

Zfx Controls the Self-Renewal of Embryonic and Hematopoietic Stem Cells

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SUMMARY

Stem cells (SC) exhibit a unique capacity for self-renewal in an undifferentiated state. It is unclear whether the self-renewal of pluripotent embryonic SC (ESC) and of tissue-specific adult SC such as hematopoietic SC (HSC) is controlled by common mechanisms. The deletion of transcription factor Zfx impaired the self-renewal but not the differentiation capacity of murine ESC; conversely, Zfx overexpression facilitated ESC self-renewal by opposing differentiation. Furthermore, Zfx deletion abolished the maintenance of adult HSC but did not affect erythromyeloid progenitors or fetal HSC. Zfx-deficient ESC and HSC showed increased apoptosis and SC-specific upregulation of stress-inducible genes. Zfx directly activated common target genes in ESC and HSC, as well as ESC-specific target genes including ESC self-renewal regulators Tbx3 and Tcl1. These studies identify Zfx as a shared transcriptional regulator of ESC and HSC, suggesting a common genetic basis of self-renewal in embryonic and adult SC.

INTRODUCTION

The development and function of multicellular organisms are based on the activity of stem cells (SC). During early embryonic development, pluripotent stem cells give rise to all cell types in the body, including the germline (Boiani and Scholer, 2005). In the adult, tissue homeostasis is maintained by multipotent adult stem cells, which continuously generate cell lineages comprising a given tissue. In adult mammals, SC have been identified in many tissues and organs, including the hematopoietic system, brain,

skin, and intestine (Weissman et al., 2001). Whereas the precise differentiation potential varies between different SC types, all SC share the fundamental property of self-renewal, i.e., the maintenance of their number and identity in an undifferentiated state.

Embryonic stem cells (ESC) can be derived from pluripotent cells of the blastocyst inner cell mass and propagated in vitro as continuous clonal lines (Chambers and Smith, 2004). These ESC lines are capable of unlimited growth in culture while retaining their undifferentiated state and full developmental potential. Murine ESC are typically cultured on fibroblast feeder cells with serum and leukemia inhibitory factor (LIF), a cytokine that activates the essential gp130/Stat3 signaling pathway. Combined with LIF, bone morphogenetic proteins (BMP) support unlimited ESC self-renewal in the absence of serum or feeders (Ying et al., 2003). The pluripotency of ESC is regulated by a unique network of ESC-specific transcription factors including Oct4, Nanog, Sox2, and their binding partners (Boyer et al., 2005; Chambers and Smith, 2004; Wang et al., 2006). Indeed, a defined combination of Oct4, Sox2, and other transcription factors is sufficient to derive ESC-like pluripotent cells from mouse fibroblasts (Takahashi and Yamanaka, 2006). In addition, a distinct genetic pathway regulating ESC self-renewal involves transcription factors Tbx3 and Esrrb and a signaling cofactor Tcl1 (Ivanova et al., 2006).

Among adult SC types, one of the best characterized is the blood-producing hematopoietic SC (HSC) of the adult bone marrow (BM) (Kondo et al., 2003). These cells sustain hematopoiesis throughout adult life and are capable of clonal, long-term hematopoietic reconstitution of irradiated recipients after serial transplantation. HSC are localized in a distinct structural and functional compartment in the BM, the HSC niche, which provides critical signals supporting HSC self-renewal (Calvi et al., 2003; Zhang et al., 2003). Upon differentiation, the bona fide long-term repopulating HSC rapidly lose their self-renewal potential, producing a hierarchy of multipotent short-term HSC and/or multilineage progenitors, common

lymphoid and myeloid progenitors, and lineage-committed precursors. In the adult mouse BM, all HSC activity is contained in the rare (<0.1%) lineage marker $(\text{Lin})^- \text{Sca-1}^+ \text{c-Kit}^+$ (LSK) cell subset, which can be further subdivided to define the homogeneous long-term HSC population. The self-renewal of adult HSC is regulated by several broadly expressed transcription factors including Bmi-1 (Lessard and Sauvageau, 2003; Park et al., 2003), Tel/Etv6 (Hock et al., 2004) and FoxO (Tothova et al., 2007).

Although both ESC and adult SC are capable of unlimited self-renewal, their cellular and molecular features are strikingly different. First, ESC can self-renew in defined culture conditions *in vitro*, whereas the self-renewal of adult SC typically depends on their *in vivo* niche (Li and Xie, 2005). Furthermore, ESC are highly proliferative and exhibit unusual features of cell-cycle control, such as the lack of a G1 checkpoint (Burdon et al., 2002). In contrast, a cardinal feature of adult SC is their low proliferation rate (Li and Xie, 2005). Indeed, genetic manipulations that increase HSC proliferation lead to HSC exhaustion and eventual failure (Cheng et al., 2000; Yilmaz et al., 2006; Zhang et al., 2006). In view of these fundamental differences between ESC and various adult SC, a major unresolved question is whether their self-renewal is regulated by common genes and/or pathways (Eckfeldt et al., 2005; Mikkers and Frisen, 2005). Such “stemness” genes have been postulated on the basis of global expression analysis of different SC populations (Ivanova et al., 2002; Ramalho-Santos et al., 2002); however, they remain to be reconciled between different studies and verified in functional assays (Sakaguchi et al., 2006). Furthermore, other studies emphasized the lack of a common gene expression program in ESC and adult SC (D’Amour and Gage, 2003; Fortunel et al., 2003).

Zfx is a zinc finger protein of the Zfy family, whose members are highly conserved in vertebrates. Mammalian Zfx is encoded on the X chromosome and contains an acidic transcriptional activation domain (AD), a nuclear localization sequence (NLS), and a DNA binding domain (DBD) consisting of 13 C₂H₂-type zinc fingers (Schneider-Gadicke et al., 1989). A targeted mutation of murine Zfx resulted in partially penetrant neonatal lethality and hypofertility in surviving females (Luoh et al., 1997). However, the possibility that the targeting created a hypomorphic Zfx allele could not be ruled out. Recently, two microarray-based expression studies suggested that Zfx might be expressed at elevated levels in several SC types (Fortunel et al., 2003; Ramalho-Santos et al., 2002), prompting us to investigate the role of Zfx in SC function.

Here, we use conditional gene targeting to examine the role of murine Zfx in two prototypical SC types, ESC and adult HSC. We report that Zfx is required for the self-renewal of both SC types, whereas it is dispensable for the growth and differentiation of their progeny. These results establish Zfx as a shared transcriptional regulator of embryonic and adult SC, suggesting a common molecular basis of SC self-renewal.

RESULTS

Conditional Targeting of Murine Zfx

To confirm the elevated expression of Zfx in SC, we measured Zfx levels in SC by quantitative PCR (qPCR). As shown in Figure 1A, the expression of Zfx was twice as high in HSC and in undifferentiated ESC compared to their respective differentiated progeny. To test the function of Zfx in SC, we generated a conditional *LoxP*-flanked (“floxed”) allele of murine Zfx by homologous recombination in ESC (Figure 1B and Figure S1 in the Supplemental Data available with this article online). Cre-mediated recombination of the only Zfx^{fllox} allele in male Zfx^{fllox}/y cells produced Zfx^{null} allele, which did not encode a detectable protein product (Figure 1C). Embryos with germline Zfx deletion developed normally until 9.5 d.p.c. and subsequently died due to extraembryonic tissue abnormalities (data not shown). The embryos with Zfx deletion in the embryo proper died neonatally but were grossly normal at 18.5 d.p.c., with Zfx^{null} cells contributing extensively to all tissues (Figure S2). Similarly, a nearly complete deletion of Zfx in the absence of obvious abnormalities was observed in embryos and newborns with early brain- or hematopoietic-specific Zfx deletion (Figure S2). These data suggest that Zfx is dispensable for cell growth and differentiation of most tissues *in vivo*.

Zfx Is Required for ESC Self-Renewal

To analyze the growth of Zfx-deficient ESC, we transiently induced Cre recombination in Zfx^{fllox} ESC and cultured the resulting mixture of recombined (Zfx^{null}) and unrecombined ESC in the conditions favoring self-renewal (serum/LIF/feeders). As shown in Figure 2A, Zfx^{null} ESC were rapidly out-competed by Zfx^{fllox} cells and disappeared after two passages. In contrast, Zfx^{null} cells persisted when ESC were allowed to differentiate in the absence of LIF/feeders. Moreover, a similar competition assay revealed normal growth of Zfx-deficient primary mouse embryonic fibroblasts (MEF), confirming a specific role of Zfx in undifferentiated ESC.

To further characterize the phenotype of Zfx-deficient ESC, we derived clonal Zfx^{null} ESC lines and then stably re-expressed Zfx in these cells (Figure 2B). The resulting Zfx^{null} ESC remained undifferentiated, as judged by the expression of ESC marker alkaline phosphatase (Figure 2C) and key ESC regulators Nanog and Oct4 (Suppl. Figure S3). Nevertheless, all Zfx^{null} clones (n = 10) exhibited abnormal colony morphology, reduced clonogenic capacity, slower growth and spontaneous apoptosis in serum/LIF (Figures 2C–2E). The impaired self-renewal of Zfx^{null} ESC was particularly evident in serum-free culture with LIF and BMP4, in which Zfx^{null} clones failed to expand and collapsed after two to three passages (Figure 2F). During the initial growth in serum-free culture, Zfx^{null} cells underwent massive apoptosis as revealed by increased proportion of Annexin V-positive and hypodiploid cells (Figures 2G and S3). Notably, nearly all (9/10) Zfx^{null} ESC clones reconstituted with Zfx (Zfx^{null} + Zfx) regained

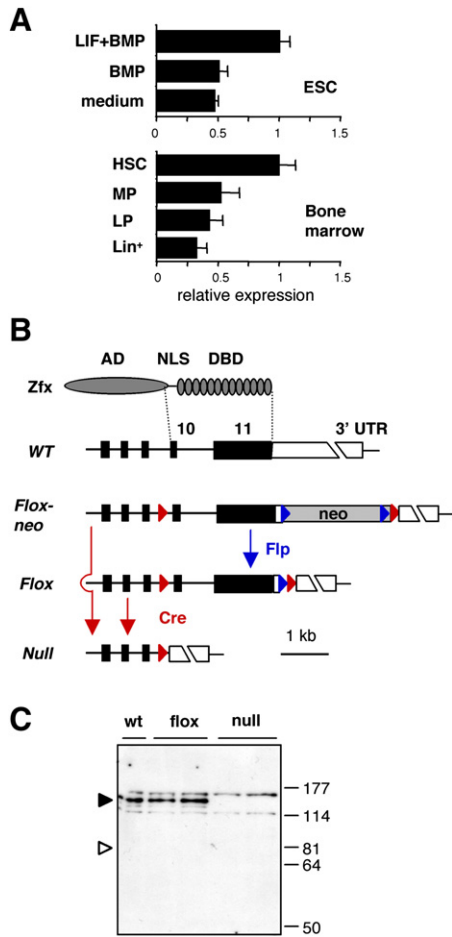


Figure 1. The Expression Analysis and Conditional Targeting of Zfx

(A) The expression of *Zfx* is elevated in undifferentiated murine ESC and HSC. The ESC were grown in serum-free culture with LIF + BMP4 (undifferentiated), BMP4 or medium alone (differentiated). Wild-type BM was sorted into lineage-positive (Lin⁺) differentiated cells and Lin⁻ populations including HSC (Sca-1⁺ c-Kit⁺), myeloid progenitors (MP, Sca-1⁻ c-Kit⁺) and lymphoid progenitors (LP, Sca-1^{low} c-Kit^{low}). Shown is normalized *Zfx* expression relative to the corresponding SC sample as measured by qPCR.

(B) The strategy of *Zfx* gene targeting. Exons 10 and 11 encoding the NLS and DBD of *Zfx* protein were flanked by *LoxP* sites (red triangles). The neomycin resistance (*neo*) cassette flanked by *FRT* sites (blue triangles) was excised from the targeted *Zfx*^{flox-neo} allele by Flp-mediated recombination. Cre-mediated recombination of either *Zfx*^{flox-neo} or *Zfx*^{flox} allele produces the *Zfx*^{null} allele.

(C) The expression of *Zfx* protein in the wild-type (wt) or targeted ESC clones, analyzed by Western blotting with antibodies against *Zfx* N-terminal AD. The positions of full-length *Zfx* and of the predicted protein encoded by the null allele (determined using the corresponding epitope-tagged expression constructs) are indicated by filled and open triangles, respectively.

normal morphology and vigorous growth (Figures 2C, 2D, 2F, and 2G). These data demonstrate a specific and reversible defect in the growth and survival of undifferentiated *Zfx*-deficient ESC.

In contrast to their impaired self-renewal, the differentiation capacity of *Zfx*-deficient ESC was intact. When injected into mice, *Zfx*^{null} ESC efficiently produced teratomas containing differentiated tissues from the three germinal layers (Figure 3A). Despite the low efficiency of chimera formation after blastocyst injection, *Zfx*^{null} ESC contributed to the majority of adult tissues in chimeric mice, including the germline (Figure 3B). The lack of contribution to the thymus and BM is consistent with the essential role of *Zfx* in adult hematopoiesis (see below). Furthermore, *Zfx*^{null} ESC clones grew normally in short-term differentiation cultures without LIF (Figures 3C and 3D) and expressed appropriate differentiation markers (Figure S3). Finally, *Zfx*^{null} ESC efficiently formed embryoid bodies (EB) that expressed the expected markers of hematopoietic differentiation (Keller, 2005) such as β -globin and Flk-1 (Figures 3D and 3E, and data not shown). These results suggest that the loss of *Zfx* impairs the self-renewal of undifferentiated ESC but does not affect their developmental potential or the growth of their differentiated progeny.

In the course of these experiments, we noticed that ESC clones expressing higher levels of *Zfx* (*Zfx*^{null} + *Zfx*^{*}, Figure 2B) were less prone to differentiation. In short-term differentiation culture without LIF, *Zfx*-overexpressing ESC grew better than *Zfx*^{null} or *Zfx*^{flox} ESC (Figure 3C) and retained undifferentiated phenotype (Figure 3D), followed by massive cell death after replating. Furthermore, *Zfx*^{null} + *Zfx*^{*} ESC generated small EB without cavities, which lost the pluripotency marker *Nanog* but failed to express hematopoietic differentiation marker β -globin (Figures 3D and 3E). Finally, *Zfx*-overexpressing ESC failed to efficiently generate teratomas or mouse chimeras (data not shown). Thus, overexpression of *Zfx* appears to facilitate ESC self-renewal in suboptimal conditions and to oppose ESC differentiation, the phenotype complementary to the loss of *Zfx*. Altogether, these data establish *Zfx* as a critical and specific regulator of ESC self-renewal.

Zfx Is Required for the Self-Renewal of Adult HSC

To examine the role of *Zfx* in adult hematopoiesis, we generated Mx1-Cre⁺ *Zfx*^{flox/y} conditional knockout (CKO) mice, in which Cre-mediated *Zfx* deletion is induced by interferonogen pl-C (Kuhn et al., 1995). Two weeks after induction, total numbers of BM cells and of c-Kit⁺ Sca-1⁻ myeloid progenitors were unaffected in CKO mice, whereas the HSC-containing LSK population was significantly ($p < 0.0001$) decreased (Figure 4A). Time course analysis revealed a progressive loss of the HSC at 2–5 weeks after induction, resulting in the decreased ratio of HSC to c-Kit⁺ Sca-1⁻ myeloid progenitors (Figure 4B). The fractions of erythroid and myeloid differentiated cells were normal, while lymphocytes were initially depleted by pl-C treatment and failed to regenerate in the CKO BM (Figure S4). A similar loss of the LSK population was observed after *Zfx* deletion induced by tamoxifen-activated Cre construct (R26-CreER, Figure S5), ruling out potential side effects of pl-C injection.

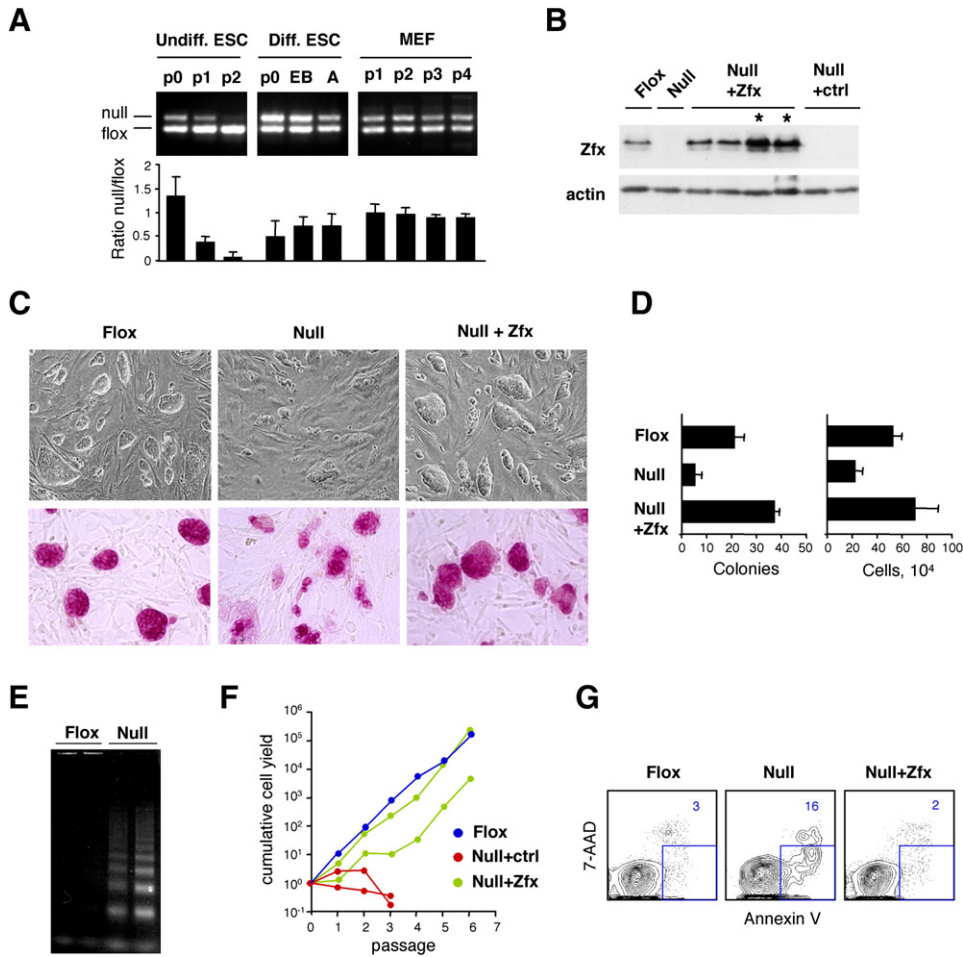


Figure 2. Defective Self-Renewal of Zfx-Deficient ESC

(A) Impaired competitive growth of Zfx-deficient ESC but not of differentiated cells. Cre recombination was transiently induced in ESC or in primary embryonic fibroblasts (MEF), and the presence of *Zfx*^{null} cells at subsequent passages (P) was monitored by genomic PCR. The ESC were grown in serum/LIF/feeders (undiff.) or differentiated (diff.) into primitive embryoid bodies (EB) followed by adherent culture (A). Shown are the simultaneous amplification of *Zfx*^{null} and *Zfx*^{flox} alleles and the ratios of separately amplified alleles determined by qPCR.

(B) Re-expression of Zfx in Zfx-deficient ESC. *Zfx*^{null} ESC stably transfected with Zfx or control constructs (ctrl) were analyzed by Western blotting with anti-Zfx Ab. The clones overexpressing Zfx are indicated with an asterisk.

(C) Defective colony morphology of Zfx-deficient ESC. Shown are live cultures (magnification 100x) and fixed cells stained for alkaline phosphatase (purple, magnification 200x) of ESC grown with serum/LIF/feeders.

(D) Defective growth and colony forming capacity of Zfx-deficient ESC clones in serum/LIF/feeders. Shown are numbers of colonies and total cell yields of ESC (average ± SD of triplicate cultures) after one passage at single-cell (10²/well) or regular (10⁴/well) densities, respectively.

(E) Increased apoptosis of Zfx-deficient ESC clones grown in serum/LIF, as measured by DNA laddering assay (two clones per genotype).

(F) Defective growth of Zfx-deficient ESC clones in serum-free culture with LIF + BMP4. The data represent average cell yields of parallel triplicate cultures for each clone.

(G) Increased apoptosis of Zfx-deficient ESC in serum-free culture. After one passage in LIF + BMP4, cells were stained for Annexin V and DNA content (7-AAD); the fraction of Annexin⁺ 7-AAD⁻ apoptotic cells is indicated.

The consequences of constitutive Zfx deletion in hematopoietic cells were analyzed using the Tie2-Cre strain, in which pan-hematopoietic Cre recombination is initiated in the early embryo (Koni et al., 2001). Zfx deletion was inefficient in most adult CKO mice, suggesting a strong selection against *Zfx*^{null} cells in adult hematopoiesis. Indeed, the rare adult CKO mice with a complete Zfx deletion (n = 5) showed the same specific loss of the HSC popula-

tion (Figure 4C). In contrast, the CKO fetal livers were uniformly *Zfx*^{null} (Figure S2), exhibited normal cellularity and contained only a slightly reduced fraction of HSC (Figure 4C). Furthermore, primitive hematopoiesis in the yolk sac of *Zfx*^{null} embryos was normal (Figure S6). Thus, the absence of Zfx abolishes the maintenance of adult BM HSC under steady-state conditions but largely spares embryonic hematopoiesis.

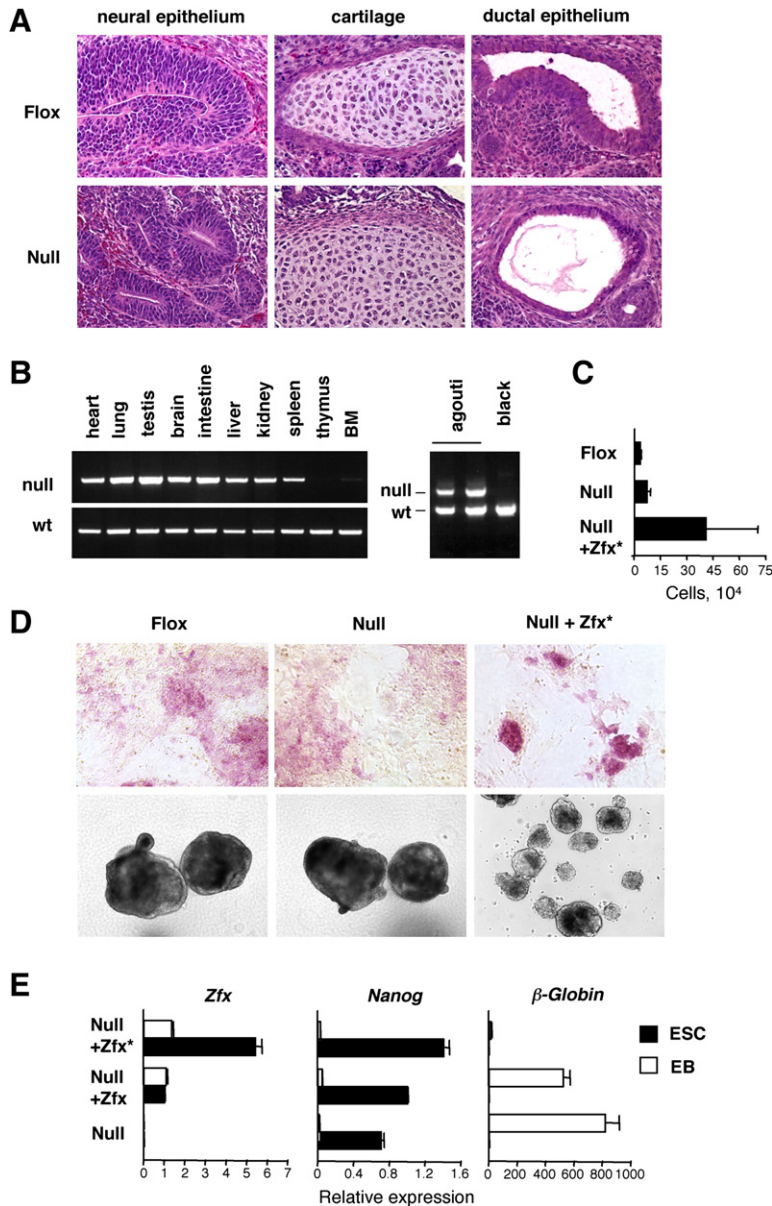


Figure 3. Normal Differentiation Capacity of Zfx-Deficient ESC

(A) Normal teratoma formation by control (*Zfx*^{Flox}) and *Zfx*^{null} ESC clones (representative of two clones per genotype). Differentiated tissues in hematoxylin/eosin-stained sections of teratomas are shown (magnification 200x).

(B) The contribution of *Zfx*^{null} ESC to tissues of chimeric mice. Left panel: null and wild-type *Zfx* alleles were separately amplified from genomic DNA of chimeric mouse tissues (representative of three chimeras). Right panel: female progeny of a chimeric mouse were genotyped, confirming germline transmission of *Zfx*^{null} allele.

(C) The growth in differentiating conditions of ESC clones lacking (*Zfx*^{null}) or overexpressing (*Zfx*^{null} + *Zfx*^{*}) *Zfx*. Shown are average cell yields of triplicate cultures plated at 2×10^4 /well and grown for one passage without LIF or feeders.

(D) The same ESC clones were grown without LIF and stained for alkaline phosphatase (purple, upper panel) or induced to form mature EB (lower panel). Magnification, 100x.

(E) The expression of pluripotency (*Nanog*) and differentiation (*β-globin*) markers in undifferentiated ESC or in EB measured by qPCR relative to the expression in *Zfx*^{null} + *Zfx* ESC.

Further analysis of the HSC/progenitor compartment in induced Mx1-Cre CKO mice (Figure 4D) and in Tie2-Cre CKO mice (Figure S7) confirmed the loss of Flt3⁻ LSK subset that contains the self-renewing long-term HSC. In addition, the Flt3⁺ LSK population containing short-term HSC and c-Kit^{low} Flt3⁺ lymphoid progenitors including IL-7Rα⁺ common lymphoid progenitors (CLP) were severely reduced (Figures 4D, S4, S5, and S7). The HSC in induced Mx1-Cre⁺ CKO mice proliferated normally, did not show increased egress from the BM, and displayed normal homing to and lodging in the BM after adoptive transfer (Figure S8). On the other hand, *Zfx*-deficient HSC showed a significant ($p = 0.0002$) increase in Annexin V-positive apoptotic cells (Figure 4E). In contrast, the Lin⁻ Sca-1⁻ c-Kit⁺ erythromyeloid progenitors were present

in normal numbers, contained common and lineage-restricted subsets (Figure 4D), did not show increased apoptosis (Figure 4E), and maintained their characteristically high proliferation rate (Figure S8). Indeed, control and CKO BM generated similar numbers of erythroid and myeloid colonies in short-term colony-forming assays (Figure 4F). These observations suggest that *Zfx* is required for the long-term survival of HSC but is dispensable in erythromyeloid progenitors.

Consistent with the defective maintenance of *Zfx*-deficient HSC, the BM from induced Mx1-Cre CKO mice failed to contribute to hematopoiesis in competitively reconstituted BM recipients (Figures 5A and 5B). To measure the maintenance of HSC independent of their homing or engraftment, we induced the loss of *Zfx* in pre-established

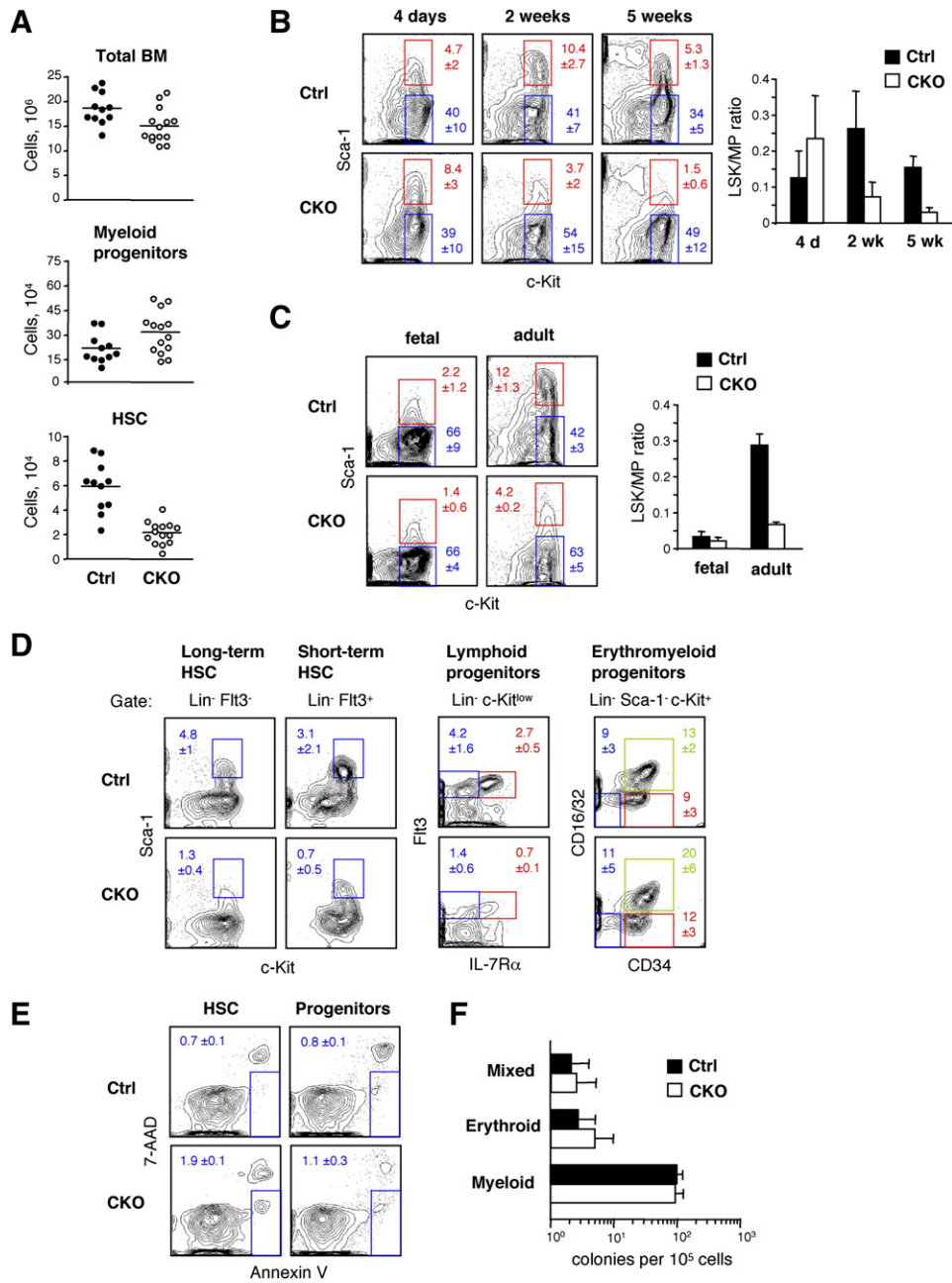


Figure 4. The Loss of Adult HSC after Zfx Deletion in Hematopoietic Cells

(A) The decrease in HSC numbers after Zfx deletion from the BM. Shown are absolute numbers of total BM cells, myeloid progenitors and the HSC-containing LSK population in control and Mx1-Cre⁺ Zfx^{flx/y} CKO mice two weeks after pl-C injection. Each symbol represents an individual mouse. (B) The loss of HSC from the stem/progenitor compartment of Mx1-Cre⁺ CKO BM at the indicated times after pl-C injection. Shown are the staining profiles of Lin⁻ cells, with the LSK population (red) and erythromyeloid progenitors (blue) and their percentages indicated (average \pm SD of 3–12 animals). The graph shows average ratios of the LSK to myeloid progenitor fractions. (C) The loss of adult HSC after constitutive Zfx deletion in the hematopoietic system. The HSC and myeloid progenitor populations in the fetal liver (n = 6–9) or in adult BM (n = 4–5) of Tie2-Cre⁺ CKO and control mice are shown as in panel (B). (D) Decreased HSC and lymphoid progenitors but normal myeloid progenitors in Mx1-Cre⁺ CKO mice two weeks after pl-C injection. Shown are Flt3⁻ and Flt3⁺ LSK populations containing long-term and short-term HSC, respectively; Flt3⁺ Lin⁻ c-Kit^{low} lymphoid progenitors including the IL-7R α ⁺ CLP subset (red); and Lin⁻ Sca-1⁺ c-Kit⁺ erythromyeloid progenitors divided into common myeloid progenitor (red), erythrocyte/megakaryocyte progenitor (blue), and granulocyte/monocyte progenitor (green). The fraction of each subset in the Lin⁻ population is indicated (average \pm SD of 5 animals). (E) Increased HSC apoptosis in Mx1-Cre⁺ CKO mice two weeks after pl-C injection. Shown are gated HSC (the LSK subset) and erythromyeloid progenitors (Lin⁻ Sca-1⁺ c-Kit⁺) stained for Annexin V and DNA content (7-AAD). The fractions of Annexin⁺ 7-AAD⁺ apoptotic cells (highlighted) are indicated (average \pm SD of 3 animals). (F) Colony formation assay showing reduced mixed, erythroid, and myeloid colonies in CKO mice compared to Ctrl mice.

BM chimeric mice (Mikkola et al., 2003). Irradiated recipient mice (CD45.1⁺ CD45.2⁺) were transplanted with a mixture of donor BM from uninduced Mx1-Cre⁺ CKO or control mice (CD45.2⁺) and competitor wild-type BM (CD45.1⁺). Four weeks later, chimeric mice were injected with pl-C to induce Zfx loss in the engrafted CKO BM, and the fraction of donor-derived peripheral blood leukocytes (PBL) was subsequently monitored (Figure 5C). In contrast to all control recipient groups, the pl-C-injected recipients of CKO BM manifested a sharp decline of donor-derived PBL (Figures 5D and 5E). The endpoint analysis 6 months after induction confirmed the virtual absence of Zfx-deficient donor cells in all hematopoietic cell types, including the HSC-containing LSK subset (Figure 5F). These results demonstrate at the functional level that Zfx is absolutely required for the long-term HSC maintenance.

A Common Gene Expression Signature of Zfx-Deficient SC

To identify Zfx target genes in SC, we compared global expression profiles of control and Zfx-deficient ESC and HSC. Several genes that were affected by Zfx loss in both ESC and HSC were identified, including 5 downregulated genes (6 probes, $p = 0.0075$) and 20 upregulated genes (22 probes, $p = 7 \times 10^{-8}$) (Figure 6A and Table S1). In the latter group, a more permissive analysis suggested several additional candidates. By qPCR (Figure 6B and Table S2), four downregulated genes (6720467C03Rik, AV340375, Phca, and Retsat) and six upregulated genes (Procr, Pkia, Ier3, Cryab, Gem, and Ptgs2) were validated as differentially expressed, revealing a common expression signature of Zfx-deficient ESC and HSC.

The predicted regulatory regions of commonly downregulated genes contain multiple potential binding sites for Zfx (Figure S9). To analyze the binding of Zfx to these genomic regions in live SC, we performed chromatin immunoprecipitation (ChIP) of epitope-tagged Zfx expressed in Zfx^{null} ESC (Zfx^{null} + Zfx^{TAG}, Figure S10). We found that promoter regions of 6720467C03Rik, AV340375 and Retsat were significantly enriched in ChIP with anti-tag antibodies; in contrast, no enrichment was seen in parental Zfx^{null} ESC that do not express tagged Zfx (Figure 6C). Finally, the expression of all three genes was increased in Zfx-overexpressing ESC clones (Table S2). These results suggest that genes commonly downregulated in Zfx^{null} ESC and HSC are direct targets of Zfx.

Among the six genes coordinately upregulated in Zfx-deficient ESC and HSC, four (Cryab, Ier3, Gem, and Ptgs2) are known to be induced by stress, inflammatory stimuli and/or growth factors, with the latter three representing immediate-early genes. Therefore, their induction could be indirect and reflect the impaired function of Zfx-deficient SC. Indeed, four genes (Pkia, Cryab, Ier3, and

Ptgs2) were upregulated in Zfx^{null} ESC and HSC, but not in their respective differentiated progeny (Figure 6D). Notably, three genes (Gem, Procr, and Pkia) display highly HSC-specific expression pattern (Ivanova et al., 2002; and this study). The specific upregulation of Procr in Zfx-deficient HSC was confirmed by cell-surface staining for its product CD201 (Figure S11). Thus, the impaired self-renewal of Zfx-deficient ESC and HSC correlates with SC-specific upregulation of stress-inducible and/or immediate-early genes.

Zfx Controls ESC-Specific Regulators Tbx3 and Tcl1

To test whether Zfx controls genes and pathways specific only for a particular SC type, we analyzed known ESC-specific regulators of self-renewal. By protein staining (Figure S3), microarray analysis (Table S3), and qPCR (Figure 7A), the components of the Nanog/Oct4/Sox2 transcriptional network were only minimally affected in Zfx^{null} ESC. On the other hand, we observed the downregulation of several ESC-specific genes including *Dub1/2*, *Ceacam1*, and particularly *Tbx3*, which encodes a transcription factor required for ESC self-renewal (Ivanova et al., 2006). Further qPCR analysis showed that another ESC-specific regulator, *Tcl1*, was similarly reduced in Zfx^{null} ESC. Short-term batch culture of ESC after Cre-mediated Zfx deletion showed a prominent reduction of *Tbx3* and a slight decrease of *Tcl1*, but not of *Nanog* (Figure 7B). Finally, Zfx-overexpressing ESC expressed higher levels of *Tbx3* and *Tcl1*, whereas *Nanog* and *Oct4* were unchanged (Figure 7C).

The predicted promoter regions of *Tbx3* and *Tcl1* contain multiple consensus binding sites for Zfx (Figure S12). In the case of *Tbx3*, ChIP analysis localized the region of prominent Zfx binding to ~0.5 Kb upstream of the transcription start site (Figure 7D). Indeed, the binding of Zfx to *Tbx3* and *Tcl1* promoters was detected only in Zfx-expressing Zfx^{null} + Zfx^{TAG} ESC, but not in the parental Zfx^{null} ESC (Figure 7E). Finally, Zfx binding to *Tbx3* promoter was observed in undifferentiated ESC but not in ESC differentiated without LIF, consistent with the rapid loss of *Tbx3* expression upon differentiation (Figure 7F). These data suggest that Zfx directly controls the expression of self-renewal regulators *Tbx3* and *Tcl1* in undifferentiated ESC.

DISCUSSION

In this paper, we examined the biological role of Zfx, one of the first identified zinc finger-encoding genes (Schneider-Gadicke et al., 1989) whose properties and function remained unclear. We found that Zfx deletion impaired the growth of ESC in vitro and increased their apoptosis rate, particularly in defined serum-free conditions. Despite the growth and survival defect, Zfx-deficient ESC

(F) Normal erythromyeloid progenitor function in Zfx-deficient BM. Shown is in vitro colony formation by BM cells from Mx1-Cre⁺ CKO or control mice two weeks after pl-C injection (average \pm SD of 4 animals).

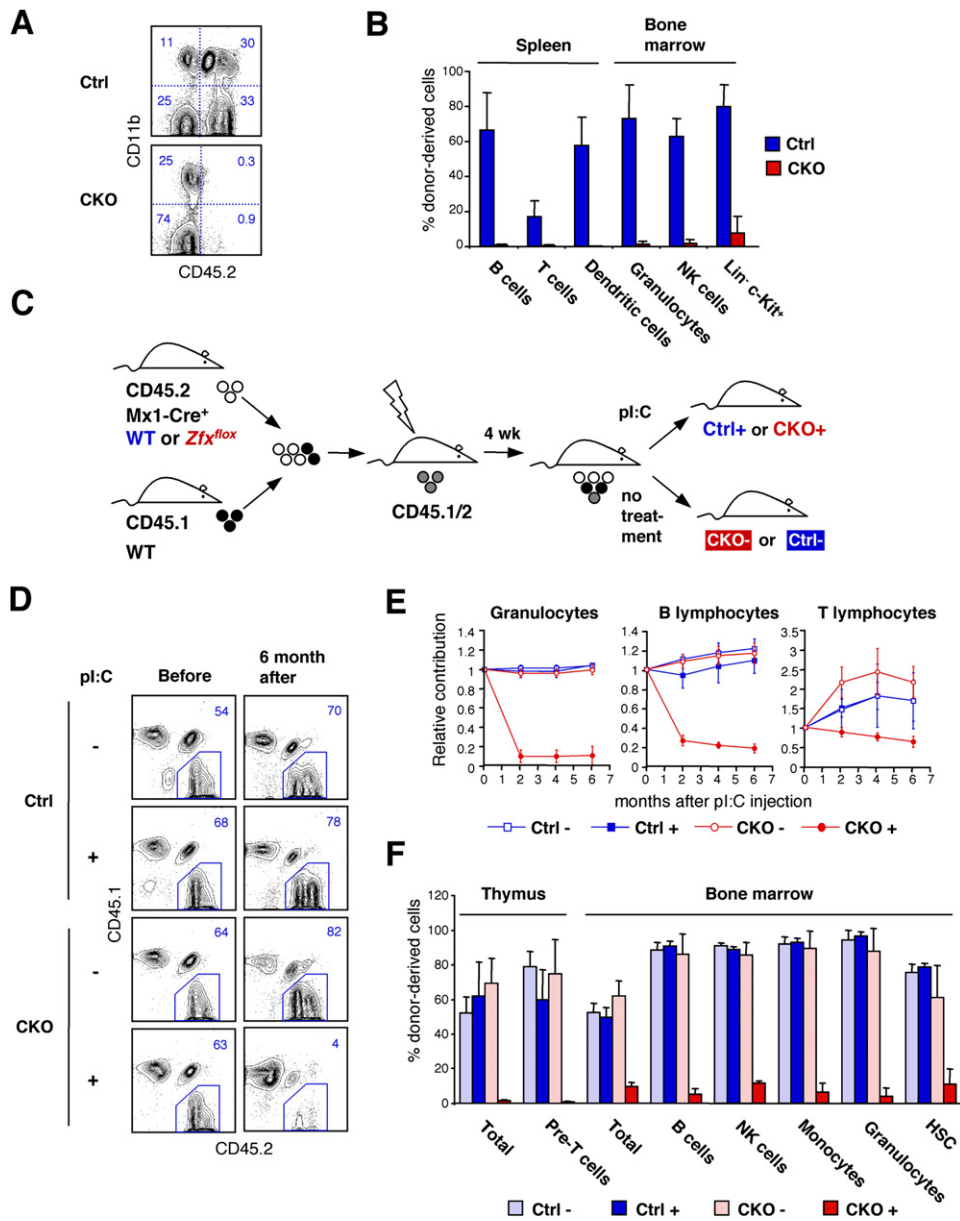


Figure 5. The Loss of Zfx Abolishes HSC Function

(A and B) Defective hematopoietic reconstitution by Zfx-deficient BM. Control or Mx1-Cre⁺ CKO mice (CD45.2⁺) were injected with pi·C to induce Zfx deletion, and their BM was mixed with wild-type competitor BM (CD45.1⁺) and transferred into irradiated CD45.1⁺ recipients. Shown are representative staining profiles of PBL (A) and the fractions of donor-derived CD45.2⁺ cells in the indicated lineages (B) two months after BM transfer (average ± SD of 3 recipients).

(C–F) The loss of HSC maintenance after Zfx deletion in pre-established BM chimeras. (C) Experimental strategy. (D) Representative staining profiles of total PBL in the recipient mice before or 6 months after pi·C injection, showing donor (CD45.2⁺CD45.1⁻, highlighted), competitor (CD45.2⁻CD45.1⁺) and residual host (CD45.2⁺CD45.1⁺) cells. (E) Relative contribution of donor-derived cells to major PBL lineages over time. The donor-derived fraction before pi·C injection (>50% in all mice) was taken as one for each recipient; the data represent average ± SD of 4 recipients per group. A representative of 3 independent reconstitution experiments is shown. (F) The percentage of donor-derived cells in the indicated cell subsets in the thymus and BM 6 months after pi·C injection.

remained undifferentiated and gave rise to all lineages, including the germline. Conversely, stable Zfx overexpression favored ESC self-renewal at the expense of differentiation. The requirement for Zfx in ESC self-renewal in vitro

is not contradicted by the relatively normal early development of Zfx^{null} embryos, which survive until midgestation. Indeed, early embryonic development is not affected by the loss of several ESC self-renewal regulators such as

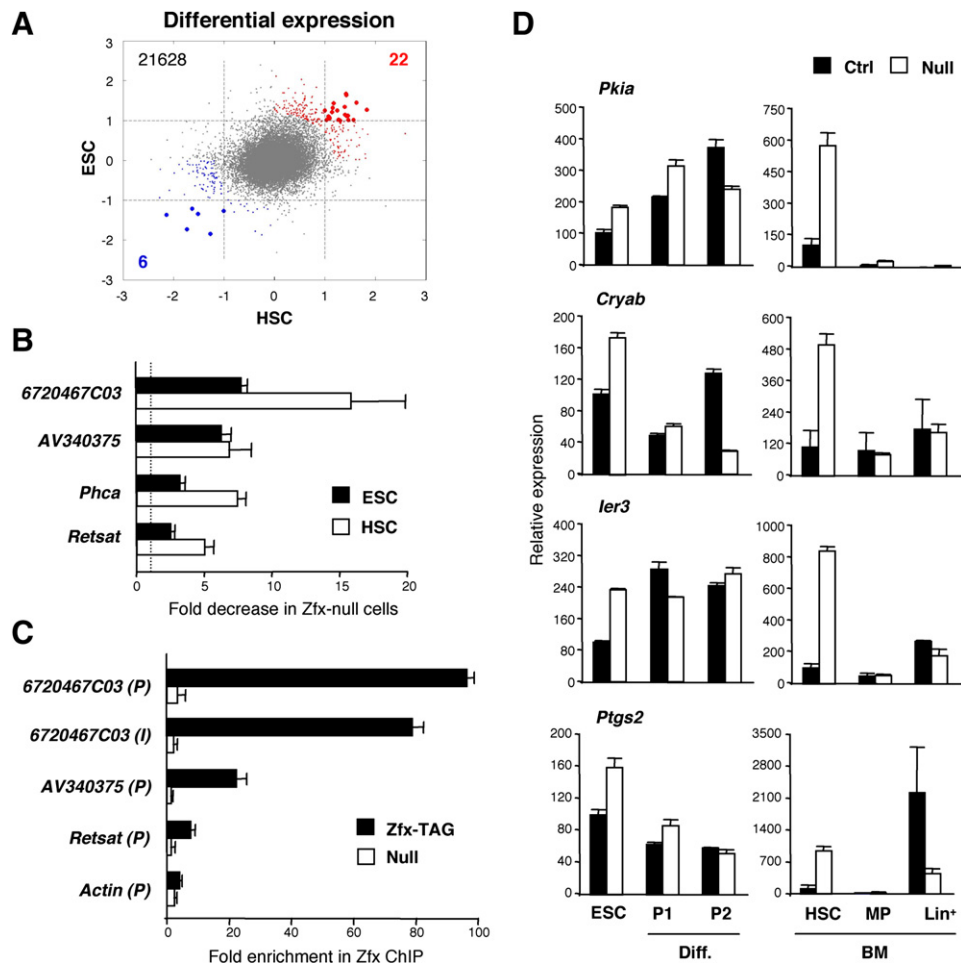


Figure 6. A Common Gene Expression Signature of Zfx-Deficient ESC and HSC

(A) Genome-wide expression analysis of Zfx-deficient ESC and HSC using microarrays. Data points represent normalized increase (>0) or decrease (<0) in individual probe levels in *Zfx*^{null} ESC and HSC relative to the respective controls. Probes showing coordinated decrease or increase in *Zfx*^{null} ESC and HSC are indicated as large blue and red dots, respectively; small colored dots represent a more permissive analysis. The numbers of total and coordinately changing probes are indicated.

(B) The downregulation of common target genes in HSC and ESC after Zfx deletion. HSC were sorted from control and Mx1-Cre⁺ CKO BM 4 days after pl-C injection. Short-term batch cultures of *Zfx*^{null} ESC were generated by sorting *Zfx*^{fllox-neo} ESC transfected with Cre-GFP. The data represent fold decrease of the indicated transcripts in *Zfx*^{null} over control SC as determined by qPCR.

(C) Binding of Zfx to its common target genes in ESC. ChIP using anti-epitope tag antibodies was performed on *Zfx*^{null} ESC reconstituted with tagged Zfx (Zfx-TAG) or on control *Zfx*^{null} ESC (null). The enrichment of promoter (P) or intron (I) regions of the indicated target genes in anti-tag antibody ChIP compared to control IgG ChIP was determined by qPCR. The promoter of β -actin was used as a negative control. The regulatory regions of *Phca* could not be reliably amplified by qPCR.

(D) A common gene induction signature of Zfx-deficient ESC and HSC. The ESC include control (*Zfx*^{fllox}) and *Zfx*^{null} clones grown as undifferentiated cells or differentiated for 1 or 2 passages without LIF (Diff. P1-P2). The HSC (LSK population), myeloid progenitors and Lin⁺ cells were sorted from control and Mx1-Cre⁺ CKO mice 4 days after the last pl-C injection. Data represent expression levels determined by qPCR relative to the respective control SC sample (assigned an arbitrary value of 100).

Eras (Takahashi et al., 2003), Tcl1, Essrb, or Tbx3 (Ivanova et al., 2006). Thus, our data suggest a critical role of Zfx in promoting ESC self-renewal, while it appears dispensable for ESC pluripotency.

In addition, we found that Zfx is not required for fetal hematopoiesis but essential in adult HSC, which failed to maintain their numbers or contribute to hematopoiesis after Zfx deletion. Zfx-deficient HSC interacted normally

with their BM niche but showed an increase in apoptosis, suggesting a defect of long-term self-renewal due to impaired survival. In contrast, the survival and function of erythromyeloid progenitors were not affected, at least in the short term. Very similar phenotypes have been observed after the inducible deletion of Tel/Etv6 (Hock et al., 2004) or FoxO1/3/4 (Tothova et al., 2007), or after germline deletion of Bmi-1 (Park et al., 2003) or Ikaros

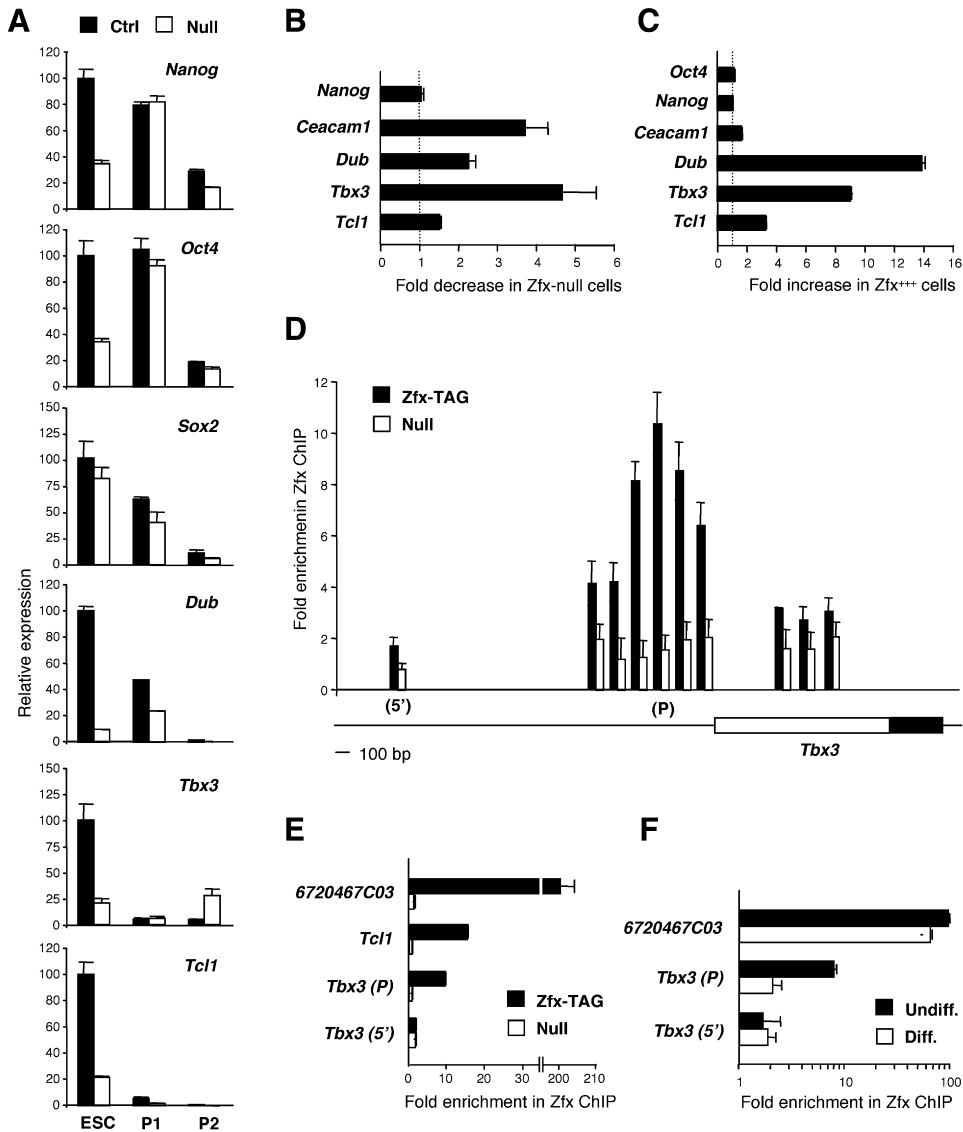


Figure 7. Zfx Controls *Tbx3* and *Tcl1* Expression in ESC

(A) The expression of ESC-specific genes in *Zfx*-deficient ESC. Control (*Zfx^{lox}*) and *Zfx^{null}* ESC clones grown in LIF or differentiated for 1 or 2 passages without LIF (P1-P2) were analyzed by qPCR. Data represent expression levels of the indicated genes relative to undifferentiated control ESC (assigned an arbitrary value of 100).

(B) The expression of *Tbx3* and *Tcl1* in polyclonal ESC after *Zfx* deletion. The data represent fold decrease of the indicated genes in *Zfx^{null}* over control ESC in short-term batch cultures as determined by qPCR.

(C) The expression of *Tbx3* and *Tcl1* in *Zfx*-overexpressing ESC as determined by qPCR. The data show fold increase of the indicated genes in *Zfx*-overexpressing ESC (*Zfx^{null}* + *Zfx**, Figure 2B) over control ESC (*Zfx^{null}* + *Zfx*).

(D) The region of *Tbx3* promoter bound by *Zfx* in ESC. The *Zfx^{null}* ESC reconstituted with tagged *Zfx* (*Zfx*-TAG) or control *Zfx^{null}* ESC were analyzed by ChIP as in Figure 6C. Data represent the enrichment of different regions of *Tbx3* upstream region, shown below with the 5' UTR (open box) and the first coding exon (black box).

(E) The binding of *Zfx* to the promoter regions of *Tbx3* and *Tcl1* in *Zfx*-expressing (*Zfx*-TAG) and *Zfx*-deficient (null) ESC. Shown is fold enrichment of the control target 6720467C03Rik, *Tcl1* promoter, and *Tbx3* promoter (P) or distal 5' region (5') indicated in panel (D).

(F) Increased binding of *Zfx* to *Tbx3* promoter in undifferentiated versus differentiated ESC. Shown is fold enrichment of control target 6720467C03Rik, *Tbx3* promoter (P) or distal 5' region (5') in *Zfx*-expressing ESC (*Zfx*-TAG) grown with LIF (undiff.) or differentiated for two passages without LIF (diff.).

(Nichogiannopoulou et al., 1999) transcription factors. Similar to the latter three models, lymphopoiesis was severely impaired after the loss of *Zfx*. Thus, *Zfx* and possibly

other HSC regulators appear to control both HSC self-renewal and lymphoid differentiation, but not erythromyeloid differentiation. Alternatively, the failure of lymphoid

development might be secondary to defective HSC function, while erythromyeloid progenitors might be less dependent on HSC input (Nichogiannopoulou et al., 1999).

Although several transcriptional regulators were shown to control multiple SC types, the common regulation of ESC and adult SC has been difficult to demonstrate. For instance, *Polycomb group* (PcG) protein Bmi-1 promotes the self-renewal of HSC and of neural SC through the repression of *Cdkn2a* products p16^{Ink4a} and/or p19^{Arf} (Les-sard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003). On the other hand, ESC are resistant to p16^{Ink4a} and to other cyclin inhibitors (Burdon et al., 2002), and are not known to depend on Bmi-1. Conversely, PcG protein Eed contributes to the repression of differentiation-associated genes in ESC (Azuara et al., 2006; Boyer et al., 2006); however, Eed promotes proliferation and antagonizes Bmi-1 in hematopoietic cells (Les-sard et al., 1999). In another case, transcription factor Sox2 controls the pluripotency of ESC and of neural stem/progenitor cells (Boyer et al., 2005; Graham et al., 2003; Ivanova et al., 2006). However, Sox2 is not expressed in other adult SC types, including HSC, and its specific role in adult neural SC remains to be established. Thus, Zfx provides a rare example of a transcription factor that is specifically required in ESC and in a canonical adult SC population such as the HSC.

Because the common transcriptional regulation of ESC and HSC has not been previously described, it is likely to be based on equally novel molecular mechanisms. Indeed, we have identified several direct target genes of Zfx in ESC and HSC, all of which are either predicted or poorly characterized. While the exact function of these direct Zfx targets is currently unknown, their coordinate regulation in ESC and HSC supports the common role of Zfx in these SC types. In addition, we identified several genes upregulated in Zfx-deficient ESC and HSC, likely as an indirect consequence of Zfx loss. Most of the coordinately induced genes are targets of stress, growth factor signaling, and/or immediate-early response, suggesting that Zfx-deficient SC undergo excessive stress. This is consistent with increased apoptosis rate of *Zfx*^{null} ESC and HSC; furthermore, both immediate-early gene induction and decreased survival occur in undifferentiated ESC and HSC, but not in their differentiated progeny. Of note, HSC are uniquely sensitive to certain forms of stress such as physiological oxidative stress (Tothova et al., 2007). Altogether, our data reveal the apoptosis and concomitant upregulation of stress-inducible genes as common features of impaired self-renewal in ESC and HSC.

In addition to the common target genes, Zfx can directly activate cell type-specific targets, such as *Tbx3* and *Tcl1* in ESC. Both genes are rapidly downregulated upon ESC differentiation and facilitate ESC self-renewal through a putative common pathway (Ivanova et al., 2006). Recently, *Tcl1* was shown to control ESC growth but not their differentiation status (Matoba et al., 2006). Thus, Zfx may promote ESC self-renewal at least in part through the direct coordinate regulation of *Tbx3* and *Tcl1*. Furthermore,

the ESC-specific expression of *Tbx3* and *Tcl1* may partially explain the requirement for Zfx in ESC but not in their differentiated progeny. Although none of the known HSC regulators were affected by the loss of Zfx (Table S3 and data not shown), Zfx may similarly regulate a yet unknown HSC-specific self-renewal pathway. At present, the ESC system provides a proof of principle that Zfx can control self-renewal pathways specific for a particular SC type.

In conclusion, our study has identified Zfx as a shared transcriptional regulator of two prototypical yet highly different murine SC types, the embryonic and hematopoietic SC. These results provide genetic evidence for a common molecular basis of self-renewal in pluripotent embryonic SC and adult tissue-specific SC. The identification of Zfx should facilitate the molecular dissection of self-renewal mechanisms in ESC, HSC, and possibly in other SC types, including tumor-initiating cancer SC. Finally, the high evolutionary conservation of Zfx raises the possibility that it may regulate SC functions in other vertebrate species, including humans.

EXPERIMENTAL PROCEDURES

Gene Targeting

Conditional targeting, deletion and re-expression of Zfx in ESC were performed in male 129/SvEv ESC as described in Figure S1 and Supplemental Experimental Procedures. The presence of Zfx protein was analyzed with an affinity-purified polyclonal antibody against the N-terminal domain. *Zfx*^{lox} and *Zfx*^{null} ESC clones were injected into C57BL/6 blastocysts, and the resulting chimeric males were crossed to wild-type 129/SvEv and Black Swiss females, respectively.

To obtain CKO mice (*Zfx*^{lox/y}, Cre⁺), *Zfx*^{lox/lox} females (pure 129/SvEv background) were crossed with males carrying Mx1-Cre (129/SvEv) or Tie2-Cre (mixed 129/B6) transgenes. Control mice in both strains included Cre⁺ wild-type and Cre⁻ *Zfx*^{lox/y} animals, which were indistinguishable by all assays. Mice carrying a tamoxifen-inducible Cre recombinase inserted into *Rosa26* locus (R26-CreER, C57BL/6) were kindly provided by Dr. Thomas Ludwig, Columbia University. The recombination in adult Mx1-Cre mice was induced with polyinosinic:polycytidylic acid (pI·C, Amersham Biosciences) administered i.p. three times (0.25 mg every third day). The deletion status of all analyzed CKO cells and tissues was monitored by genomic PCR. All animal studies were performed according to the investigator's protocol approved by the Institutional Animal Care and Use Committee.

Cell Culture and Analysis

Undifferentiated ESC were cultured in complete DMEM containing 15% FCS and recombinant LIF (Chemicon) on mitomycin-treated embryonic fibroblast feeders. For gene expression studies and ChIP, ESC were grown on gelatin-coated plates in 5%–15% Knockout SR serum replacement (Invitrogen) and LIF. Serum- and feeder-free culture in the presence of LIF and 10 ng/ml recombinant BMP4 (R&D Systems) was performed as described (Ying et al., 2003). To measure cell growth or colony formation, ESC were seeded into triplicate wells of 24-well plates and cultured as indicated. Apoptosis of ESC was measured by gel fractionation of low molecular weight DNA or by staining live cells with Annexin V-FITC conjugate (BD Pharmingen) and 7-amino-actinomycin D (7-AAD). For competitive growth assay, *Zfx*^{lox-neo} ESC were transiently transfected with Cre expression vector and grown as indicated. For the batch culture of *Zfx*^{null} ESC shortly after Zfx deletion, *Zfx*^{lox-neo} ESC were transiently transfected with Cre-GFP expression vector, and GFP⁺ cells were sorted 24 hr later and

cultured for 3 days with LIF on gelatin. Control ESC were generated in parallel by transfection with an empty expression vector.

For differentiation, ESC were grown in 15% FCS without LIF/feeders on gelatin-coated plates. Where indicated, ESC were cultured in suspension for 4 days to form primitive embryoid bodies (EB), and then returned to adherent culture or allowed to form mature EB in suspension for additional 4–9 days. For teratoma formation, undifferentiated ESC grown on gelatin were injected s.c. into syngeneic 129/SvEv mice, and teratomas were harvested 3 weeks later, sectioned, and stained with hematoxylin-eosin.

Mouse embryonic fibroblasts (MEF) were derived from 14.5 d.p.c. R26-CreER⁺, *Zfx^{lox}/y* embryos. After the first passage, half of the culture was incubated with 4-hydroxytamoxifen to induce *Zfx* deletion, and then equal numbers of treated and untreated cells were mixed and propagated by the 3T3 protocol.

Hematopoietic Cell Analysis

Single-cell suspensions from hematopoietic organs were stained with the indicated fluorochrome-conjugated antibodies (BD Pharmingen and eBiosciences) for 4- or 5-color analysis. The lineage cocktail included antibodies to CD11b, Gr-1, Ter119, B220, CD3, and DX5. For apoptosis detection, enriched lineage-negative BM cells were stained with antibody conjugates, Annexin V TACS reagent (Trevigen) and 7-AAD. The samples were acquired using FACSCalibur or LSR II flow cytometers or sorted on FACSaria flow sorter (BD Immunocytometry Systems), and analyzed using FlowJo software (Treestar Inc.). Statistical significance was estimated using unpaired, two-tailed Student's *t* test. For competitive reconstitution, BM cells from control (Mx1-Cre⁺) or CKO (*Zfx^{lox}/y*, Mx1-Cre⁺) mice 7 days after pl-C injection was mixed 2:1 with the competitor BM cells from B6.SJL mice (C57BL/6 congenic for CD45.1), and 2×10^5 total cells were injected i.v. into lethally irradiated B6.SJL recipients. For *Zfx* deletion in pre-established BM chimeras, BM cells from untreated Mx1-Cre⁺ control or CKO mice were mixed 2:1 with competitor B6.SJL BM and injected i.v. into lethally irradiated (129/SvEv \times B6.SJL)F1 recipients. Four weeks later, one half of each recipient group was treated with pl-C. For progenitor assays, total BM was plated in triplicates (10^5 cells/35 mm dish) into cytokine-supplemented methylcellulose medium (Methocult, Stem Cell Technologies), and the number and morphology of colonies were scored 10 days later.

Global Gene Expression Analysis

For ESC, *Zfx^{null}* and control *Zfx^{lox-neo}* clones were grown on gelatin in medium with 15% Knockout SR and LIF. For HSC, BM cells were pooled from 4–6 Mx1-Cre⁺ *Zfx^{lox}/y* CKO and control *Zfx^{lox}/y* Cre⁻ littermates 4 days after the last pl-C injection, and Lin⁻ Sca-1⁺ c-Kit⁺ cells were flow-sorted. RNA was amplified, labeled and hybridized to Mouse Genome 430 2.0 microarrays containing probes for ~37,000 genes (Affymetrix). Details of the sample preparation and analysis are described in [Supplemental Experimental Procedures](#). Microarray data have been deposited in the GEO database under accession number GSE7069.

Quantitative PCR

Total cell RNA was reverse-transcribed and assayed by quantitative real-time PCR using SYBR Green incorporation. The expression of all genes was normalized to that of β -actin and expressed relative to the indicated reference sample (average \pm SD of triplicate reactions). For competition experiments (Figure 2A), *Zfx^{lox}* and *Zfx^{null}* alleles were amplified separately and normalized using qPCR reaction that detects all targeted *Zfx* alleles but not the feeder cell-derived wild-type allele. The measurements of *Zfx* expression (Figure 1A) were performed using the calibration curve method. All other cDNA and genomic DNA measurements were performed using the $\Delta\Delta C_T$ method on MX3000P instrument (Stratagene).

Chromatin Immunoprecipitation

An ESC clone expressing FLAG-HA epitope-tagged *Zfx* protein (*Zfx*-TAG) was generated as described in [Supplemental Experimental Procedures](#). *Zfx*-TAG cells or parental *Zfx^{null}* cells were crosslinked with formaldehyde and subjected to chromatin immunoprecipitation (ChIP) using anti-FLAG M2 antibody (Sigma) or control IgG as described (Boyer et al., 2005). After crosslink reversal, precipitated chromatin was analyzed by qPCR for genomic fragments of interest. For experiments shown in Figure 7E, ChIP was performed using anti-*Zfx* antibody or control IgG, followed by whole genome ligation-mediated PCR amplification as described (Boyer et al., 2005). All results were normalized using a control genomic DNA fragment from murine *Snai3* gene, which was not enriched in *Zfx* ChIP.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, twelve figures, and three tables and can be found with this article online at <http://www.cell.com/cgi/content/full/129/2/345/DC1/>.

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Accession Numbers

Microarray data have been deposited in the GEO database under accession number GSE7069.