeloid and lymphoid) were *Tet2*^{-/-} (Figure 5C). To define in detail the developmental stage in which *Tet2*^{-/-} cells out-compete *Tet2*-expressing cells, we have analyzed transplanted recipients 23 weeks post polyI-polyC injection. Analysis of the LT-HSC compartment indicated that the vast majority (>90%) of these cells were *Tet2*^{-/-}, suggesting that TET2 deficient HSC have the ability to outcompete wild type counterparts. Once established, this bias towards the *Tet2*^{-/-} cells was also observed in downstream bone marrow multipotent progenitors (MPP1,2), myeloid progenitors (CMP), bone marrow mature CD11b⁺ monocytes and B220⁺ lymphocytes (Figure 5D–E). Competitive transplant studies using *VavCre*⁺-*Tet2*^{-/-} HSC demonstrated TET2 deficient cells outcompeted wild-type cells to a similar extent, further supporting our previous findings (Figure S4). These studies confirm that *Tet2* function regulates the self-renewal of adult HSC.

Tet2 deficient animals develop CMML-like disease

We next performed a detailed phenotypic analysis of *Tet2*^{-/-}*VavCre*⁺ mice 20 weeks after deletion. We found that *Tet2*^{-/-} animals but not *Tet2*^{+/+} littermates were characterized by progressive leukocytosis; this was associated with neutrophilia and a marked increase in peripheral monocyte counts (Figure 6A,B, Table 1 and Table S1). Peripheral blood analysis revealed that by week 20, approximately 70% of the *Tet2*^{-/-} *VavCre*⁺ mice developed significant peripheral blood monocytosis (Figure S5). Myeloid dysplasia was apparent in both bone marrow and peripheral lymphoid tissue, as defined by myeloid left shift (presence of blasts, promyelocytes, myelocytes, or metamyelocytes). In addition, presence of hypogranular cytoplasm or abnormal segmentation of the nuclei was detected in the *Tet2* deficient animals (Figure 6 B, D and not shown).

Moreover, *Tet2*^{-/-} mice had significantly enlarged spleens (Figure 6C) compared to littermate controls ($p < 0.05$). Detailed pathologic analysis revealed that approximately all *Tet2*^{-/-} mice by week 20 developed significant splenomegaly (spleen weight >250mg) consistent with significant myeloproliferation. Histopathologic analysis revealed that *Tet2*^{-/-} mice, but not *Tet2*^{+/+} littermate controls, had prominent histologic evidence of disease, including significant destruction of spleen architecture, infiltration of the liver and lung as well as bone marrow neutrophilia and monocytosis (Figure 6D). FACS analysis of the spleen of these mice revealed a significant enlargement of both the CD11b⁺ and CD11b⁺Gr1⁺ populations consistent with granulocytic and monocytic expansion (Figure 6E). Collectively, these studies demonstrate that *Tet2* loss is associated with myeloproliferation *in vivo*, and suggest that *TET2* mutations contribute to increased stem-cell self-renewal and to progressive myeloid expansion *in vivo*. Although splenomegaly and leukocytosis are observed in a spectrum of myeloid malignancies, the constellation of splenomegaly, extramedullary hematopoiesis, neutrophilia, and monocytosis is most consistent with human CMML. *TET2* mutations are most commonly observed in human CMML, with somatic *loss-of-function* mutations in more than 40% of well-annotated CMML patient cohorts (Abdel-Wahab et al., 2009; Jankowska et al., 2009). In agreement with the murine data, detailed clinical and molecular analysis of CMML patients revealed that *TET2*-mutant CMML patients presented with splenomegaly, elevated peripheral blood WBC, and monocytosis (Figure S1B). Moreover, *TET2*-mutant CMML was characterized by a paucity of co-occurring cytogenetic abnormalities; 5.9% of *TET2*-mutant patients had cytogenetic abnormalities versus 61.3% of *TET2*-wildtype CMML patients ($p = 0.018$, Figure S1B–C). These studies suggested that *Tet2* deletion leads to progressive myeloproliferation *in vivo* with cardinal features of human CMML.

Tet2 haploinsufficiency is sufficient to initiate aberrant hematopoiesis *in vitro* and *in vivo*

Although a small subset of leukemia patients present with biallelic *TET2* mutations and/or deletions, the majority of patients present with monoallelic *TET2* loss (Abdel-Wahab et al., 2009; Jankowska et al., 2009). We performed DNA and cDNA sequencing of patient samples with monoallelic *TET2* mutations and demonstrated these patients continue to express the wild type allele (Figure S6). These data suggest the possibility that haploinsufficiency for *TET2* may result in alterations in self-renewal, hematopoietic differentiation, and susceptibility to myeloid transformation. We therefore performed phenotypic analysis of *Tet2*^{+/-} (*Vav-Cre*⁺*Tet2*^{wt/f}) mice. We first purified CD150⁺ HSC from the bone marrow of *Tet2* heterozygotes and plated them in methylcellulose cultures. Similar to *Tet2*^{-/-} cells, *Tet2*^{+/-} cells were able to serially replat (Figure 7A), unlike wild-type HSC that failed to generate colonies after the second plating. Likewise, *Tet2*^{+/-} cells generated colonies with a similar immunophenotype as *Tet2*^{-/-} cells as they upregulated cell surface expression of c-Kit, CD34 and FcγR and exhibit an immature, pro-myeloblastic morphology (Figure 7B–C and data not shown) Furthermore, competitive reconstitution

experiments, performed as described in Figure 5A, demonstrated that *Tet2*^{+/-}, but not *Tet2*^{+/+} cells were able to outcompete wild-type counterparts, albeit with slower kinetics than *Tet2*^{-/-} cells (Figure 7D–7H). Indeed, 23 weeks post deletion more than 70% of peripheral blood cells were *Tet2*^{+/-} consistent with increased long-term self-renewal with loss of a single *Tet2* allele. Moreover, in a non-competitive setting, 20 week old *Vav-Cre*⁺*Tet2*^{wt/f} mice showed increased circulating monocytes (Figure 7I) and extramedullary hematopoiesis (Figure 7J–K) with a significant increase of both Lineage^{neg} c-Kit⁺ and LSK cells (Figure 7J and data not shown). These data clearly demonstrate that *Tet2* loss leads to dose-dependent effects on hematopoiesis and on myeloid transformation, and that monoallelic *TET2* loss is an important pathogenetic event in myeloid malignancies.

DISCUSSION

The identification of recurrent somatic *loss-of-function* mutations in *TET2* provides genetic evidence that mutational inactivation of enzymes which regulate cytosine hydroxymethylation is a common pathogenetic event in myeloid malignancies (Delhommeau et al., 2009; Ko et al., 2010; Langemeijer et al., 2009). In addition, genetic and functional studies suggest that neomorphic *IDH* mutations contribute to myeloid transformation, at least in part, by inhibiting TET enzymatic function (Figuroa et al., 2010; Xu et al., 2011). Although genetic and *in vitro* studies suggested a role for TET2 in regulating hematopoietic differentiation and stem/progenitor cell expansion, the *in vivo* effects of *Tet2* loss had not previously been described. Here we report the effects of *Tet2* loss in the hematopoietic compartment, which has allowed us to make several important observations. First, we found that *Tet2* loss leads to increased replating capacity *in vitro*, and that *Tet2* deficient cells had enhanced repopulating activity in competitive reconstitution assays consistent with enhanced HSC function *in vivo*. Second, *Tet2* loss led to progressive myeloproliferation *in vivo*, with features characteristic of human CMML. In addition, we found that *Tet2* haploinsufficiency confers increased self-renewal to stem/progenitor cells and to extramedullary hematopoiesis, suggesting that heterozygous loss of *TET2*, as is commonly observed in myeloid malignancies, is sufficient to contribute to myeloid transformation *in vivo*.

Our *in vivo* investigation of the effects of *Tet2* loss is consistent with several observations emanating from previous genetic and functional studies. The presence of *TET2* mutations in a significant proportion of MPN, MDS, and AML patients is consistent with the notion that *TET2* loss confers increased self-renewal to stem/progenitor cells *in vivo*, which then leads to acquisition of mutations which direct the phenotype of *TET2*-mutant hematopoietic cells (Haeno et al., 2009; Tefferi, 2010). These data are also consistent with previous xenotransplantation studies of a small cohort of *TET2* mutant primary MPN samples that demonstrated that *TET2*-mutant MPN cells could engraft NOD-SCID mice (Delhommeau et al., 2009). In addition, genetic studies of MPN patients who subsequently transformed to AML identified *TET2* mutations as a recurrent somatic mutation during progression from MPN to AML (Abdel-Wahab et al., 2010; Schaub et al., 2010). Our data implicate increased self-renewal as a mechanism of transformation by TET2 mutations, which contributes to disease initiation and progression. Our transcriptional data suggest that *Tet2* loss leads to increased expression of myeloid-specific and self-renewal gene programs. However, subsequent studies will have to functionally investigate which downstream targets of *Tet2* loss are required to confer enhanced self-renewal on stem/progenitor cells. Recent work suggested that TET proteins regulate embryonic stem cell self-renewal and differentiation (Ito et al., 2010; Koh et al., 2011). Taken together, the data suggest that *Tet2* deletion likely has effects at multiple stages in hematopoietic differentiation, and that regulation of hydroxymethylation by TET enzymes controls stem cell self-renewal and differentiation in different cellular contexts. However, the exact mechanisms by which perturbations in TET

enzyme function remain to be delineated. This is particularly evident given recent studies suggesting a dynamic interplay between changes in TET1-mediated hydroxymethylation and other epigenetic marks in regulating gene expression (Ficz et al., 2011; Williams et al., 2011; Wu et al., 2011), and it is likely additional alterations in the epigenetic state cooperate with TET2 mutations in malignant transformation.

In addition to effects of *Tet2* loss on self-renewal, we found that *Tet2* loss led to progressive myeloproliferation *in vivo*. Two recent studies used RNAi-mediated *Tet2* knock-down *in vitro* to suggest that TET2 depletion led to impaired hematopoietic differentiation and to preferential myeloid commitment (Figuroa et al., 2010; Ko et al., 2010). These data are consistent with our *in vivo* data, as we observe progressive hematopoietic stem/progenitor cell expansion and myeloproliferation *in vivo* with *Tet2* deletion or haploinsufficiency notable for neutrophilia, monocytosis, and splenomegaly. Although these features are seen, to a varying degree, in different myeloid malignancies, they are most commonly observed in human CMML suggesting that *Tet2* loss in the absence of other genetic lesions favors progressive myelomonocytic expansion. This is consistent with the observations that *TET2* mutations are most common in CMML (Abdel-Wahab et al., 2009; Jankowska et al., 2009) and that *TET2*-mutant CMML is characterized by fewer additional somatic cytogenetic alterations compared to *TET2*-wild-type CMML (Figure S1B–C). Most importantly, we demonstrate that *Tet2* haploinsufficiency is sufficient to confer increased self-renewal to stem/progenitor cells and to promote myeloproliferation *in vivo*. Given the vast majority of patients with *TET2*-mutant hematopoietic malignancies retain a wild-type copy of *TET2*, our data provide functional evidence that loss of a single *TET2* allele can contribute to transformation.

Collectively, the presented data implicate *TET2* as a master regulator of normal and malignant hematopoiesis. It is likely that *TET2* has distinct roles in other hematopoietic lineages, and may contribute to transformation in lymphoid malignancies or even in epithelial tumors. These data also suggest that dysregulation of hydroxymethylation by mutations in the TET family of enzymes and by other somatic mutations may contribute to malignant transformation in other contexts. In addition, it is possible that therapies that modulate hydroxymethylation levels might be of benefit in malignancies characterized by loss of TET enzyme function by inhibiting malignant stem cell self-renewal. Moreover, our studies provide insights into mechanisms of transformation by a novel class of mutations found in myeloid malignancies and in other tumors, and we predict that subsequent studies will identify additional mutations in epigenetic modifiers, which contribute to neoplasia by similar mechanisms.

EXPERIMENTAL PROCEDURES

Animals

All animals were housed at New York University School of Medicine or at Memorial Sloan Kettering Cancer Center. All animal procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees (IACUC) at New York University School of Medicine and Memorial Sloan Kettering Cancer Center.

Generation of *Tet2* deficient mice

The *Tet2* allele was deleted by targeting exon 3. Briefly, we have inserted two LoxP sites flanking Exon 3, as well as a Frt-flanked Neomycin selection cassette in the upstream intron (Figure 3A). Ten micrograms of the targeting vector was linearized by NotI and then transfected by electroporation of BAC-BA1 (C57BL/6 × 129/SvEv) hybrid embryonic stem

cells. After selection with G418 antibiotic, surviving clones were expanded for PCR analysis to identify recombinant ES clones. Secondary confirmation of positive clones identified by PCR was performed by Southern Blotting analysis. DNA was digested with ScaI, and electrophoretically separated on a 0.8% agarose gel. After transfer to a nylon membrane, the digested DNA was hybridized with a probe targeted against the 3' or 5' external region. DNA from C57Bl/6 (B6), 129/SvEv (129), and BA1 (C57Bl/6 × 129/SvEv) (Hybrid) mouse strains were used as wild type controls. Positive ES clones were expanded and injected into blastocysts.

The generated mice (*Tet2^{fl/fl}*) were initially crossed to a germline Flp-deleter (Jackson Laboratories), to eliminate the neomycin cassette and subsequently to the IFN α -inducible Mx1-cre (Jackson Laboratories), the hematopoietic specific Vav-cre and the germline EIIa-cre (Lakso et al., 1996). The Vav-Cre transgenic line was generated by the Graf laboratory (Stadtfeld and Graf, 2005) and were a gift from Virginia Shapiro (Mayo Clinic).

***in vivo* Studies**

Tet2 conditional and control mice received 5 intra-peritoneal injections of poly(I:C) every other day at a dose of 20ug/g of body weight starting at 2 weeks post birth. For the hematopoietic specific Vav-cre line, *Tet2^{fl/fl} VavCre⁺*, *Tet2^{fl/w} VavCre⁺* and *Tet2^{w/w} VavCre⁺* mice were analyzed between 2 and 20 weeks of age. Thymus, bone marrow, spleen and peripheral blood were analyzed by flow cytometry and formalin-fixed paraffin embedded tissue sections stained with hematoxylin and eosin. Peripheral blood was smeared on a slide and stained using the Giemsa-Wright staining method. Tissue sections and blood smears were evaluated by a hematopathologist. Deletion of the *Tet2* allele and transcript were measured by genomic PCR and quantitative real-time PCR.

Gene Expression Analysis

LSK and CMP cells were sorted from *Tet2^{+/+} Mx-1⁺*, *Tet^{-/-} Mx-1⁺* littermate mice 4 weeks post deletion via pIC injection. Additionally cells were obtained from serial replating of CD150⁺ sorted *Tet2* deficient cells. RNA was isolated using RNeasy RNA isolation kit (Qiagen) according to manufacturer's protocol. A portion of total RNA (5ng) from target cell populations was amplified and then labeled with the Ovation RNA Amplification System v2 and Ovation cDNA Biotin System (Nugen). The resulting cDNA was hybridized to GeneChip MG 430 2.0 arrays according to the array manufacturer's recommendations (Affymetrix). The Affymetrix gene expression profiling data was normalized using the previously published Robust Multi-array Average (RMA) algorithm using the GeneSpring GX software (Agilent, Palo Alto, CA). The gene expression intensity presentation was generated with GeneSpring GX software and Multi Experiment Viewer software. Hierarchical clustering on genes and samples was performed using Euclidean Distance as a metric and average linkage method (Bolstad et al., 2003).

GeneSet Enrichment Analysis

Geneset Enrichment Analysis was performed using GSEA (Subramanian et al., 2005) <http://www.broadinstitute.org/gsea/>, using Gene set as permutation type, 1000 permutations and log₂ ratio of classes as metric for ranking genes. Independent stem cell gene expression signatures are obtained by <http://franklin.imgen.bcm.tmc.edu> and Ng et al. (Ng et al., 2009).

The "CMP signature" genesets were generated using a systematic approach based on the comparison of gene expression arrays from WT LSK and WT GMP. Genes that were significantly modulated in CMP compared to LSK (over 1.33 fold, p-value<0.05) were used to define CMP vs LSK_UP geneset containing upregulated genes and CMP vs LSK_DWN containing downregulated genes.

In vitro colony forming assays

CD150⁺ LSK as well as ckit⁺ cells were sorted from the bone marrow of *Tet2*^{-/-} *VavCre*⁺, *Tet2*^{+/-} *MxCre*⁺, *Tet2*^{-/-} *Mx-1Cre*⁺, *Tet2*^{+/+} *VavCre*⁺, or *Tet2*^{+/+} *Mx-1Cre*⁺ mice and seeded at a density of 500 cells/replicate for the CD150⁺ subset and 2000 cells/replicate for the cKit⁺ subset into cytokine supplemented methylcellulose medium (Methocult, M3434, Stem Cell Technologies). Colonies propagated in culture were scored at day 7.

Representative colonies were isolated from the plate for cytopins. Remaining cells were resuspended, counted, and a portion was stained for c-kit (clone 2B8, Biolegend), and re-plated (4000 cells/replicate) for a total of 5 platings (7, 14, 21, 28 and 35 days). Cytopins were performed by resuspending in warm PBS and spun to the slides at 350g for 5 minutes. Slides were air-dried and stained using the Giemsa Wright method.

Bone marrow transplantation

Freshly dissected femurs and tibias were isolated from *Tet2*^{w/w} *Mx-1Cre*⁺ CD45.2⁺, *Tet2*^{f/w} *Mx-1Cre*⁺ CD45.2⁺, or *Tet2*^{f/f} *Mx-1Cre*⁺ CD45.1⁺/CD45.2⁺ mice. Hence, *Tet2* deficient animals are expressing both CD45.2 and CD45.1 markers while *Tet2* heterozygous animals and *Tet2* WT control animals express only the CD45.2 marker. Bone marrow was flushed with a 1cc insulin syringe into PBS supplemented with 3% fetal bovine serum. The bone marrow was spun at 0.5g by centrifugation at 4 C and following red blood cells were lysed in ammonium chloride-potassium bicarbonate lysis buffer for 3 minutes on ice. After centrifugation, cells were re-suspended in PBS + 3%FBS, passed through a cell strainer and counted. Finally, 0.5×10^6 total bone marrow cells of *Tet2*^{w/w} *Mx-1Cre*⁺ CD45.2⁺, *Tet2*^{f/w} *Mx-1Cre*⁺ CD45.2⁺, or *Tet2*^{f/f} *Mx-1Cre*⁺ CD45.1⁺/CD45.2⁺ cells were mixed with 0.5×10^6 wild-type CD45.1⁺ supporting bone marrow and transplanted via retro-orbital injection into lethally irradiated (2x 550 Rad) CD45.1⁺ host mice. Chimerism was measured by FACS in peripheral blood at 4 weeks post-transplant (week 0, pre-polyI-polyC). Deletion of *Tet2* was initiated at this time point via 5 intra-peritoneal injections of polyI-polyC at a dose of 20ug/g of body weight. Chimerism for the duration of the experiment was subsequently followed via FACS in the peripheral blood every two weeks (week 0, 2, 4, 6, 8, 10, 12, 14 and 16 post polyI-polyC injection). Additionally, for each bleeding, whole blood cell counts were measured on a blood analyzer and peripheral blood smears were scored. Chimerism in the bone marrow, spleen and thymus was evaluated at 10 and 14 weeks via animal sacrifice and subsequent FACS analysis.

Mouse bone marrow infections and shRNA Colony Assays

Bone marrow was harvested from the femur of wild-type C57Bl/6 mice. After red cell lysis, the bone marrow was cultured in media containing RPMI/10% FBS and IL-3 (7ng/ml), IL-6 (10ng/ml), and stem cell factor (10ng/ml). The next day, cells were infected with MSCV retroviral vectors for control and *Tet2* shRNAs containing IRES GFP. Targets for shRNAs can be found in supplemental experimental procedures. Cells were infected twice in the presence of polybrene and then sorted for GFP⁺ cells 48hrs following second infection. 1×10^4 cells were plated per well in Methocult (StemCell Technologies) supplemented with IL-3 (20ng/ml), IL-6 (10ng/ml), SCF (10ng/ml), GMCSF (10ng/ml), TPO (50ng/ml), and Flt3L (100ng/ml). Colonies were scored 14 days after seeding, recollected in PBS, and then recultured in Methocult at 1×10^4 cells. *Tet2* knockdown was confirmed by qRT-PCR using Thermo Scientific Verso cDNA kit and SYBR green quantification in an ABI 7500 sequence detection system.

5hmC quantification

Genomic DNA was purified using Purgene DNA purification kit (Qiagen). Genomic DNA was hydrolyzed and analyzed in triplicate for the relative levels of 5-hydroxymethylcytosine

by liquid chromatography-electrospray ionization tandem mass spectrometry (Shah et al., 2010).

Patient samples and sequencing

Approval was obtained from the institutional review boards of Memorial Sloan-Kettering Cancer Center, Dana-Farber Cancer Institute, and MD Anderson Cancer Center. Patients provided written informed consent in all cases at time of enrollment. DNA sequencing methods and primer sequences for *TET2* have been described previously (Abdel-Wahab et al., 2009). 389 AML samples were obtained at diagnosis from patients enrolled in the Eastern Cooperative Oncology Group's (ECOG) E1900 clinical trial. Samples were deidentified at the time of inclusion. 354 MPN, 69 CMML, and an additional 119 AML samples were analyzed with characteristics previously described (Abdel-Wahab et al., 2009). Approval was obtained from the institutional review boards of Memorial Sloan-Kettering Cancer Center, Dana-Farber Cancer Institute, and MD Anderson Cancer Center. DNA sequencing methods and primer sequences for *TET2* have been described previously (Abdel-Wahab et al., 2009).

Human CD34⁺ stem cell isolation

Peripheral blood was collected from normal bone marrow transplant donors who underwent stem cell mobilization with GCSF. Remainder excess aliquots were used for CD34⁺ cell isolation. CD34⁺ HSPCs were purified by positive selection using the Midi-magnetic-activated cell sorting LS⁺ separation columns and isolation kit (Miltenyi). RNA was collected using the Qiagen RNeasy kit.

Accession Number

Microarray data can be accessed under GEO accession number GSE27816.

Highlights

Tet2 expression silencing leads to increased self-renewal ability

Tet2 deletion leads to progressive defects in hematopoiesis.

Tet2 deficient hematopoietic stem cells show increased repopulating ability.

Tet2 deficient animals develop CMML-like disease.

SIGNIFICANCE

Recurrent somatic mutations in *TET2* and in other genes that regulate the epigenetic state have been identified in patients with myeloid malignancies and in other cancers.

However, the *in vivo* effects of *Tet2* loss have not been delineated. We report here that *Tet2* loss leads to increased stem-cell self-renewal and to progressive stem cell expansion. Consistent with human mutational data, *Tet2* loss leads to myeloproliferation *in vivo*, notable for splenomegaly and monocytic proliferation. In addition, haploinsufficiency for *Tet2* confers increased self-renewal and myeloproliferation, suggesting that the monoallelic *TET2* mutations found in most *TET2*-mutant leukemia patients contribute to myeloid transformation. This work demonstrates that reduction in *TET2* expression or function leads to enhanced stem cell function *in vivo* and myeloid transformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Hans-Guido Wendel and Dino Mavrikakis for assistance with Tet2 shRNA construction. Also, we thank the NYU Genome Technology Center (supported in part by NIH/NCI P30 CA016087-30 grant) for expert assistance with micro-array experiments, and the NYU Flow Cytometry facility (supported in part by NIH/NCI 5 P30CA16087-31) for expert cell sorting, the NYU Histology Core (5P30CA16087-31), and the Transgenic Mouse Core (NYU Cancer Institute Center Grant (5P30CA16087-31). IA is supported by the National Institutes of Health (RO1CA133379, RO1CA105129, R21CA141399, RO1CA149655, RO1GM088847), the Leukemia & Lymphoma Society (TRP grant), the American Cancer Society (RSG0806801), the Irma T. Hirsch Trust, and the Dana Foundation. This work was supported in part by grants from the National Institutes of Health (U54CA143798-01, Physical Sciences Oncology Center) and 1R01CA138234-01 to RLL, and by grants from the Starr Cancer Consortium and Howard Hughes Medical Institute to RLL. LR is supported by a NIH Ruth L. Kirchstein Award (F31-AG039991). MEF is funded by a Leukemia and Lymphoma Society Special Fellow award. AM is funded by a Leukemia and Lymphoma Society SCOR and TRP award, and is a Burroughs Wellcome Clinical Translational Scholar and Scholar of the Leukemia and Lymphoma Society. AM and MEF are also supported by the Sackler Center for Biomedical and Physical Sciences. LAG was supported by the NIH grants CA129831 and CA129831-03S1. XZ and SDN are supported by a Leukemia and Lymphoma Society SCOR award and FP by an American Italian Cancer Foundation award. RLL is a Geoffrey Beene Junior Faculty Chair at Memorial Sloan Kettering Cancer Center. I.A is a Howard Hughes Medical Institute Early Career Scientist.

REFERENCES

- Abdel-Wahab O, Levine RL. EZH2 Mutations: Mutating the Epigenetic Machinery in Myeloid Malignancies. *Cancer Cell*. 2010; 18:105–107.
- Abdel-Wahab O, Manshour T, Patel J, Harris K, Yao J, Hedvat C, Heguy A, Bueso-Ramos C, Kantarjian H, Levine RL, Verstovsek S. Genetic analysis of transforming events that convert chronic myeloproliferative neoplasms to leukemias. *Cancer Res*. 2010; 70:447–452. [PubMed: 20068184]
- Abdel-Wahab O, Mullally A, Hedvat C, Garcia-Manero G, Patel J, Wadleigh M, Malinger S, Yao J, Kilpivaara O, Bhat R, et al. Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. *Blood*. 2009; 114:144–147. [PubMed: 19420352]
- Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics*. 2003; 19:185–193. [PubMed: 12538238]
- Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature*. 2009; 462:739–744. [PubMed: 19935646]
- Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, Kosmider O, Le Couedic JP, Robert F, Alberdi A, et al. Mutation in TET2 in myeloid cancers. *N Engl J Med*. 2009; 360:2289–2301. [PubMed: 19474426]
- Dorrance AM, Liu S, Yuan W, Becknell B, Arnoczky KJ, Guimond M, Strout MP, Feng L, Nakamura T, Yu L, et al. Mll partial tandem duplication induces aberrant Hox expression in vivo via specific epigenetic alterations. *J Clin Invest*. 2006; 116:2707–2716. [PubMed: 16981007]
- Ernst T, Chase AJ, Score J, Hidalgo-Curtis CE, Bryant C, Jones AV, Waghorn K, Zoi K, Ross FM, Reiter A, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. *Nat Genet*. 2010; 42:722–726. [PubMed: 20601953]
- Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W. Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature*. 2011
- Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, Li Y, Bhagwat N, Vasanthakumar A, Fernandez HF, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell*. 2010; 18:553–567. [PubMed: 21130701]
- Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. *Blood*. 2002; 100:1532–1542. [PubMed: 12176867]
- Haeno H, Levine RL, Gilliland DG, Michor F. A progenitor cell origin of myeloid malignancies. *P Natl Acad Sci USA*. 2009; 106:16616–16621.

- Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature*. 2010; 466:1129–1133. [PubMed: 20639862]
- Jankowska AM, Szpurka H, Tiu RV, Makishima H, Afable M, Huh J, O'Keefe CL, Ganetzky R, McDevitt MA, Maciejewski JP. Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. *Blood*. 2009
- Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, An J, Lamperti ED, Koh KP, Ganetzky R, et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature*. 2010; 468:839–843. [PubMed: 21057493]
- Koh KP, Yabuuchi A, Rao S, Huang Y, Cunniff K, Nardone J, Laiho A, Tahiliani M, Sommer CA, Mostoslavsky G, et al. Tet1 and tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. *Cell Stem Cell*. 2011; 8:200–213. [PubMed: 21295276]
- Krivtsov AV, Feng Z, Lemieux ME, Faber J, Vempati S, Sinha AU, Xia X, Jesneck J, Bracken AP, Silverman LB, et al. H3K79 methylation profiles define murine and human MLL-AF4 leukemias. *Cancer Cell*. 2008; 14:355–368. [PubMed: 18977325]
- Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice. *Science*. 1995; 269:1427–1429. [PubMed: 7660125]
- Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, Lee E, Alt FW, Westphal H. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. *Proc Natl Acad Sci U S A*. 1996; 93:5860–5865. [PubMed: 8650183]
- Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M, Stevens-Linders E, van Hoogen P, van Kessel AG, Raymakers RA, et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. *Nat Genet*. 2009; 41:838–842. [PubMed: 19483684]
- Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, Kandoth C, Payton JE, Baty J, Welch J, et al. DNMT3A mutations in acute myeloid leukemia. *N Engl J Med*. 2010; 363:2424–2433. [PubMed: 21067377]
- Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K, Koboldt DC, Fulton RS, Delehaunty KD, McGrath SD, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med*. 2009; 361:1058–1066. [PubMed: 19657110]
- Metzeler KH, Maharry K, Radmacher MD, Mrozek K, Margeson D, Becker H, Curfman J, Holland KB, Schwind S, Whitman SP, et al. TET2 Mutations Improve the New European LeukemiaNet Risk Classification of Acute Myeloid Leukemia: A Cancer and Leukemia Group B Study. *J Clin Oncol*. 2011
- Morin RD, Johnson NA, Severson TM, Mungall AJ, An JH, Goya R, Paul JE, Boyle M, Woolcock BW, Kuchenbauer F, et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. *Nature Genetics*. 2010; 42 181-NIL_124.
- Ng SY, Yoshida T, Zhang J, Georgopoulos K. Genome-wide lineage-specific transcriptional networks underscore Ikaros-dependent lymphoid priming in hematopoietic stem cells. *Immunity*. 2009; 30:493–507. [PubMed: 19345118]
- Nikoloski G, Langemeijer SM, Kuiper RP, Knops R, Massop M, Tonnissen ER, van der Heijden A, Scheele TN, Vandenberghe P, de Witte T, et al. Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. *Nat Genet*. 2010; 42:665–667. [PubMed: 20601954]
- Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science*. 2008; 321:1807–1812. [PubMed: 18772396]
- Schaub FX, Looser R, Li S, Hao-Shen H, Lehmann T, Tichelli A, Skoda RC. Clonal analysis of TET2 and JAK2 mutations suggests that TET2 can be a late event in the progression of myeloproliferative neoplasms. *Blood*. 2010; 115:2003–2007. [PubMed: 20061559]
- Shah MY, Vasanthakumar A, Barnes NY, Figueroa ME, Kamp A, Hendrick C, Ostler KR, Davis EM, Lin S, Anastasi J, et al. DNMT3B7, a truncated DNMT3B isoform expressed in human tumors, disrupts embryonic development and accelerates lymphomagenesis. *Cancer Res*. 2010; 70:5840–5850. [PubMed: 20587527]

- Stadtfield M, Graf T. Assessing the role of hematopoietic plasticity for endothelial and hepatocyte development by non-invasive lineage tracing. *Development (Cambridge, England)*. 2005; 132:203–213.
- Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005; 102:15545–15550. [PubMed: 16199517]
- Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science*. 2009; 324:930–935. [PubMed: 19372391]
- Tefferi A. Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. *Leukemia*. 2010; 24:1128–1138. [PubMed: 20428194]
- van Haften G, Dalgliesh GL, Davies H, Chen L, Bignell G, Greenman C, Edkins S, Hardy C, O'Meara S, Teague J, et al. Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. *Nat Genet*. 2009; 41:521–523. [PubMed: 19330029]
- Varela I, Tarpey P, Raine K, Huang D, Ong CK, Stephens P, Davies H, Jones D, Lin ML, Teague J, et al. Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. *Nature*. 2011; 469:539–542. [PubMed: 21248752]
- Ward PS, Patel J, Wise DR, Abdel-Wahab O, Bennett BD, Collier HA, Cross JR, Fantin VR, Hedvat CV, Perl AE, et al. The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell*. 2010; 17:225–234. [PubMed: 20171147]
- Williams K, Christensen J, Pedersen MT, Johansen JV, Cloos PA, Rappsilber J, Helin K. TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature*. 2011
- Wu H, D'Alessio AC, Ito S, Xia K, Wang Z, Cui K, Zhao K, Eve Sun Y, Zhang Y. Dual functions of Tet1 in transcriptional regulation in mouse embryonic stem cells. *Nature*. 2011
- Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, Ito S, Yang C, Xiao MT, Liu LX, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. *Cancer Cell*. 2011; 19:17–30. [PubMed: 21251613]
- Yamashita Y, Yuan J, Suetake I, Suzuki H, Ishikawa Y, Choi YL, Ueno T, Soda M, Hamada T, Haruta H, et al. Array-based genomic resequencing of human leukemia. *Oncogene*. 2010; 29:3723–3731. [PubMed: 20400977]
- Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, Kos I, Batinic-Haberle I, Jones S, Riggins GJ, et al. IDH1 and IDH2 mutations in gliomas. *N Engl J Med*. 2009; 360:765–773. [PubMed: 19228619]

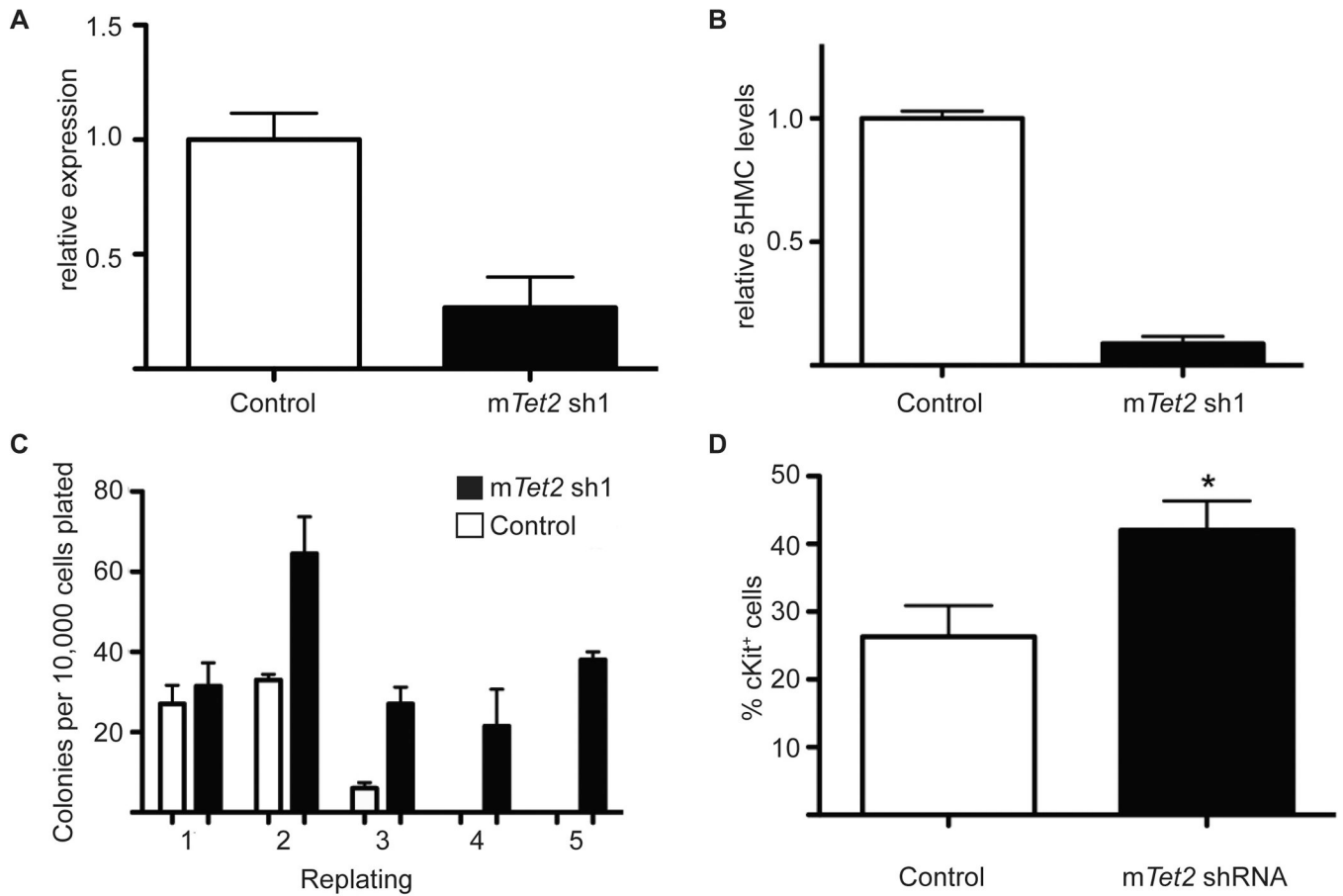


Figure 1. *In vitro* Tet2 expression silencing leads to increased serial replating

A) Quantification of the shRNA-mediated *Tet2* knock-down efficiency using qRT-PCR, **B)** Quantification of 5-hmC by LC/MS of control (MSCV-IRES-GFP) versus *Tet2* knockdown derived cells from after retroviral transduction and GFP sorting. **C)** CFU assay of bone marrow derive cells infected with control or *Tet2* shRNA virus. Colony counts were scored every 14 days. **D)** Upregulation of surface c-Kit expression in progenitors expressing *Tet2*-specific shRNA. * $p < 0.026$. Error bars represent \pm SD.

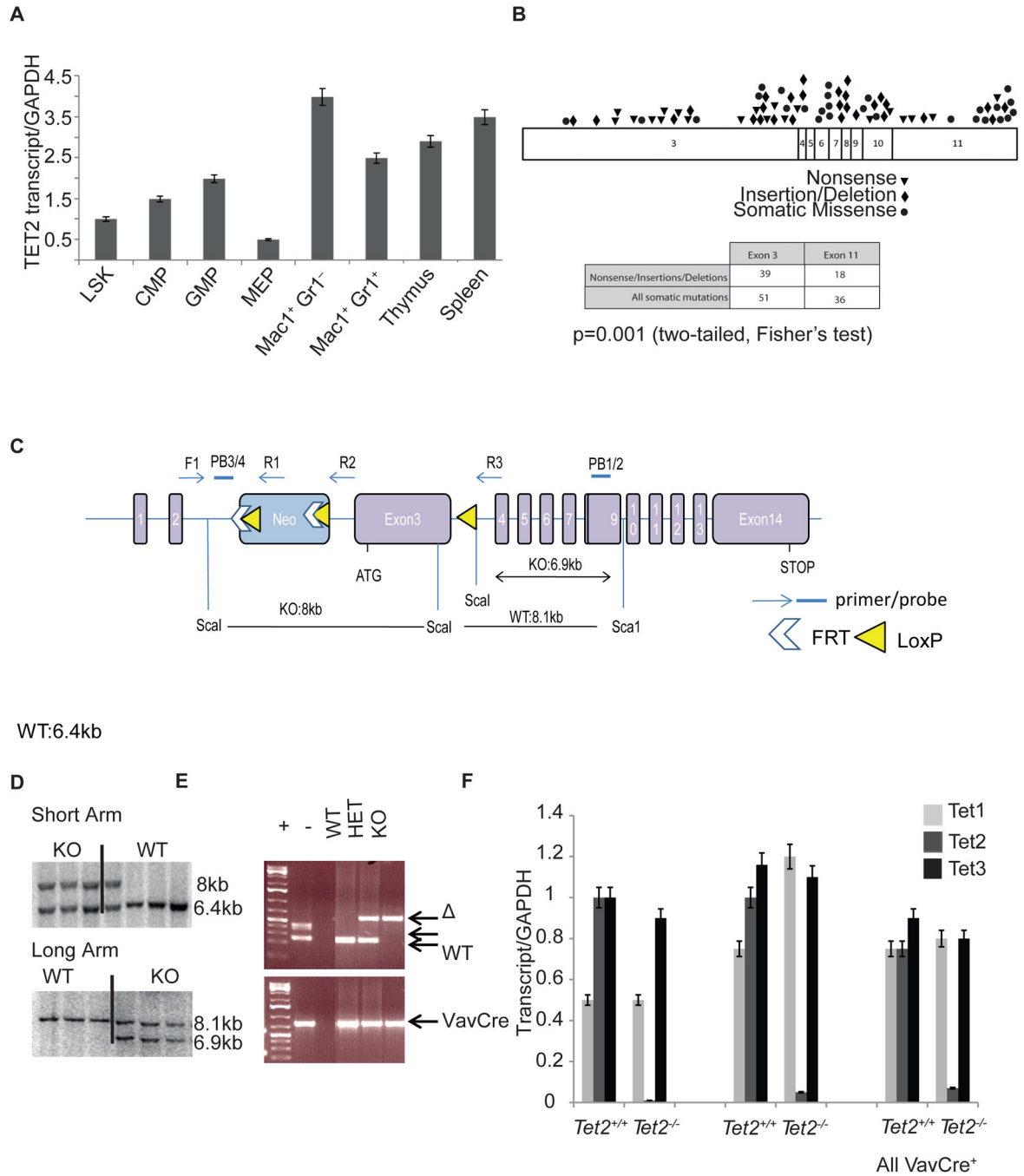


Figure 2. Generation of a conditional *Tet2* allele

A) qRT-PCR showing relative expression levels of *Tet2* in purified progenitor and mature mouse hematopoietic stem and progenitor subsets. **B)** Exon distribution of *TET2* mutations found in AML and CMML patients. **C)** Schematic depiction of the targeted *Tet2* allele. Exon 3 is targeted and flanked by LoxP sites upon Frt-mediated deletion of the NEO cassette. **D)** Verification of correct homologous recombination using Southern Blots on targeted ES cells. **E)** Verification of VavCre-mediated excision using genomic PCR. Recombined (Δ), Floxed and wild-type *Tet2* alleles are shown. **F)** Efficiency of *Tet2* knock-out using qRT-PCR in purified bone marrow c-Kit⁺ cells, total thymus and spleen. Expression of *Tet1* and *Tet3* in wild-type and *Tet2* deficient bone marrow cells reveals no

differences in *Tet1* and *Tet3* expression with *Tet2* deletion. Error bars represent \pm SD. See also Figure S1.

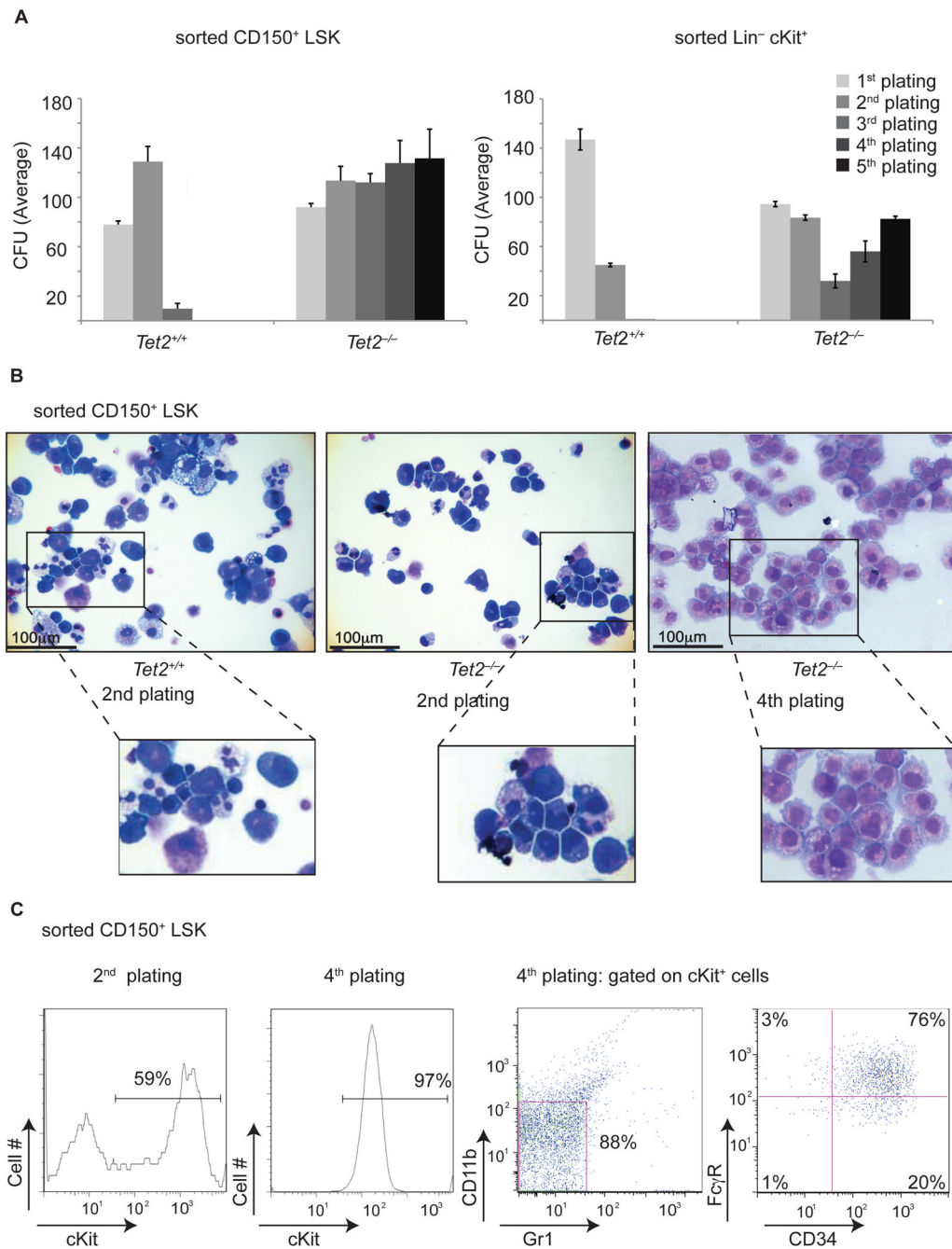


Figure 3. *Tet2* deficiency leads to increased serial replating ability *in vitro*

A) Left Panel: Methylcellulose CFU assay using LSK CD150⁺ purified cells. Absolute number of colonies is shown at different platings. A similar CFU assay using Lineage⁻c-Kit⁺ cells is shown in the right panel. **B)** Morphology of generated colonies either at the 2nd or 4th plating. Cytospins of the colonies are shown. **C)** Left panel: Upregulation of surface c-Kit expression at the 2nd and 4th plating. Right Panel: Cell surface expression of CD34 and FcγR on Lin⁻cKit⁺ *Tet2*^{-/-} cells by the 4th plating. A representative example of at least three independent experiments is shown for each assay. Error bars represent ± SD. See also Figure S2.

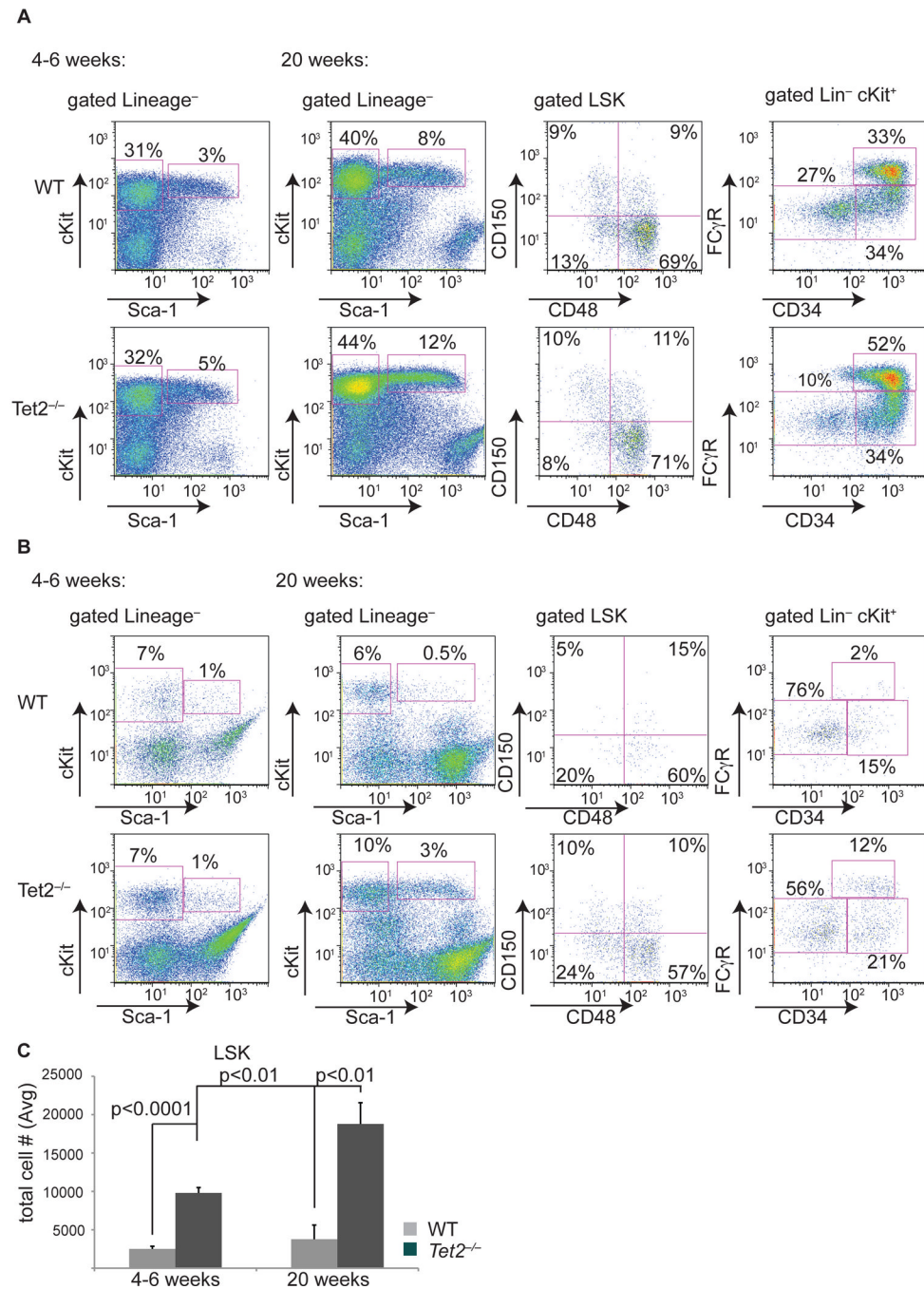


Figure 4. *Tet2* deletion leads to progressive defects in bone marrow and extramedullary hematopoiesis

A) FACS analysis of bone marrow stem and progenitor populations of *Tet2*^{-/-} (*vav-Cre*⁺*Tet2*^{fl/fl}) and WT (*vav-Cre*⁺*Tet2*^{wt/wt}) at two different ages (4–6 and 20 weeks). Antibody stainings are as indicated. **B)** Identical antibody labeling as in **A)** but using spleen cells from *Tet2*^{-/-} (*vav-Cre*⁺*Tet2*^{fl/fl}) and WT (*vav-Cre*⁺*Tet2*^{wt/wt}) mice. **C)** Absolute numbers of spleen LSK cells in *Tet2*^{-/-} (*vav-Cre*⁺*Tet2*^{fl/fl}) and WT (*vav-Cre*⁺*Tet2*^{wt/wt}) mice at two different ages. P values are shown for each comparison. For all experiments shown in this figure n=8 for each genotype. Error bars represent ± SD. See also Figure S3.

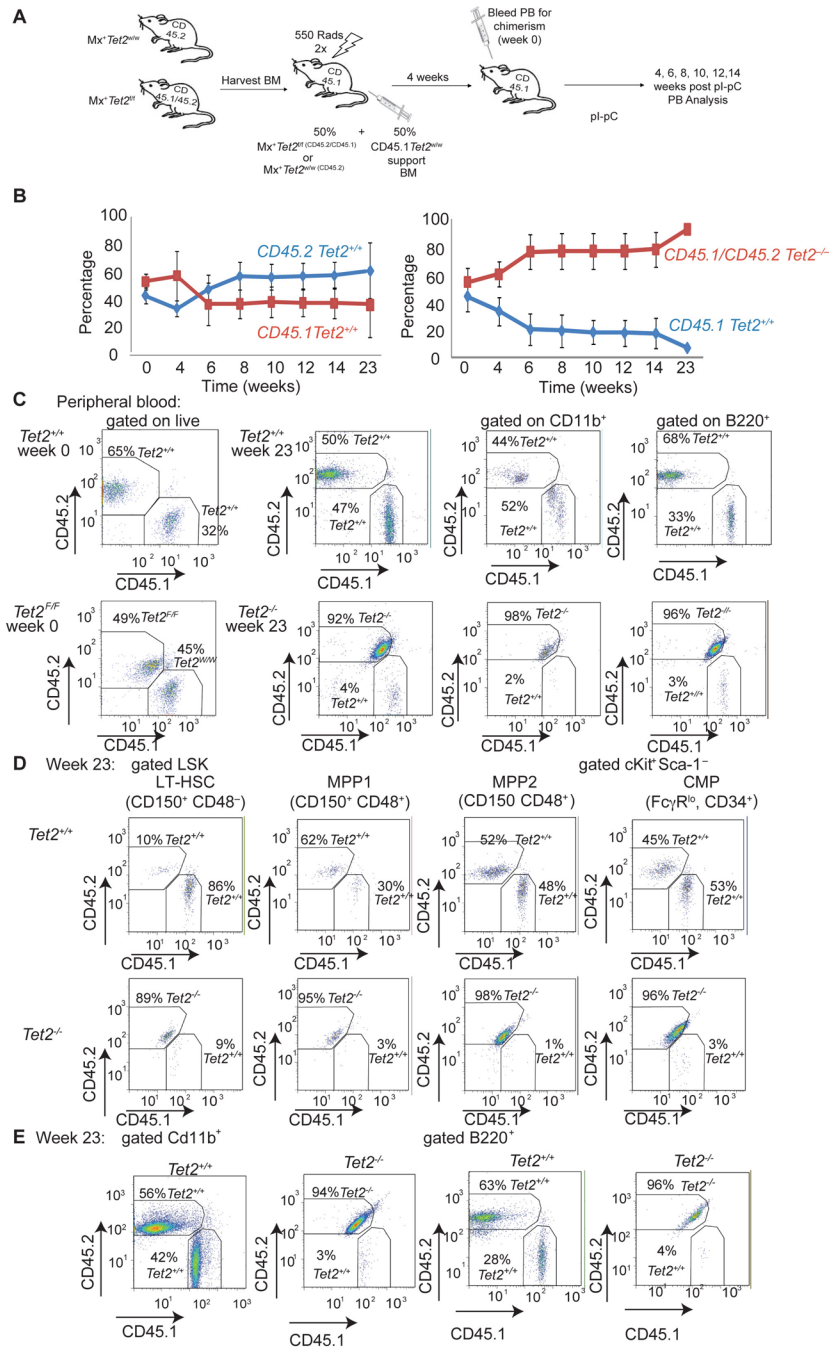


Figure 5. *Tet2*^{-/-} hematopoietic stem cells show increased re-populating ability consistent with increased self-renewal

A) Schematic depiction of the competitive transplantation scheme. *Tet2*^{-/-} cells are double positive for CD45.1/CD45.2 markers. *Tet2* WT cells are CD45.2 single positive cells. **B)** Percentage of CD45.1 vs CD45.2 total chimerism in the peripheral blood of recipient animals (n=15 for each genotype). Time (weeks) denotes the time post the termination of polyI-polyC injections. **C)** Representative FACS plots showing CD45.1/CD45.2 (*Tet2*^{-/-}) and CD45.2 (*Tet2*^{+/+}) stainings and chimerism in whole peripheral mononuclear cells, myeloid CD11b⁺ and lymphoid B220⁺ cells. Two time points (week 0 and week 23) are shown. **D)** Representative FACS plots showing CD45.1/CD45.2 (*Tet2*^{-/-}) and CD45.2

(*Tet2*^{+/+}) stainings and chimerism within the bone marrow stem (LT-HSC) and progenitor (MPP1, MPP2 and CMP) compartments. Analysis performed at week 23 post-polyI-polyC injection. **E**) Similar stainings quantifying CD45.1/CD45.2 (*Tet2*^{-/-}) and CD45.2 (*Tet2*^{+/+}) expression in bone marrow myeloid (CD11b⁺) and lymphoid (B220⁺) mature populations. Week 23 stainings are shown (n=5 for each genotype). Error bars represent ± SD. See also Figure S4.

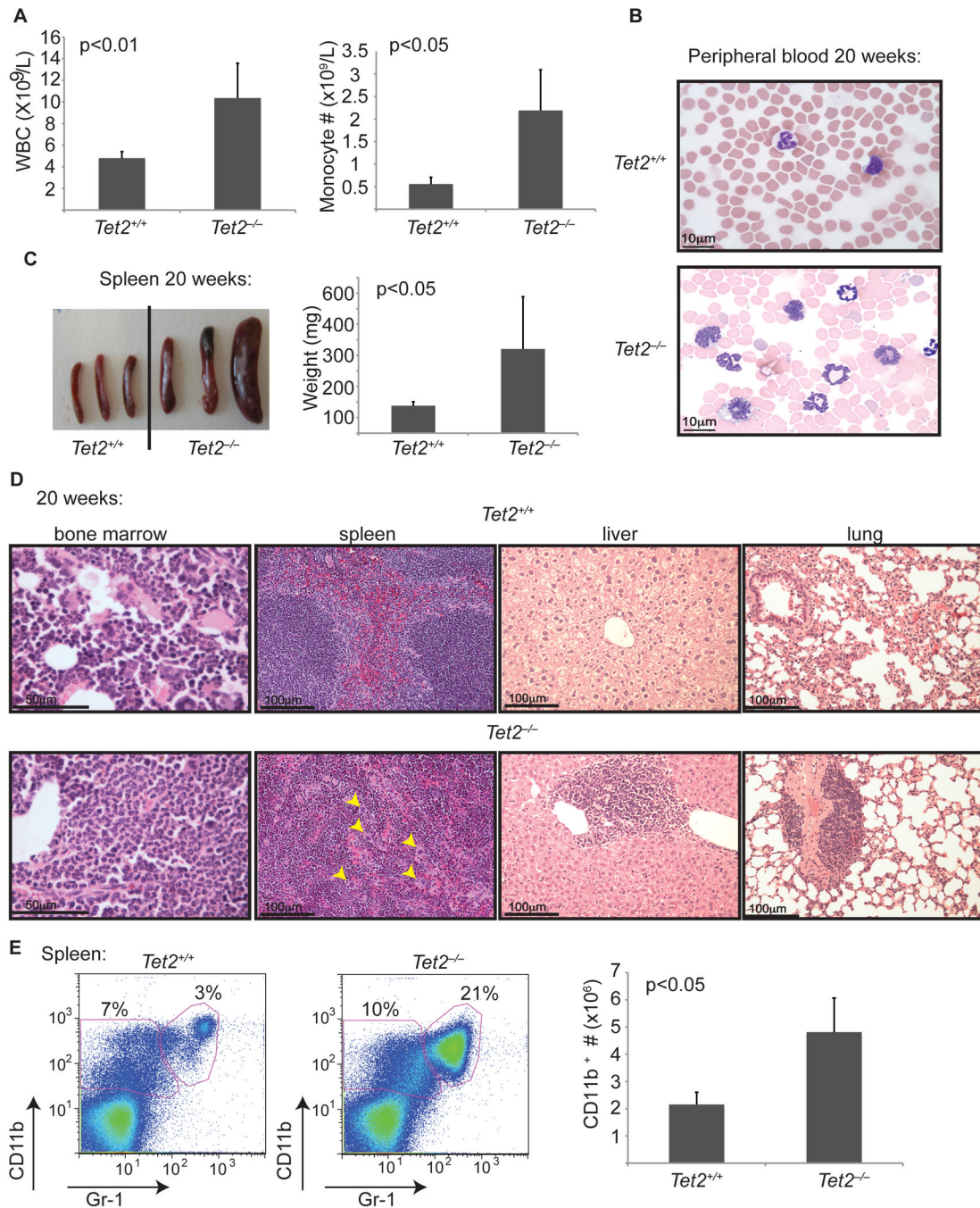


Figure 6. *Tet2*^{-/-} animals develop myeloid neoplasia reminiscent of human CMML

A) Left panel: Automated peripheral blood enumeration of whole blood cells (WBC). Right panel: Manual differential counts of neutrophils and monocytes showing blood monocytosis ($n \geq 3$ for each genotype). **B)** Representative images of peripheral blood smears from *Tet2*^{+/+} and *Tet2*^{-/-} mice at 20 weeks of age ($n \geq 3$ for each genotype). **C)** Splenomegaly in 20-week old *Tet2*^{-/-} (*Vav-Cre*⁺*Tet2*^{f/f}) animals. Littermate WT (*Vav-Cre*⁺*Tet2*^{w/w}) controls at 20 weeks are used. $n=5$ mice **D)** Histologic (H&E) analysis of *Tet2*^{-/-} and control tissues (bone marrow, spleen, liver and lung) are shown, illustrating disrupted spleen architecture, monocytic infiltrations in liver and lung and bone marrow neutrophilia ($n=5$ for each genotype). **E)** Left panel: FACS analysis of spleen CD11b and Gr-1 expressing populations.

A representative FACS plot from each genotype is shown. Right panel: total cell number of CD11b⁺ cells in the spleen of *Tet2*^{+/+} and *Tet2*^{-/-} mice at 20 weeks of age. Error bars represent \pm SD. See also Figure S5.

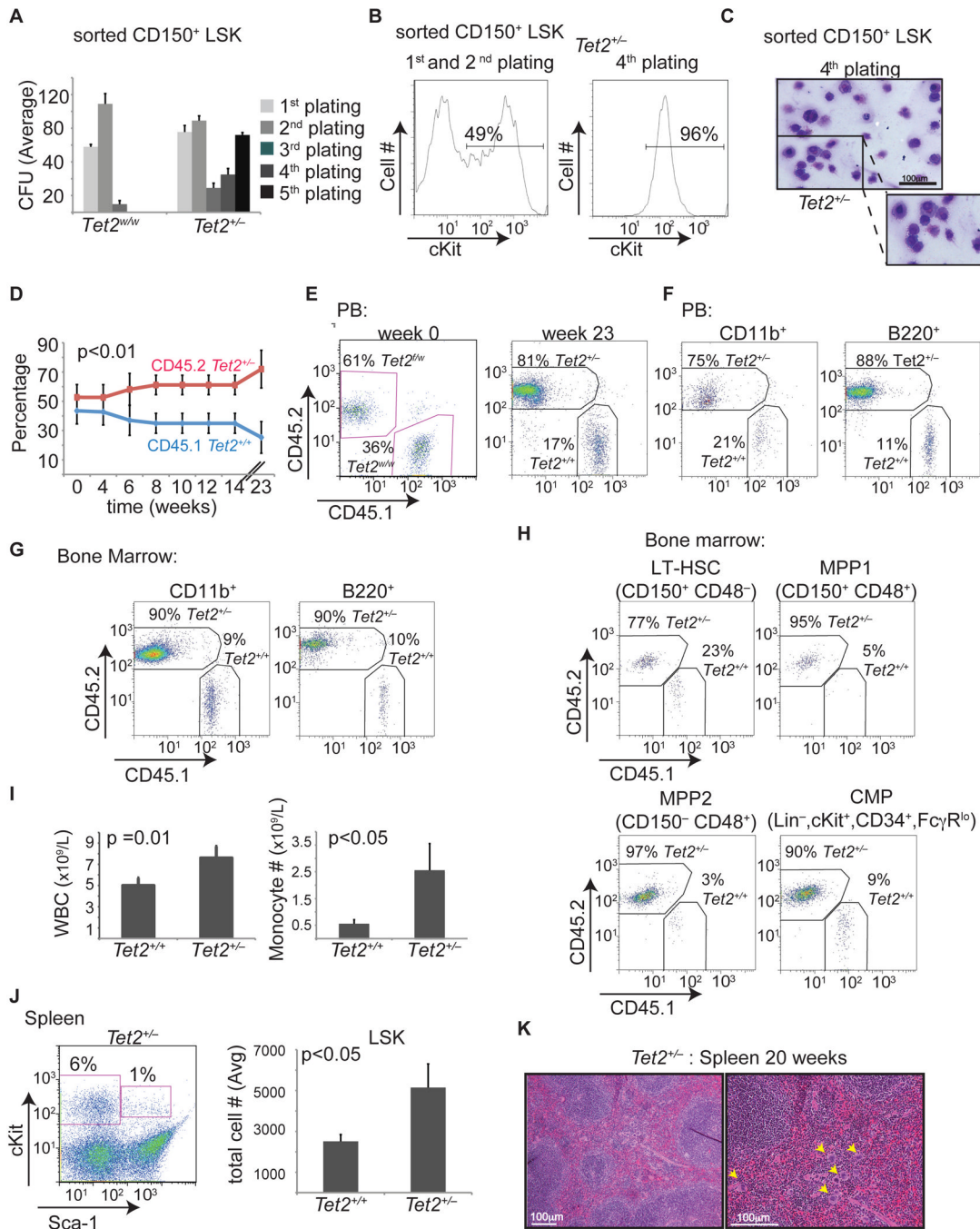


Figure 7. *Tet2* haploinsufficiency is able to initiate aberrant *in vitro* and *in vivo* hematopoiesis
A) Methylcellulose CFU assay using LSK CD150⁺ cells (Lin⁻, cKit⁺, Sca-1⁺, CD150⁺). An average of the absolute number of colonies is shown for sequential platings (1–5) (n= 5) **B)** Representative FACS plot of colony forming assay showing c-Kit expression at the 2nd and 4th plating. **C)** Wright-Giemsa staining of colonies formed from $Tet2^{+/-}$ CD150⁺ LSKs ($Vav-Cre^{+}Tet2^{f/w}$). Images of cytopspins are shown. **D)** Graph represents chimerism between CD45.2 ($Tet2^{+/-}$) and CD45.1 ($Tet2^{+/+}$) in the peripheral blood from a competitive transplantation assay over time (0–23 weeks). Experimental strategy is described in Figure 5A. Blue line indicates WT:WT cohorts and red indicates $Tet2^{+/-}$:WT cohorts. (n= 5 mice) **E)** Representative FACS plots showing CD45.1/CD45.2 chimerism in the peripheral blood

from CD45.1 (*Tet2^{+/+}*) and CD45.2 (*Tet2^{+/-}*) cohorts pre-(wk 0) and post-deletion (23 wk post-polyI-polyC). **F–H**) Representative FACS plots showing CD45.1 (*Tet2^{+/+}*)/CD45.2 (*Tet2^{+/-}*) chimerism at 23 weeks post deletion in myeloid and lymphoid populations in the peripheral blood (**F**), in the bone marrow (**G**), and in the bone marrow stem and progenitor cell compartment (**H**). **I**) Left panel: Automated peripheral blood enumeration of whole blood cells (WBC). Right panel: Manual differential counts of neutrophils and monocytes showing monocytosis ($n \geq 3$ for each genotype). **J**) Representative FACS plot showing increased frequency of LSKs in the spleen of *Tet2^{+/-}* mice. Right panel depicts absolute number of LSKs in the spleen of WT and *Tet2^{+/-}* mice ($n=5$ for each genotype). **K**) Hematoxylin and Eosin staining of spleen from 20 week old *Tet2^{+/-}* mice. Error bars represent \pm SD. See also Figure S6.

Table 1

Tet2 deficiency leads to progressive peripheral leukocytosis. Peripheral blood analysis (automated) at 4–6 weeks of age and at 20 weeks of age.

Genotype	WBC ($\times 10^9/L$)	HCT (%)	PLT ($\times 10^9/L$)	
4–6wk				
<i>Tet2</i> ^{+/+}	5.48 \pm 1.54	40.32 \pm 8.70	367 \pm 66	n=11
<i>Tet2</i> ^{+/-}	6.61 \pm 1.95	49.63 \pm 5.90	365 \pm 59	n=7
<i>Tet2</i> ^{-/-}	6.66 \pm 2.04	50.06 \pm 4.70	352 \pm 85	n=9
20wk				
<i>Tet2</i> ^{+/+}	5.43 \pm 1.35	46.29 \pm 5.56	498 \pm 171	n=8
<i>Tet2</i> ^{+/-}	* 8.77 \pm 1.90	52.55 \pm 4.42	559 \pm 119	n=10
<i>Tet2</i> ^{-/-}	* 10.00 \pm 2.28	52.45 \pm 11.18	517 \pm 124	n=13

* $p \leq 0.01$ See also Table S1.