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ZFX controls propagation and prevents differentiation of acute T-lymphoblastic and myeloid leukemia

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Summary

Tumor-propagating cells in acute leukemia maintain a stem/progenitor-like immature phenotype and proliferative capacity. Acute myeloid leukemia (AML) and acute T-lymphoblastic leukemia (T-ALL) originate from different lineages through distinct oncogenic events such as MLL fusions and Notch signaling, respectively. We found that Zfx, a transcription factor that controls hematopoietic stem cell self-renewal, controls the initiation and maintenance of AML caused by MLL-AF9 fusion and of T-ALL caused by Notch1 activation. In both leukemia types, Zfx prevents differentiation and activates gene sets characteristic of immature cells of the respective lineages. In addition, endogenous Zfx contributes to gene induction and transformation by Myc overexpression in myeloid progenitors. Key Zfx target genes include the mitochondrial enzymes *Ptprmt1* and *Idh2*, whose overexpression partially rescues the propagation of Zfx-deficient AML. These results show that distinct leukemia types maintain their undifferentiated phenotype and self-renewal by exploiting a common stem cell-related genetic regulator.

Introduction

Acute leukemia is characterized by the rapid overproduction of malignant immature hematopoietic cells that inhibit normal hematopoiesis in the bone marrow (BM) and invade

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peripheral organs. T cell acute lymphoblastic leukemia (T-ALL) accounts for 15–20% of acute leukemia cases in adults and children, whereas acute myeloid leukemia (AML) is the most common acute leukemia found in adults. Both types of leukemia are associated with a high risk of relapse after chemotherapy treatment. The development of T-ALL and AML is driven by distinct oncogenic pathways that "hijack" normal molecular mechanisms operating in the respective T cell and myeloid progenitors.

Aberrant activation of the NOTCH1 receptor plays a major role in the pathogenesis of T-ALL, with activating NOTCH1 mutations occurring in >50% of human T-ALL cases (Weng et al., 2004). Notch1 is essential for early development of T cell progenitors in the thymus, but becomes dispensable for T cell development after the CD4⁺CD8⁺ double-positive (DP) stage (Pui et al., 1999; Radtke et al., 1999; Wolfer et al., 2001). The activation of Notch receptor releases its intracellular domain (NotchIC), which translocates to the nucleus, forms a complex with transcription factor CSL and activates transcription of target genes. *Hes1* is a canonical direct target of Notch/CSL that is required both for normal T cell development and for Notch-induced T-ALL (Wendorff et al., 2010). Overexpression of NotchIC in murine hematopoietic progenitors is sufficient to initiate transplantable T-ALL, which originates from highly proliferative CD4⁻CD8⁻ double-negative (DN) stage 4 (DN4) and CD4⁻CD8⁺ immature single-positive (ISP) thymocytes (Li et al., 2008).

Chromosomal translocations involving the mixed lineage leukemia gene (MLL) with multiple fusion partners are common in human AML (Liedtke and Cleary, 2009). Experimental overexpression of MLL fusion proteins such as MLL-AF9 (MA9) causes transformation of murine myeloid progenitors (Krivtsov et al., 2006; Somervaille and Cleary, 2006). The resulting AML cells can be propagated in cytokine-supplemented cultures and cause serially transplantable AML in recipient mice. These leukemias are hierarchically organized and include cells with immature c-Kit⁺ phenotype that can propagate the disease. MLL is a histone methyltransferase that is required for normal HSC function (Jude et al., 2007; McMahon et al., 2007). Oncogenic MLL fusion proteins recruit endogenous nuclear protein complexes to facilitate the transcription of target genes such as *Hoxa9* and *Meis1* (Muntean et al., 2010), which are necessary (Ayton and Cleary, 2003; Wong et al., 2007) and sufficient for the transformation (Kroon et al., 1998). Additional transcription factors that facilitate MLL-induced transformation, such as Myb, have also been identified (Zuber et al., 2011a).

A common feature of many cancers, including acute leukemia, is their dependence on the cellular proto-oncogene c-Myc (Myc). Myc is a transcription factor that induces multiple target genes such as metabolic enzymes and cell cycle regulators to promote the survival and proliferation of transformed cells. Myc and its regulator Brd4 have been shown to be important for AML propagation (Wong et al., 2010; Zuber et al., 2011b). In T-ALL, Myc represents a direct target of Notch signaling that contributes to leukemia growth (Palomero et al., 2006; Weng et al., 2006) and maintains the leukemia-initiating capacity of undifferentiated leukemic cells (King et al., 2013). However, common factors that cooperate with and/or act downstream of Myc in different leukemia types have not been fully elucidated.

ZFX is a transcription factor that is encoded on the X chromosome and contains an acidic transcriptional activation domain and a DNA-binding zinc finger domain. Murine and human ZFX are expressed ubiquitously, yet the function of Zfx appears cell type-specific. Thus, murine Zfx is generally dispensable for embryonic development and for the growth of multiple cell types including embryonic fibroblasts, myeloid progenitors and neural stem/progenitor cells (Galan-Caridad et al., 2007). However, Zfx is necessary for the self-renewal and survival of adult hematopoietic stem cells (HSCs) *in vivo* and of embryonic stem cells

(ESCs) *in vitro*. Zfx is highly conserved in vertebrates, and similarly controls the self-renewal of human ESCs (Harel et al., 2012). Given its essential and specific role in normal stem cell self-renewal, we hypothesized that Zfx might regulate the aberrant self-renewal of leukemia cells in T-ALL and AML.

Results

Zfx contributes to the development of Notch1-induced T-ALL

First, we tested the role of Zfx in normal T cell development in the thymus. Pan-hematopoietic Zfx deletion using *Tie2-Cre* (Fig. S1A) delayed the DN to DP transition in the fetal thymus, and reduced proliferation of DN4 and ISP thymocytes; however, it did not preclude normal thymocyte development (Fig. S1B and S1C). Furthermore, Zfx deletion at the DN to DP transition using *CD4-Cre* did not impair thymocyte development in any way (Fig. S1D and S1E). We conclude that Zfx facilitates the massive proliferation of DN4 and ISP thymocytes, but is largely dispensable for this process and for T cell development in general.

To study the role of Zfx in the development of spontaneous T-ALL *in vivo*, we used the *CD4-Cre* deleter combined with a Cre-inducible *Eef1a1*-NotchIC allele (Buonamici et al., 2009). These mice were crossed with a conditional null Zfx allele (*Zfx^{fl/y}*), so that Cre would induce NotchIC and delete Zfx in the same cell. Cre-induced NotchIC induction in both control *Zfx^{wt/y}* and *Zfx^{fl/y}* mice initiated in DN thymocytes and was complete by the DP stage (Fig. 1A). The *Eef1a1*-NotchIC⁺ *CD4-Cre*⁺ *Zfx^{wt/y}* mice had abnormal DP T cells in the blood (Fig. 1B), developed extreme splenomegaly (~750×10⁶ splenocytes, Fig. 1C), and 100% of them succumbed to T-ALL by 2–4 months of age (Fig. 1D). In contrast, the *Eef1a1*-NotchIC⁺ *CD4-Cre*⁺ *Zfx^{fl/y}* mice never showed DP T cells in the periphery (Fig. 1B), had spleens of the normal size (~60×10⁶ splenocytes, Fig. 1C), and ~30% of them survived for >7 months. The remaining animals succumbed to an inflammatory disease characterized by wasting and skin inflammation, which was caused by NotchIC activation (Fig. 1D) but was clearly distinct from T-ALL. This phenotype likely reflects the pro-inflammatory effector T cell differentiation induced by activated Notch1 (Alam et al., 2010). We conclude that the loss of Zfx completely abrogates the development of Notch-induced T-ALL from immature thymocytes.

Zfx facilitates propagation and prevents differentiation of T-ALL

To examine the role of Zfx in the maintenance of pre-established T-ALL, we transduced retroviral NotchIC-IRES-GFP into hematopoietic progenitors carrying the *Zfx^{wt/y}* or *Zfx^{fl/y}* allele and the tamoxifen-inducible Cre recombinase (*R26-CreER*) (Fig. 2A). Each transduced culture was transferred into an individual recipient animal to generate multiple independent leukemia lines of each genotype. Four months after the transfer, all recipients succumbed to GFP⁺ T-ALL with extensive infiltration into the BM and spleen (Fig. S2A). These primary T-ALL lines were then transplanted into secondary recipient mice, which were treated 2 days later with either tamoxifen (Tmx) or vehicle (oil). Tmx treatment led to efficient recombination of *Zfx^{fl}* into the null *Zfx^Δ* allele (Fig. S2B), and did not affect leukemia development from the control *Zfx^{wt/y}* cells (Fig. 2B). All seven transplanted T-ALL lines produced fulminant leukemia in vehicle-treated recipients, but only one produced a delayed leukemia with the recombined *Zfx^Δ* allele in Tmx-treated recipients (Fig. 2B). The remaining leukemias were also delayed and carried the unrecombined *Zfx^{fl}* allele, revealing strong selection for the rare T-ALL cells that escaped Zfx deletion. The expansion of T-ALL was abrogated by Tmx treatment as late as 6 days post-transfer (Fig. 2C), confirming that the loss of Zfx impairs T-ALL propagation independently of its initial engraftment.

The analysis of *ex vivo* T-ALL cells shortly after *Zfx* deletion revealed normal expression of functional Notch target genes *Hes1* and *Myc* (Fig. S2C), suggesting that *Zfx* does not directly affect the Notch pathway activity. To analyze the fate of T-ALL after *Zfx* deletion, the recipients of primary *R26-CreER⁺Zfx^{fl/y}* T-ALL were treated with vehicle or Tmx and analyzed 1–2 weeks later. All vehicle-treated recipients were moribund by 14 days, with a high fraction of T-ALL in the blood (Fig. 2C), BM and spleen. Although Tmx treatment abrogated T-ALL expansion in the peripheral blood (Fig. 2C), a small fraction of GFP⁺ T-ALL cells could be detected in the BM and spleens at these time points. One week after Tmx, *Zfx*-deficient T-ALL cells in the BM manifested the same immature phenotype, high proliferation rate and minimal apoptosis as control T-ALL cells (Fig. S2D-E). Two weeks after *Zfx* deletion, the residual T-ALL cells shifted from the CD4^{low} to DP phenotype (Fig. 2D) and upregulated DP marker CD5 (Fig. 2E). Furthermore, they showed a dramatic reduction of DN marker CD25 and of the forward scatter parameter indicative of cell size (Fig. 2E). The reduction of CD25 could be detected as early as one week after *Zfx* deletion (Fig. 2E and data not shown). Thus, defective propagation of *Zfx*-deficient T-ALL cells is associated with their progressive differentiation into the more mature DP thymocyte-like cells.

Zfx facilitates the initial transformation and propagation of AML

To test the role of *Zfx* in AML, we induced *Zfx* deletion *in vivo* and transduced myeloid progenitors with a retrovirus encoding the MA9 oncogene (Fig. 3A). MA9-transduced common myeloid progenitors (CMPs) and granulocyte/macrophage progenitors (GMPs) from control animals displayed efficient serial replating in semi-solid media (Fig. 3B). In contrast, CMPs and GMPs from *Zfx*-deficient BM formed normal colonies on the first passage but failed at serial replating (Fig. 3B). These data suggest that *Zfx* is dispensable in normal myeloid progenitors as described (Galan-Caridad et al., 2007), but becomes essential in MA9-transformed progenitors that acquire the capacity for self-renewal.

To assess the role of *Zfx* in the propagation of pre-established AML, we used retroviral MA9 to transduce hematopoietic progenitors from *Zfx^{wt/y}* or *Zfx^{fl/y}* *R26-CreER⁺* mice. Upon transfer into primary recipients, these cells caused fatal AML that could be transplanted into secondary recipients or propagated in cytokine-enriched medium (Fig. 3C). AML cells from moribund primary recipients were cultured with 4-hydroxytamoxifen (4-OHT) for 72 hours to induce *Zfx* deletion, and then transplanted into secondary recipients. Prior to the transplantation, the *Zfx^{Δ/y}* cells displayed no significant changes in the cell cycle or surface marker expression (Fig. S3A-B). The treatment had no effect on leukemia development from the *Zfx^{wt/y}* cells, but it significantly delayed AML development from the *Zfx^{fl/y}* cells (Fig. 3D). Next, we transferred untreated primary AML cells directly into secondary recipients, and administered Tmx *in vivo* 10 days later. The development of AML was significantly delayed in the recipients of *Zfx^{fl/y}* cells (Fig. 3E), confirming that *Zfx* controls the propagation rather than engraftment of AML. We also tested the role of *Zfx* downstream of *Hoxa9* and *Meis1*, the key transcriptional targets of MA9. The deletion of *Zfx* did not affect the expression of *Hoxa9*, *Meis1* or *Myb* in MA9 AML cells (Fig. S3C). Furthermore, *Zfx* deletion significantly delayed leukemic outgrowth from primary *Hoxa9/Meis1*-induced AML cells (Fig. 3F), suggesting that *Zfx* does not act upstream of these factors.

Among the 7–8 independent primary *Zfx^{fl/y}* leukemia lines analyzed in each experiment, the majority either did not grow after *Zfx* deletion or gave rise to delayed AML that retained the unrecombined *Zfx* allele. The 2 MA9 AML lines and 4 *Hoxa9/Meis1* AML lines that grew in the absence of *Zfx* showed delayed growth kinetics and more differentiated phenotype (Fig. 3D-F and data not shown). The analysis of one such line showed unique

overexpression of several genes including transcription factor *Six1* (Fig. S3D), which has been implicated in MLL-mediated transformation (Wang et al., 2011). Thus, partial resistance to *Zfx* deletion in rare AML lines is associated with potential compensatory changes in their expression profile. Altogether, our data suggest that *Zfx* is important for the propagation of AML caused by MA9 or its effectors *Meis1/Hoxa9*.

Zfx maintains clonogenic growth and prevents differentiation of AML

To test the *in vitro* clonogenic growth of *Zfx*-deficient AML, MA9-transformed *Zfx^{wt/y}* and *Zfx^{fl/y}* *R26-CreER⁺* AML cells were treated with 4-OHT and propagated in semi-solid medium with cytokines (Fig. 4A). Colonies derived from single *Zfx^{Δ/y}* cells displayed less compact morphology in the first passage (Fig. 4B), and the frequency of colony-forming cells was decreased ~5-fold in subsequent passages (Fig. 4C). Thus, *Zfx* supports the optimal frequency of clonogenic cells in cytokine-driven AML cultures.

To model AML propagation in a native BM environment, we grew 4-OHT-treated *Zfx^{wt/y}* and *Zfx^{fl/y}* *R26-CreER⁺* MA9 AML cells on a BM stromal cell layer in the absence of exogenous cytokines (Sykes et al., 2011). After several days of culture, control AML cultures maintained a prominent fraction of c-Kit⁺ cells that lacked the myeloid marker CD14. *Zfx* deletion caused the loss of this c-Kit⁺CD14⁻ subset, whereas differentiated c-Kit⁻CD14⁺ cells accumulated (Fig. 4D,E). Furthermore, the expression levels of several myeloid differentiation markers (CD11b, MHC class II, CD80) progressively increased in *Zfx*-deficient cells (Fig. 4F). The resulting *Zfx*-deficient c-Kit⁻CD14⁺ cells grew poorly in cytokine-supplemented liquid culture, confirming the loss of proliferative capacity (Fig. 4G). These results suggest that *Zfx* opposes the differentiation of murine AML cells grown in the presence of BM stroma.

Human leukemia samples show a significant increase in the expression of *ZFX* and its expressed human gametolog *ZFY* (Fig. S4A). Several *Zfx*-specific lentiviral shRNA constructs impaired cell growth in the human Notch1C-dependent T-ALL line RPMI-8402, MA9-expressing AML cell line NOMO-1 and in three additional leukemia cell lines (Fig. S4B-E). Like murine MA9 AML cells, a distinct fraction of NOMO-1 cells express c-Kit; *ZFX* knockdown depleted this population and increased the expression of myeloid marker CD14 (Fig. S4F). Thus, *ZFX* facilitates the growth of human leukemia cell lines and helps prevent their phenotypic differentiation.

Zfx controls the gene expression programs of undifferentiated cells

To establish the transcriptional program controlled by *Zfx* in leukemic cells, we performed genome-wide expression profiling on murine T-ALL and AML cells shortly after *Zfx* deletion. Genes that were positively and negatively regulated by *Zfx* were defined by comparing *Zfx^{Δ/y}* samples to the respective control *Zfx^{fl/y}* samples (and, in the case of AML, to *Zfx^{wt/y}* samples) (Table S1). In parallel, we performed ChIP-seq analysis of *ZFX* binding to chromatin in the human *ZFX*-dependent cell lines RPMI-8402 (T-ALL) and NOMO-1 (AML). *ZFX* binding regions in both lines showed striking enrichment for the proximal (<1 Kb) promoters and 5' UTRs of genes (Table S2), suggesting that *ZFX* binds close to the transcription start site (TSS). As outlined in Fig. 5A, the sets of *Zfx*-regulated genes in T-ALL and AML were compared to ChIP targets in the respective human leukemia, as well as to ChIP targets in murine ESCs (Chen et al., 2008) (Table S3). The majority (>80%) of genes that were decreased in *Zfx*-deficient T-ALL or AML had *Zfx* binding regions within 1 Kb of the TSS in either murine ESCs or in the corresponding human leukemia line (Fig. 5B). Of these *Zfx* binding regions, 44% and 78% were conserved between murine ESCs and RPMI-8402 and NOMO-1 cells, respectively (Fig. 5B). Most of *ZFX* binding sites within *Zfx*-regulated genes were tightly clustered within 300 bp of the

TSS (Fig. S5A). In contrast, the majority of genes that were increased in *Zfx*-deficient T-ALL or AML did not contain *Zfx* binding sites within 1 Kb of the TSS (Fig. 5B). Thus, *Zfx* in leukemic cells directly activates its target genes by binding within their proximal promoter regions, whereas *Zfx*-mediated gene repression appears indirect.

The overlap of expression analysis in murine leukemias and ChIP-Seq in the corresponding human leukemia lines yielded sets of direct target genes that were activated by *Zfx* in T-ALL and AML (Table S4). Most of these genes were also reduced in *Zfx*-deficient ESCs and HSCs, suggesting a shared regulation by *Zfx* in normal stem cells and leukemic cells (Fig. 5C). Importantly, *Zfx* target gene sets in T-ALL or AML did not overlap with the proliferation-associated gene sets (Fig. S5B and Table S5), indicating that the *Zfx* gene expression program is not a mere reflection of cell proliferation. Within the normal differentiation hierarchy, the expression of *Zfx* T-ALL target set was enriched in normal DN thymocytes compared to the more differentiated DP thymocytes (Fig. 5D). This enrichment was not due to the higher proliferation rate of DN cells, because it was observed both in the quiescent (DN3a) and in proliferating (DN3b/DN4) DN subsets (Fig. S5C). Similarly, the expression of the *Zfx* AML target set was enriched in the HSC/myeloid progenitor BM compartment compared to mature myeloid cells (Fig. 5D). In contrast, the genes that were increased in *Zfx*-deficient T-ALL and AML were enriched in mature T cells and myeloid cells, respectively (Fig. 5D). Thus, in both T-ALL and AML cells, *Zfx* directly activates gene sets enriched in immature cells of the respective lineages, and indirectly prevents the induction of differentiation programs.

***Zfx* facilitates Myc-induced gene expression and transformation**

Given the important role of *Myc* in both T-ALL and AML, we asked whether *Zfx* cooperated with *Myc* in leukemia development. Retroviral overexpression of *Myc* in myeloid progenitors is sufficient to induce their transformation and serial replating capacity (Luo et al., 2005). To test the role of *Zfx* in *Myc*-induced transformation, we treated *Zfx^{fl/y}/R26-CreER⁺* mice or control *Zfx^{w^t/y}/R26-CreER⁺* mice with Tmx to induce *Zfx* deletion in the BM. Myeloid progenitors from these mice were transduced with retroviral vectors encoding GFP alone or GFP and *Myc*, and GFP⁺ cells were sorted and plated in semi-solid medium (Fig. 6A). *Myc*-encoding retrovirus supported serial replating of control progenitors, whereas *Zfx*-deficient progenitors failed to propagate beyond the first passage (Fig. 6B). Thus, endogenous *Zfx* facilitates the propagation of *Myc*-transformed progenitors, suggesting that it acts downstream or in parallel to *Myc* in leukemogenesis.

To test whether *Zfx* modulates the *Myc*-dependent gene expression program, we performed microarray analysis of wild-type or *Zfx*-deficient progenitors at the first passage after transduction with *Myc* or GFP. This analysis identified a distinct set of genes that were induced by *Myc* in wild-type progenitors (Fig. 6C, circled dots, and Table S6). Nearly a quarter of these genes (21 out of 94, 22%) were induced to a lower extent in *Zfx*-deficient progenitors (Fig. 6C, blue circled dots below the diagonal), compared to only 3 genes (3.2%) that were over-induced (blue circled dots above the diagonal). Conversely, the decrease of genes in response to *Myc* overexpression was affected weakly and non-directionally in *Zfx*-deficient progenitors (Fig. S6A and Table S6). Furthermore, the decrease of *Zfx* target genes in *Zfx*-deficient progenitors was not directionally affected by *Myc* overexpression (Fig. S6B and Table S7). These results suggest that endogenous *Zfx* facilitates the optimal induction of *Myc*-induced expression program that may ultimately lead to progenitor transformation.

Mitochondrial enzymes PTPMT1 and IDH2 are functional Zfx target genes

Direct Zfx target sets in T-ALL and AML included both lineage-specific as well as common target genes, the latter comprising 43% of Zfx targets in AML (Fig. 7A and Table S4). This incomplete overlap suggests the tissue specificity of some Zfx targets, but may also reflect technical limitations of target identification. Common Zfx targets included mitochondrial isocitrate dehydrogenase (*IDH2*) and mitochondrial protein tyrosine phosphatase 1 (*PTPMT1*) genes, which were also activated by Zfx in normal HSCs and ESCs (Fig. 5C). The deletion of Zfx decreased the expression of *Idh2* and *Ptpmt1* in murine T-ALL (5-fold and 12-fold, respectively) and in AML (2-fold and 4-fold, respectively) (Fig. 7B). The expression of *Idh2* and *Ptpmt1* was increased by Myc overexpression in wild-type progenitors, but did not reach the same levels in the absence of Zfx (Fig. 7C). The promoters of *Idh2* and *Ptpmt1* are bound by Myc along with Zfx in murine ESC and in human leukemia cells (Fig. S7A,B), raising the possibility of direct regulation by both factors.

IDH2 was shown to be important for human leukemia growth (Ward et al., 2010), and we further confirmed that its knockdown impaired the growth of murine MA9 AML cells (Fig. S7C). *IDH2* may drive biosynthesis in leukemia cells in part by facilitating conversion of glutamine into citrate (Ward et al., 2010; Wise et al., 2011). Zfx-deficient AML cells showed reduced glucose consumption and lactate production rates, consistent with impaired glycolytic flux in the mitochondria (Fig. 7D). These defects were partially rescued by *Idh2* overexpression, suggesting that Zfx-dependent *Idh2* expression facilitates glycolysis in AML cells. *Ptpmt1* facilitates the production of mitochondrial structural lipid cardiolipin by dephosphorylating its precursor phosphatidylglycerophosphate (PGP) (Zhang et al., 2011). Indeed, Zfx-deficient AML cells expressed lower levels of *Ptpmt1* protein (Fig. 7E), and showed the accumulation of PGP (Fig. 7F), consistent with rapid PGP accumulation in *Ptpmt1*-deficient cells (Zhang et al., 2011).

To test whether the overexpression of *Idh2* or *Ptpmt1* could rescue the propagation of Zfx-deficient AML, we transduced *Zfx^{fl/y} R26-CreER⁺* AML cells with GFP-marked retroviral vectors expressing these genes. Transduced cells were treated with vehicle or 4-OHT for 72 hours to delete Zfx prior to transplantation into recipients. Zfx-deficient AML cells with retroviral overexpression of either *Ptpmt1* or *Idh2* caused leukemia more quickly than those transduced with GFP-only retrovirus (Fig. 7G), while remaining Zfx-negative (Fig. S7D). Even though retroviral overexpression results in supraphysiological expression levels, the observed partial rescue of Zfx-deficient AML cells identifies *Idh2* and *Ptpmt1* as functional targets of Zfx in leukemia.

Discussion

We report that the transcription factor Zfx is important for the emergence and propagation of two acute leukemia types, T-ALL and AML. In normal conditions, Zfx is necessary for the long-term propagation of self-renewing HSCs and ESCs, but is dispensable in a variety of proliferating cells that do not self-renew. Indeed, Zfx plays a limited role in the immature DN4/ISP thymocytes (this study) and in myeloid progenitors (Galan-Caridad et al., 2007), the normal counterparts of leukemia-propagating cells in T-ALL and AML. Thus, Zfx is not universally required for the proliferation of hematopoietic progenitors but becomes important for the self-renewal of their transformed counterparts. The dependence of NotchIC- and MA9-driven leukemias on Zfx is notable given the strength of these oncogenic stimuli; for instance, the propagation of MA9 AML appears independent of *Bmi1*, an important regulator of normal and leukemic stem cell self-renewal (Smith et al., 2011). Our results reveal a common molecular network operating in distinct types of acute leukemia and highlight the importance of “non-oncogene addiction” of transformed cells to a non-oncogenic, native cellular factor (Luo et al., 2009).

Acute leukemia cells appear “locked” in an undifferentiated state characterized by immature phenotype and clonogenic growth. These characteristics are often restricted to a subset of leukemic cells, which can initiate leukemia upon adoptive transfer and are therefore termed “leukemia-initiating cells” (LICs). The LIC compartment in AML comprises c-Kit⁺ cells, and the blockade of major oncogenic pathways often leads to their depletion and/or myeloid differentiation. For instance, AML differentiation was observed after the genetic or pharmacological disruption of MLL-containing protein complexes (Bernt et al., 2011; Harris et al., 2012) or of FOXO transcription factors (Sykes et al., 2011). The LICs in Notch-induced T-ALL were recently shown to comprise cells with the immature DN phenotype (King et al., 2013); however, the differentiation of T-ALL into the more mature DP-like cells has not been commonly observed. We found that the loss of Zfx did not directly affect cell survival, proliferation or a proliferation-associated gene expression signature. On the other hand, it induced the differentiation of AML and T-ALL, along with the depletion of cells with the respective immature phenotypes. In each leukemia type, Zfx directly activated sets of genes that were enriched in immature myeloid cells and thymocytes. These gene sets were also regulated by Zfx in the normal ESCs and HSCs, revealing a common Zfx-dependent gene expression program. These observations further support the similarity of gene expression programs underlying the propagation of cancers and normal ESCs (Kim et al., 2010; Somervaille et al., 2009) and HSCs (Eppert et al., 2011).

We found that Zfx contributes to the transformation of myeloid progenitors by Myc, and facilitates the induction of Myc-dependent genes at the early stages of transformation. Zfx binds many actively transcribed promoters in murine ESCs jointly with Myc (Chen et al., 2008), and was proposed as a component of the Myc-driven network that facilitates the growth of ESCs and cancer cells (Kim et al., 2010). However, the specificity of Zfx-Myc cross-talk remains to be elucidated, given that Myc binds broadly to the chromatin and generally amplifies multiple expression programs (Lin et al., 2012; Nie et al., 2012). Furthermore, Zfx has been recently implicated as a potential tumor suppressor in Myc-induced experimental hepatomas (O'Donnell et al., 2012), suggesting that the effect of Zfx on Myc activity may be context-dependent. Importantly, Myc facilitates LIC maintenance in Notch1-induced T-ALL (King et al., 2013), and opposes differentiation of MLL fusion-driven AML (Schreiner et al., 2001; Zuber et al., 2011b). Thus, Myc maintains the propagation and opposes differentiation of both T-ALL and AML, and its function in acute leukemia appears to be dependent on endogenous Zfx.

Among the genes activated by Zfx in the two leukemia types and in normal stem cells, we focused on mitochondrial enzymes Idh2 and Ptpmt1. Because Idh2 is important for the production of citrate from glutamine in cancer cells (Ward et al., 2010; Wise et al., 2011), Zfx-mediated activation of Idh2 in leukemic cells may contribute to their Warburg metabolism and facilitate growth in hypoxic conditions in vivo. Ptpmt1 catalyzes the synthesis of mitochondrial cardiolipin, and is required for HSC/progenitor maintenance (Yu et al., 2013) and for the growth of multiple cancer cell lines (Niemi et al., 2013). It is therefore likely that reduced Ptpmt1 expression, such as observed after Zfx loss, would be particularly detrimental in leukemic cells that undergo continuous self-renewal. Altogether, Zfx-induced expression of Idh2 and Ptpmt1, and likely other targets, facilitates the function and integrity of mitochondria to promote the sustained growth of leukemic cells.

In conclusion, our results establish Zfx as an important endogenous regulator of two disparate, highly aggressive acute leukemia types. While the loss of Zfx also impairs the self-renewal of normal HSCs, the resulting gradual loss of HSCs allows erythro/myelopoiesis to proceed for weeks (Galan-Caridad et al., 2007). This is in contrast to rapid and pronounced effects of induced Zfx deletion on the mortality from acute T-ALL and

AML. This kinetic difference, further amplified by combination with other therapy forms, might permit ample window for potential therapeutic targeting of Zfx in acute leukemia.

Experimental Procedures

Animal studies

All mouse studies were performed according to the investigator's protocol approved by the Institutional Animal Care and Use Committee. Mice with the conditional *Zfx*^{fl} allele and their crosses to pan-hematopoietic *Tie2*-Cre, interferon-inducible *Mx1*-Cre and tamoxifen-inducible *R26*-CreER deleters have been described previously (Galan-Caridad et al., 2007). Spontaneous T-ALL was induced by crossing *Eef1a1*-Notch1C strain (Buonamici et al., 2009) with *CD4*-Cre deleter (Jackson Labs), with or without the *Zfx*^{fl} allele. For in vitro transformation of Zfx-deficient BM progenitors, Zfx deletion was induced by poly-I:C in *Zfx*^{fl/y} *Mx1*-Cre⁺ animals or with Tmx in *Zfx*^{fl/y} *R26*-Cre⁺ animals as described (Galan-Caridad et al., 2007). For *in vivo* Cre induction in recipients of *R26*-CreER⁺ AML or T-ALL, they were administered Tmx at the indicated days after leukemia transfer. For leukemia propagation, sublethally irradiated B6129F1 recipients syngeneic to the *R26*-CreER⁺ AML cells were used.

Cell culture and analysis

Murine MA9 and Hoxa9/Meis1 AML cells were maintained in medium with 20% fetal calf serum (FCS) and recombinant murine IL-3, IL-6 (10 ng/ml) and SCF (20 ng/ml) (Peprotech) (R20 AML media). Human NOMO-1 and RPMI-8402 cells were obtained from the Leibniz Institut DSMZ (Braunschweig, Germany) and cultured in medium with 10% FCS.

To induce recombination in *R26*-CreER⁺ AML cells, they were treated with 10 nM 4-OHT (Sigma-Aldrich) for 72 hours. BM stromal cells for co-culture experiments were obtained by serially passaging adherent cells isolated from crushed bones of GFP transgenic mice. For assays of clonogenic growth, AML cells were plated in semi-solid Methocult media (M3234, StemCell Technologies) supplemented with cytokines as above, and colony formation was evaluated 5–7 days later. For the analysis of mitochondrial metabolism, murine AML cells grown in cytokine-supplemented liquid culture were incubated with 4-OHT as above or left untreated, and analyzed 4 days later. Glucose consumption and lactate production were analyzed by incubating murine AML cells at high density (2×10^6 /ml) and measuring changes in supernatant glucose and lactate concentration using colorimetric assays (Sigma-Aldrich) over a period of 6 hours. Western blotting of Ptpmt1 and thin layer chromatography of mitochondrial lipids were performed as described (Zhang et al., 2011).

MSCV-based retroviral constructs encoding oncogenes are described in Supplemental Methods. Bicistronic retroviral constructs encoding Ptpmt1 and Idh2 were constructed by cloning open reading frames of mouse Ptpmt1 and Idh2 into MSCV-IRES-GFP. Lentiviral constructs expressing shRNAs to human ZFX have been described (Harel et al., 2012).

Hematopoietic progenitors or primary MA9 AML cells were transduced with these retroviruses and injected i.v. into sublethally irradiated recipient mice or cultured as above. For shRNA expression in human cells, concentrated lentiviral supernatants were applied in triplicate at MOI of 1 followed by selection in puromycin or (for GFP-expressing constructs) by FACS.

Flow cytometry analysis was performed on an LSR II flow cytometer and cell sorting was performed on a FACSAria or Influx flow sorters (BD Immunocytometry Systems). Data were analyzed using FlowJo software (Treestar Inc.). Myeloid progenitor populations were isolated by FACS for the following immunophenotypes: CMP, c-Kit⁺, Sca1⁻, Lin⁻, CD34⁺,

CD16/32^{lo}; GMP, c-Kit⁺, Sca1⁻, Lin⁻, CD34⁺, CD16/32^{hi}. LICs in AML were sorted based on the following immunophenotypes: Lin^{lo} CD16/32^{hi}, CD34⁺ c-Kit⁺ and Lin^{lo} CD16/32^{hi} CD34⁻ c-kit⁺.

Expression analysis and ChIP

Genome-wide expression analysis was done using Mouse Gene 1.0 ST microarrays (Affymetrix). Microarray hybridization, scanning and data extraction using the Expression Console software package was according to the manufacturer's instructions. Quantitative RT-PCR analysis was performed using open reading frame-specific primers (sequences available upon request) and the $\Delta\Delta_{CT}$ method as described (Galan-Caridad et al., 2007).

For ChIP, nuclei from 10⁷ formaldehyde-fixed NOMO-1 and RPMI-8402 cells were isolated, lysed and ultrasonically sheared using the TRUChIP High Cell Chromatin Shearing Kit (Covaris). ChIP was performed using anti-ZFX rabbit polyclonal antibody or non-specific rabbit IgG as described (Galan-Caridad et al., 2007). After eluting the sheared immunoprecipitated chromatin, crosslinking were reversed and DNA was recovered by phenol-chloroform extraction. Samples of 'input' (sheared but not immunoprecipitated) chromatin were used as controls. Library construction and sequencing were performed by the Yale Center for Genome Analysis (New Haven, CT). The analysis of microarray and ChIP data is described in Supplemental Methods.

Statistical analysis

Statistical significance was estimated using log-rank test for Kaplan-Meier survival plots; two-tailed Student's *t*-test for the comparison of two groups; or two-way ANOVA for multivariate analysis such as gene expression levels in 4 samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- * Zfx is important for the initiation and propagation of AML and T-ALL
- * The loss of Zfx caused differentiation in both leukemia types
- * Zfx facilitated leukemic transformation and gene induction by Myc
- * Key Zfx target genes included the mitochondrial enzymes *Ptpmt1* and *Idh2*

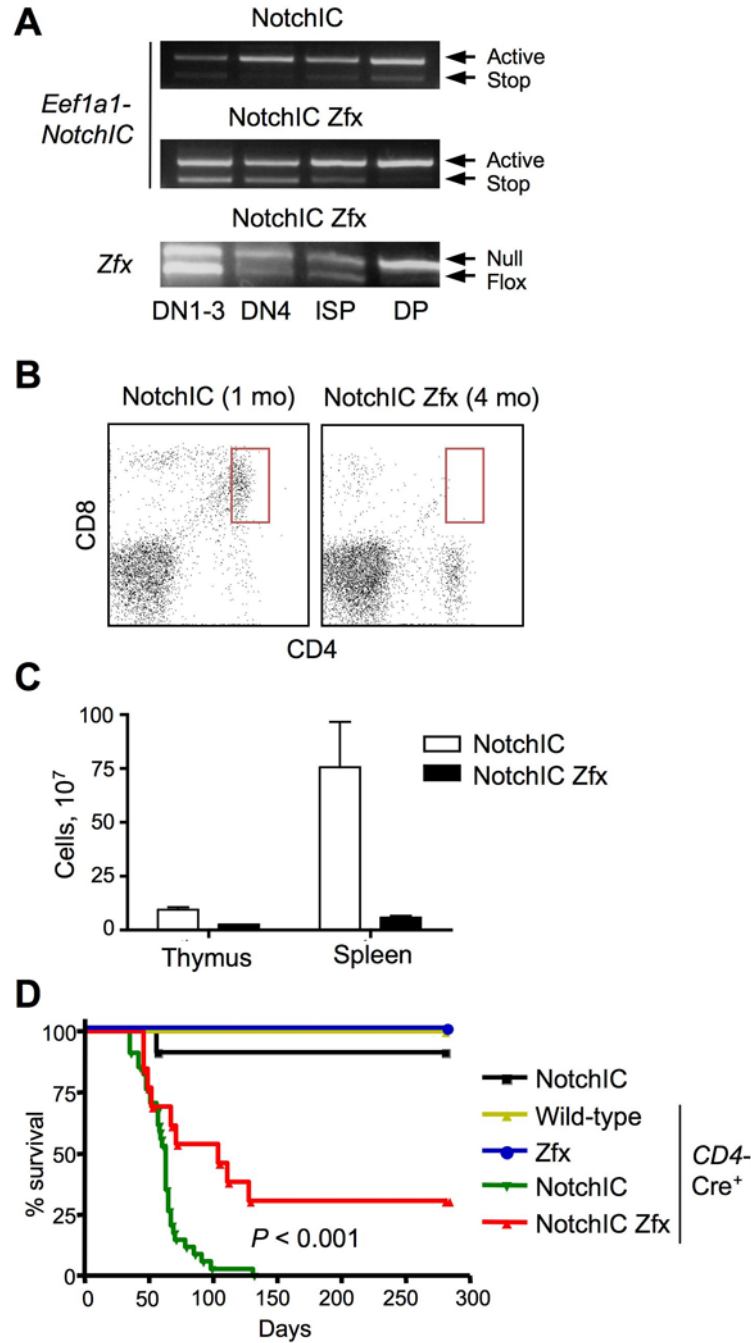


Figure 1. Zfx contributes to the development of Notch-driven T-ALL

Mice carrying T cell-specific Cre transgene (*CD4-Cre*) and Cre-inducible activated Notch1 (*Eef1a1-NotchIC*) with (*NotchIC Zfx*) or without (*NotchIC*) the conditional *Zfx*^{f1} allele were analyzed.

(A) Recombination kinetics of the *Eef1a1-NotchIC* allele during T cell development. The indicated thymocyte subsets from pre-leukemic 4-wk old animals were sorted and analyzed by genomic PCR.

(B) Representative staining profiles of T cells in the peripheral blood; the abnormal DP population associated with T-ALL is highlighted.

(C) The cellularity of the thymus and spleen from moribund mice (mean \pm SEM of 5–6 animals).

(D) The survival of experimental animals and of the indicated control mice (*Eef1a1*-Notch1C only; *CD4*-Cre only; *CD4*-Cre⁺ *Zfx*^{fl} without *Eef1a1*-Notch1C) (n =12–30). See also Fig. S1.

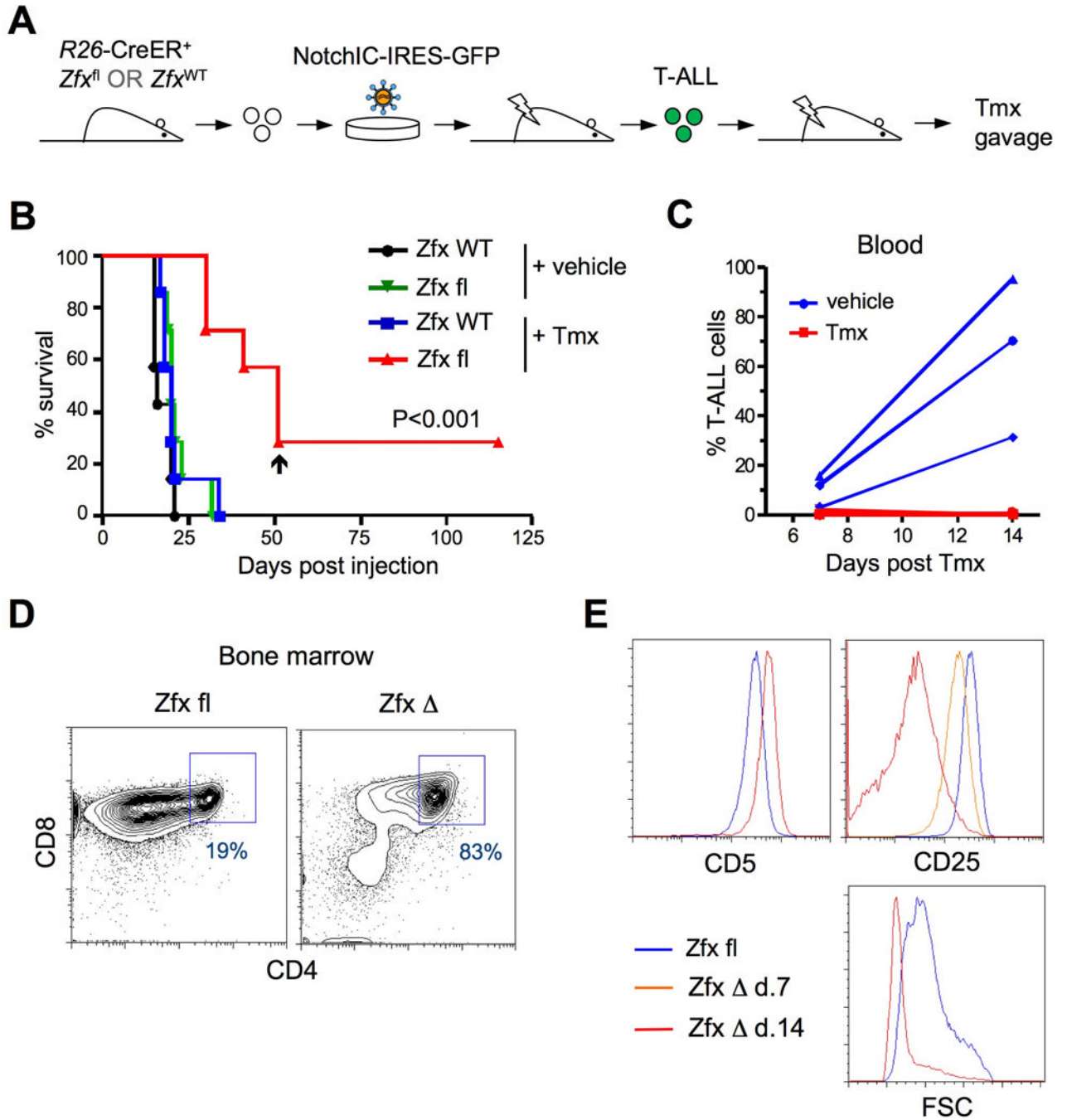


Figure 2. Zfx contributes to the propagation of pre-established T-ALL

(A) Schematic of experimental approach to test the role of Zfx in pre-established Notch1C dependent T-ALL.

(B) The survival of mice transplanted with T-ALL cells followed by inducible Zfx deletion. Independent primary *R26-CreER⁺* T-ALL lines carrying wild-type (*Zfx^{WT}*) or conditional (*Zfx^{fl}*) Zfx allele (7 of each genotype) were transplanted into recipient mice, which were treated 2 days later with either Tmx or vehicle. Arrowhead indicates the only recipient of *R26-CreER⁺ Zfx^{fl}* cells that died from *Zfx*-deficient leukemia.

(C) The propagation of secondary T-ALL after delayed Zfx deletion. Three primary *R26-CreER⁺ Zfx^{fl/y}* T-ALL lines were transplanted into recipient mice, which were treated 6 days later with either Tmx or vehicle. Shown is the fraction of GFP⁺ T-ALL cells in the peripheral blood at the indicated time points after Tmx treatment.

(D–E) The phenotype of T-ALL cells after Zfx deletion. Secondary recipients of T-ALL cells described in panel C were sacrificed at 7 or 14 days after vehicle/Tmx treatment, and their BM were analyzed by flow cytometry. Panel D shows the CD4/CD8 expression profile of gated GFP⁺ T-ALL 14 days after treatment with vehicle (Zfx fl) or Tmx (Zfx Δ). Panel E shows the staining level of indicated markers or forward scatter (FSC) of gated GFP⁺ T-ALL after the treatment with vehicle on day 14 or with Tmx on day 7 (for CD25) or 14. See also Fig. S2.

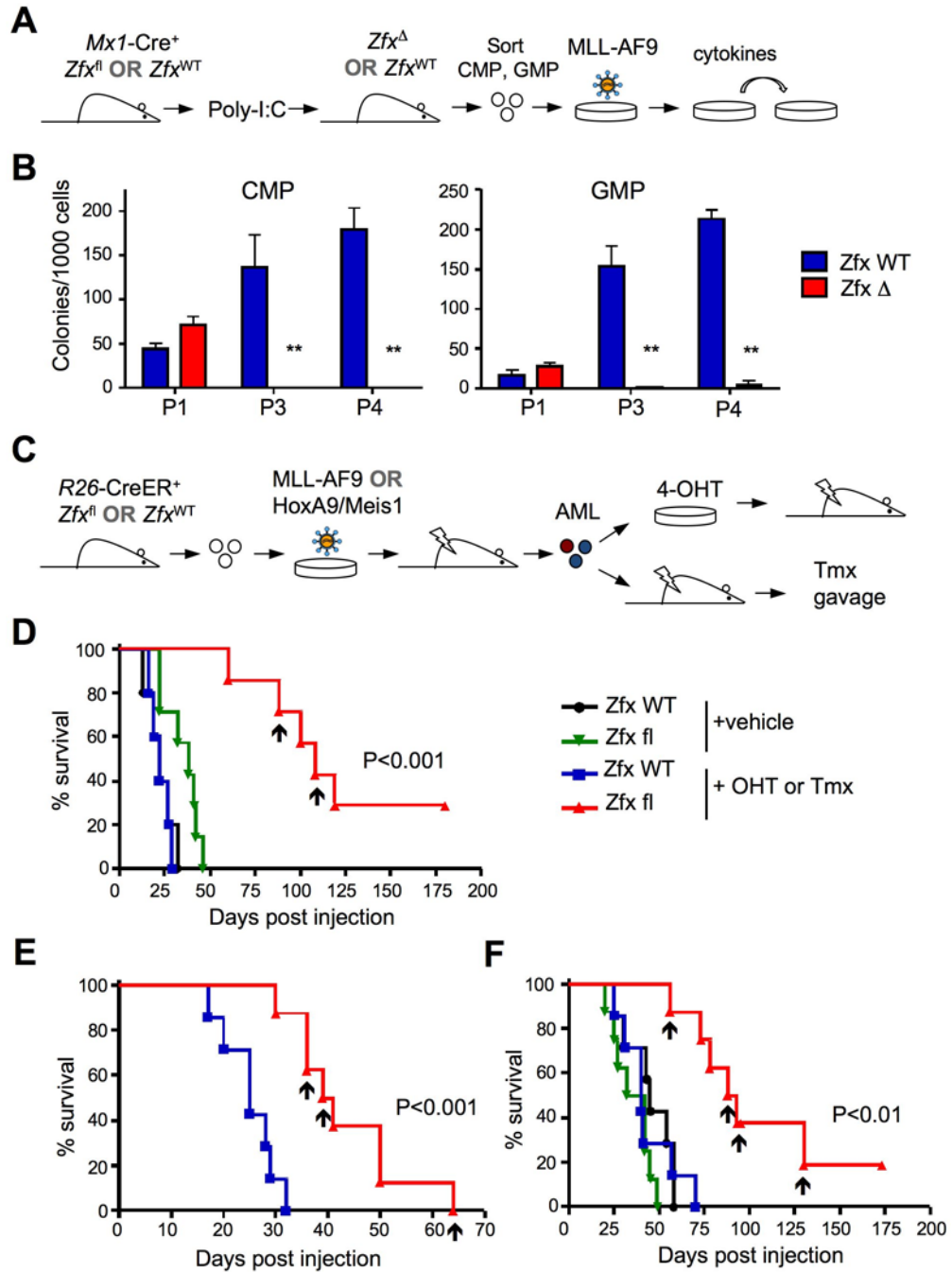


Figure 3. Zfx contributes to the initiation and propagation of MA9-induced AML

(A) Schematic of experiment to test the transformation of Zfx-deficient myeloid progenitors by the MLL-AF9 (MA9) retrovirus.

(B) Clonal outgrowth of $Zfx^{wt/y}$ and $Zfx^{\Delta/y}$ common myeloid progenitors (CMP) and granulocyte-monocyte progenitors (GMP) transduced with MA9. Shown are colony yields at the indicated passages (P) in semi-solid medium (mean S.E.M. of 3–5 independent parallel cultures). ** $P < 0.01$.

(C) Schematic of experimental approach to test the role of Zfx in pre-established AML *in vivo*.

(D) The effect of *Zfx* deletion on leukemia initiation by primary MA9-induced AML. Independent primary *R26-CreER*⁺ AML lines carrying wild-type (*Zfx*^{WT}) or conditional (*Zfx*^{fl}) *Zfx* allele (7–8 of each genotype) were incubated with 4-OHT or vehicle for 3 days and transplanted into secondary recipients. Shown is the Kaplan-Meier survival plot of recipient mice; arrowheads indicate recipients of *R26-CreER*⁺ *Zfx*^{fl} cells that died from *Zfx*-deficient leukemia.

(E) The effect of *Zfx* deletion *in vivo* on the progression of MA9-induced AML. Untreated primary *R26-CreER*⁺ AML lines carrying *Zfx*^{WT} or *Zfx*^{fl} alleles were transplanted into secondary recipients, which were treated with Tmx 10 days later. The results are shown as in panel (D).

(F) The effect of *Zfx* deletion on leukemia initiation by *Hoxa9/Meis1*-induced AML. The experiment was performed and is presented as in panel (D), except that AML was induced by *Hoxa9/Meis1* instead of MA9 retrovirus. The results are shown as in panel (D).

See also Fig. S3.

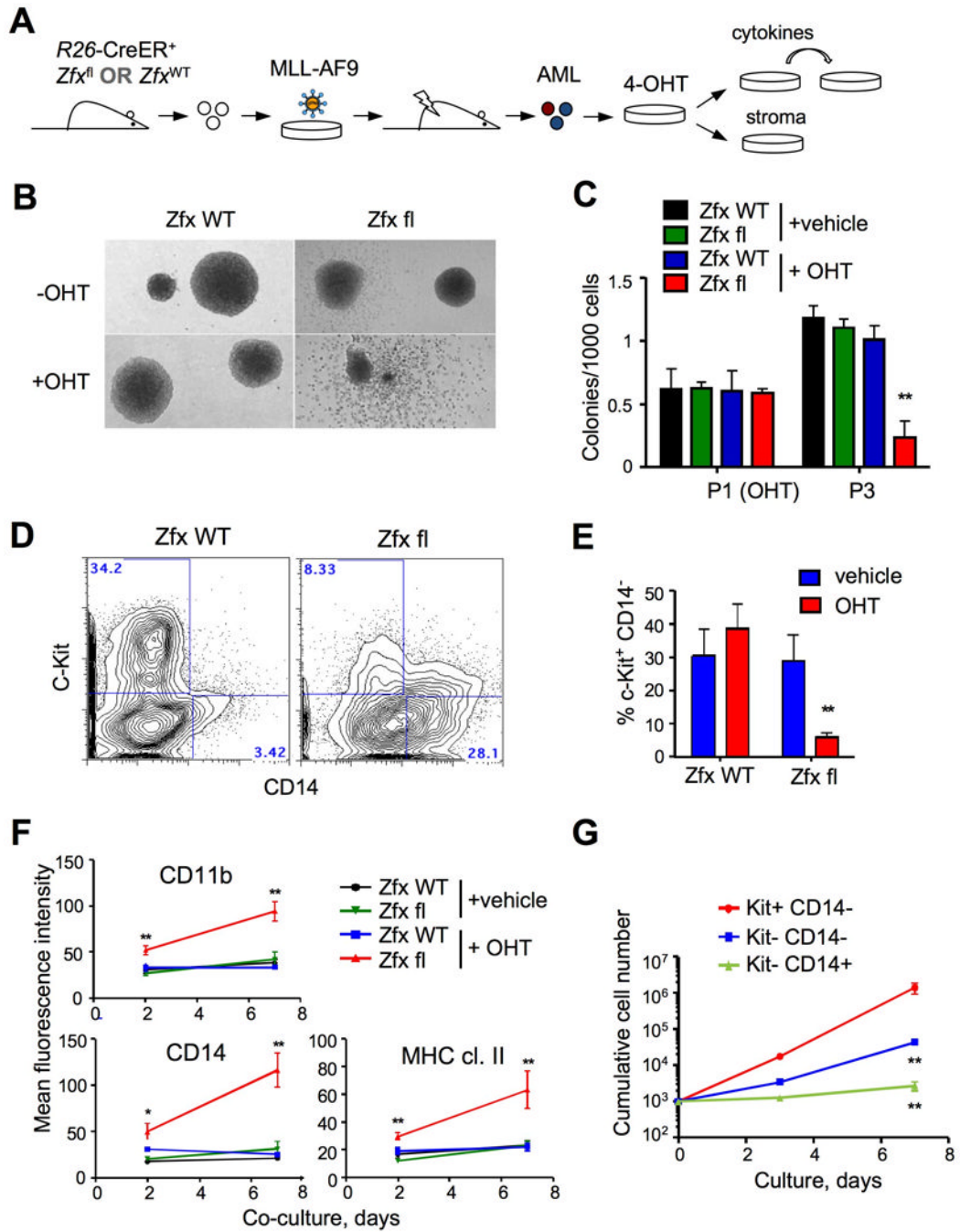


Figure 4. Zfx controls the clonogenic growth and immature phenotype of murine MA9 AML
 (A) Schematic of experimental approach to test the role of Zfx in the growth and phenotype of pre-established AML.

(B–C) The effect of Zfx loss on MA9 AML cells grown in cytokine-supplemented culture. Primary $R26\text{-CreER}^+$ AML lines carrying wild-type (Zfx^{WT}) or conditional (Zfx^{fl}) Zfx allele were incubated with 4-OHT or vehicle for 4 days (passage 1, P1) and passaged in semi-solid medium. Shown are representative microphotographs of colonies at P1 (panel B) and colony yields at P3 (mean \pm SEM of 6 independent cultures). ** $P < 0.01$.

- (D–G) The effect of Zfx loss on MA9 AML cells co-cultured with BM stromal cells. Primary AML lines described above were cultured with 4-OHT or vehicle in cytokine-supplemented liquid culture and plated on stromal cells without cytokines.
- (D) Representative staining profiles of OHT-treated cells after 4 days of stromal co-culture
- (E) The fraction of cells with progenitor phenotype (c-Kit⁺ CD14⁻) after 7 days of stromal co-culture (mean ± SEM of 6 independent cultures). ***P*<0.01.
- (F) Mean fluorescence intensity of myeloid differentiation markers during stromal co-culture (mean ± SEM of 7–8 independent cultures). **P*<0.05, ***P*<0.01.
- (G) The growth potential of Zfx-deficient AML cells after stromal co-culture. Cell fractions of the indicated phenotype were sorted from OHT-treated *R26-CreER⁺ Zfx^{fl}* AML cells grown in stromal co-culture, and replated into cytokine-supplemented liquid culture (mean ± SEM of 3 independent cultures). ***P*<0.01.
- See also Fig. S4.

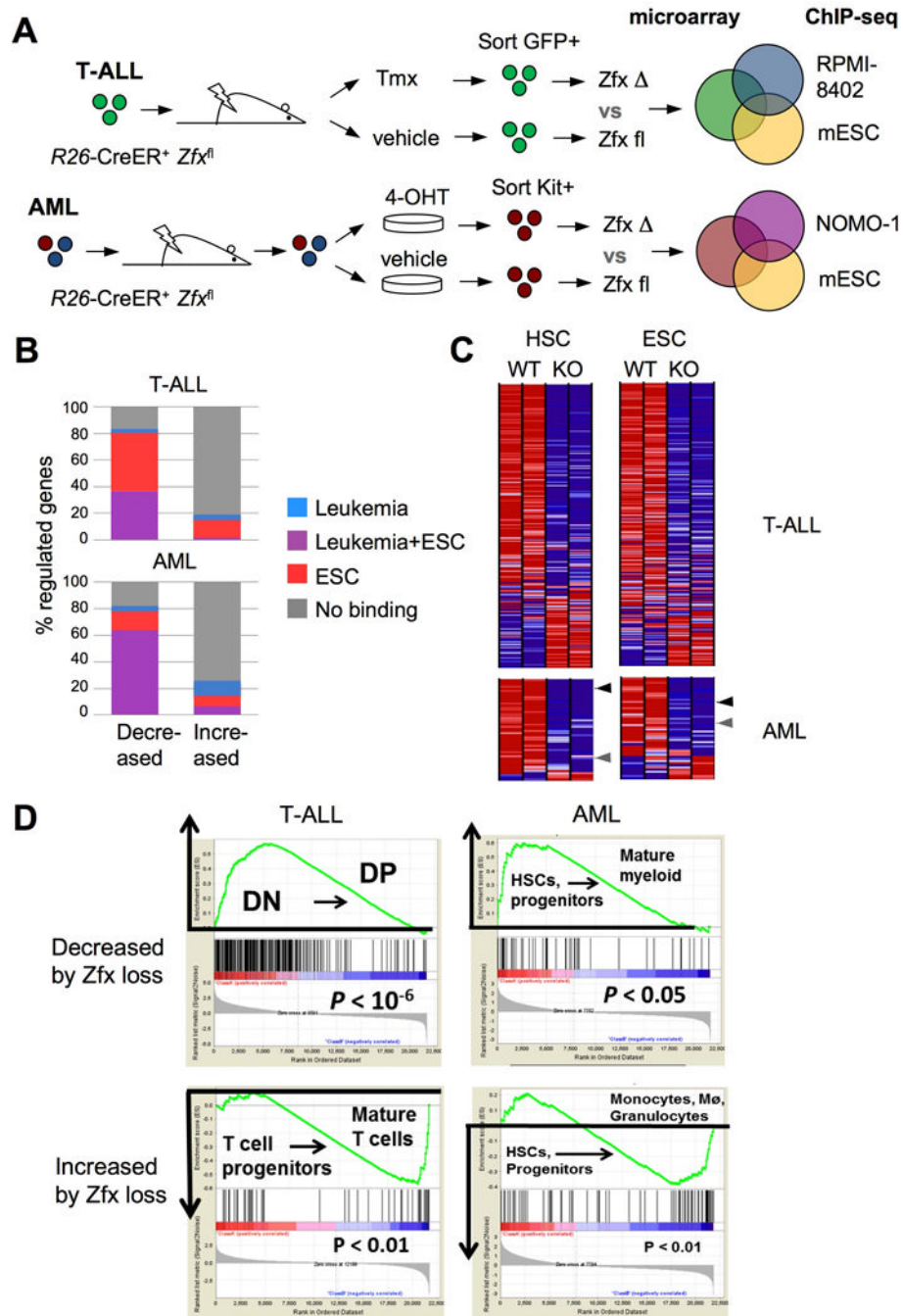


Figure 5. Characterization of gene expression program controlled by Zfx in T-ALL and AML (A) Schematic of the approach to define Zfx-controlled gene expression programs in leukemia, using the overlap between Zfx-regulated gene sets and genomic Zfx binding regions (as determined by microarrays and ChIP-seq, respectively).

(B) The identification of direct Zfx target genes in T-ALL and AML. Shown is the percentage of Zfx-regulated genes in murine T-ALL and AML that had significant Zfx binding regions within 1 Kb of the TSS in murine ESCs and/or the respective human leukemia cell line.

(C) The expression of leukemia-derived Zfx target gene sets in normal Zfx-deficient stem cells. Shown are expression levels (as heat maps) in control vs Zfx-deficient HSCs and

ESCs (Galan-Caridad et al., 2007) of direct Zfx target genes defined in T-ALL and AML. Arrowheads highlight Zfx target genes *Ptpmt1* (black) and *Idh2* (grey).

(D) The expression of leukemia-derived Zfx target genes during normal development of the respective lineages. Genes that were decreased or increased by Zfx loss in T-ALL were analyzed for their expression in normal DN versus DP thymocytes; genes that were decreased or increased by Zfx loss in AML were analyzed for their expression in normal HSCs and myeloid progenitors versus mature myeloid cells. Shown is the output of gene set enrichment analysis (GSEA), with the enrichment score graphs on top. See also Fig. S5 and Tables S1–S5.

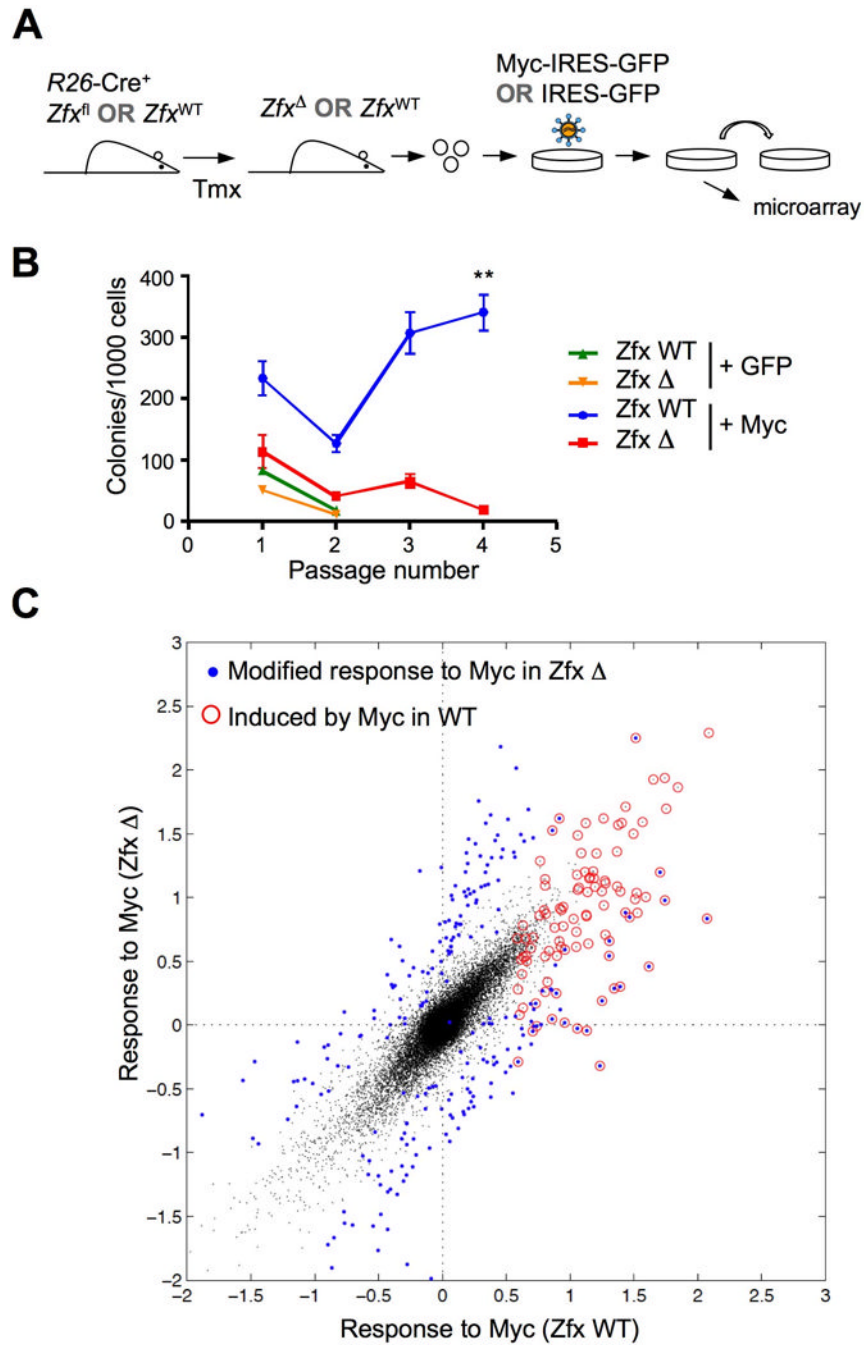


Figure 6. Zfx contributes to Myc-induced progenitor transformation

(A) Schematic of experiment to test the role of Zfx in the transformation of myeloid progenitors by Myc.

(B) Effect of Zfx deletion on clonogenic growth of Myc-transformed myeloid progenitors. Myeloid progenitors from mice with Tmx-induced Zfx deletion ($Zfx^{\Delta/y}$) or from control Tmx-treated mice ($Zfx^{WT/y}$) were transduced with Myc-GFP or GFP only retroviruses, and GFP⁺ progenitors were sorted and propagated in semi-solid medium. Shown are the colony yields over serial passages (mean \pm SEM of 6 independent cultures) ** $P < 0.01$.

(C) Response to Myc overexpression in Zfx-deficient progenitors. Wild-type or Zfx-deficient myeloid progenitors transduced with Myc/GFP or GFP only as above were cultured for 4

days and analyzed by microarray (3 independent cultures for each sample). Average differential expression in Myc-expressing versus GFP-expressing cells of each Zfx genotype (Myc response) was calculated for each probe. Shown is the pairwise comparison of Myc responses in wild-type versus Zfx-deficient cells, with the probes showing differential responses in blue. The probes whose levels were increased by Myc in wild-type cells are circled in red.

See also Fig. S6 and Tables S6–S7.

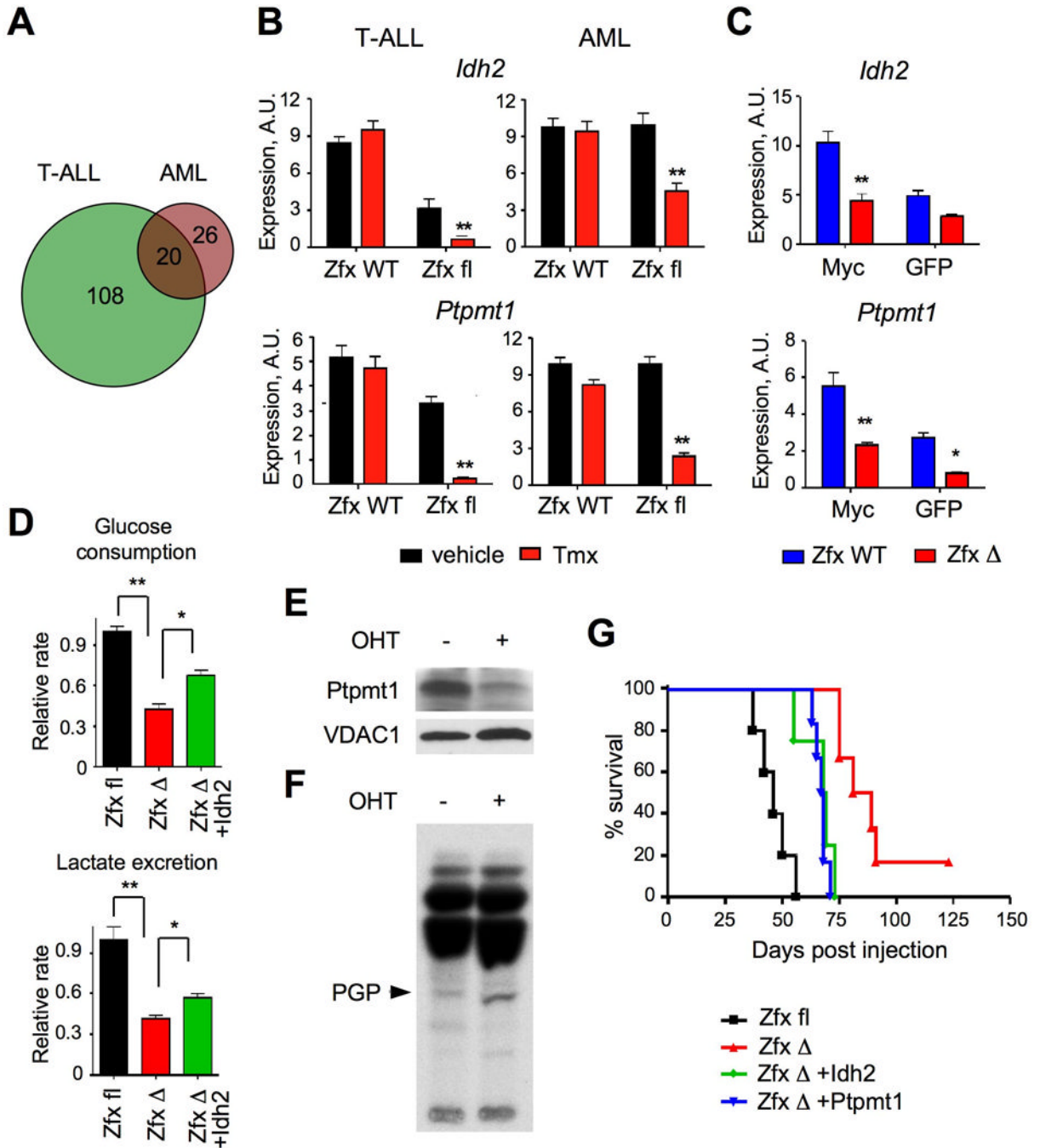


Figure 7. Zfx activates the expression of *Ptpmt1* and *Idh2* in leukemia cells

(A) The overlap between conserved direct targets of Zfx activation in T-ALL and AML. (B) The expression of *Idh2* and *Ptpmt1* in murine leukemia cells after Zfx deletion. The deletion was induced by Tmx (or vehicle as a control) in the *R26-CreER*⁺ conditional (*Zfx*^{fl/y}) or control (*Zfx*^{wt/y}) leukemia cells. Notch1C-transformed T-ALL cells were sorted from secondary recipients 5 days after Tmx treatment; MA9-transformed primary AML were incubated with 4-OHT for 3 days. Shown are relative expression levels as determined by qRT-PCR (mean ± SEM of 5 independent lines for T-ALL and 7–8 independent lines for AML). ***P*<0.01.

(C) The expression of *Idh2* and *Ptpmt1* in *Zfx*-deficient progenitors during transformation by *Myc*. Shown are relative transcript levels determined by qRT-PCR in *Zfx*^Δ/*y* or *Zfx*^{wt}/*y* progenitors 4 days after transduction by *Myc*-GFP or GFP only (mean ± SEM of 6 independent cultures) * *P*<0.05, ** *P*<0.01.

(D) Effect of *Zfx* deletion on the glycolysis rate of murine AML cells grown in liquid culture with cytokines. MA9-transformed *R26*-CreER⁺ *Zfx*^{fl}/*y* AML line was transduced with retroviral vectors expressing *Idh2* or GFP alone. The cells were incubated with 4-OHT to induce *Zfx* deletion, propagated for 4 days in liquid culture and analyzed for the steady-state glucose consumption and lactate excretion rates (mean ± SEM of triplicate cultures). **P*<0.05, ***P*<0.01.

(E–F) Effect of *Zfx* deletion on the expression and function of *Ptpmt1*. MA9-transformed *R26*-CreER⁺ *Zfx*^{fl}/*y* AML line was incubated with 4-OHT for 5 days to induce *Zfx* deletion, and analyzed 4 days later by Western blotting for *Ptpmt1* (panel D) and by thin layer chromatography for ³²P-labeled mitochondrial lipids (panel E). The position of PGP was confirmed by parallel analysis of pure ¹⁴C-labeled PGP.

(G) Effect of *Idh2* and *Ptpmt1* overexpression on the growth of *Zfx*-deficient AML. MA9-transformed *R26*-CreER⁺ *Zfx*^{fl}/*y* AML line was transduced with retroviral vectors expressing *Idh2*, *Ptpmt1* or GFP alone. The cells were incubated with 4-OHT to induce *Zfx* deletion, and the resulting *Zfx*^Δ/*y* cells were transferred into secondary recipients. Each recipient received one cell line resulting from an independent transduction with the respective vector. Shown are Kaplan-Meier survival plots of the recipient groups; the difference between *Idh2* or *Ptpmt1*-expressing and control GFP-expressing *Zfx*^Δ/*y* AML is significant (*P*<0.01). The results represent a summary of 3 independent experiments involving 1–3 lines of each genotype. See also Fig. S7.