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Loss of c-kit and bone marrow failure upon conditional removal of the GATA-2 C-terminal zinc finger domain in adult mice

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Abstract

Heterozygous mutations in the transcriptional regulator GATA-2 associate with multilineage immunodeficiency, myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). The majority of these mutations localize in the zinc finger (ZnF) domains, which mediate GATA-2 DNA binding. Deregulated hematopoiesis with GATA-2 mutation frequently develops in adulthood, yet GATA-2 function in the bone marrow remains unresolved. To investigate this, we conditionally deleted the GATA-2 C-terminal ZnF (C-ZnF) coding sequences in adult mice. Upon *Gata2 C-ZnF* deletion, we observed rapid peripheral cytopenia, bone marrow failure and decreased c-Kit expression on hematopoietic progenitors. Transplant studies indicated GATA-2 has a cell autonomous role in bone marrow hematopoiesis. Moreover, myeloid lineage populations were particularly sensitive to *Gata2* hemizygosity, while molecular assays indicated GATA-2 regulates c-Kit expression in multilineage progenitor cells. Enforced c-Kit expression in *Gata2 C-ZnF*-deficient hematopoietic progenitors enhanced myeloid colony activity, suggesting GATA-2 sustains myelopoiesis via a cell intrinsic role involving maintenance of c-Kit expression. Our results provide insight into mechanisms regulating hematopoiesis in bone marrow and may contribute to a better understanding of immunodeficiency and bone marrow failure associated with GATA-2 mutation.

Keywords

GATA-2 mutation; c-kit; hematopoiesis; myelopoiesis

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DISCLOSURE of POTENTIAL CONFLICTS of INTEREST

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INTRODUCTION

Inherited and acquired immunodeficiencies are devastating yet these diseases provide opportunities to uncover regulatory pathways of the immune system, which is necessary to advance efforts to modulate pathological symptoms. Heterozygous germline or acquired *GATA2* mutations have been identified in individuals with lymphedema or bone marrow failure including MDS, congenital neutropenia and the MonoMAC/DCML syndrome of reduced monocytes, dendritic cells (DCs), B and NK cells, which lead to compromised immunity and enhanced propensity for AML (1–6); reviewed in (7). Notable features of these diseases include onset in early to late adulthood, variable penetrance and severe effects on myeloid lineage populations. These discoveries link *GATA2* mutations and multilineage immunodeficiency, underscoring a critical need to evaluate mechanisms by which GATA-2 sustains adult hematopoiesis.

Global *Gata2* deletion in mice triggers severe yolk sac anemia, leading to early lethality (E10-11) (8). Experiments with blastocyte chimeras showed *Gata2*^{-/-} embryonic stem cells failed to contribute to fetal liver or bone marrow hematopoiesis, suggesting GATA-2 operates at early stages (e.g., stem/progenitor) of definitive hematopoiesis. Consistently, hemizygous *Gata2*^{+/-} hematopoietic stem (HSC) and progenitor (HSPC) cells show defective repopulating activity upon transplantation and attenuated myeloid colony forming activity *ex vivo* (9, 10). Recently, GATA-2 was found to be required for lymphatic vascular development, emergence of long-term repopulating HSCs from hemogenic endothelium and survival of adult mouse HSCs in fetal liver (11, 12). These results highlight key roles for GATA-2 in murine HSC specification, HSC survival and progenitor/mature lineage expansion. Furthermore, taken in light of human immunodeficiency with *GATA2* mutation, the data indicate conserved GATA-2 function between humans and mice.

Currently available mouse models have limited investigation of GATA-2 function in adult bone marrow hematopoiesis, in large part due to embryonic lethality as a consequence of rapid HSC/HPSC failure during development. To address mechanisms of immunodeficiency and bone marrow failure mediated by mutant GATA-2 in adults, we developed inducible murine systems to remove coding sequences of the ZnF domain located proximal to the GATA-2 C-terminus (*Gata2 C-ZnF*), which is frequently mutated in disease (7). Upon *Gata2 C-ZnF* deletion from adult mice, we observed rapid neutropenia, monocytopenia and mortality. Bone marrow transplantation assays and subsequent *Gata2 C-ZnF* deletion indicated hematopoietic cell-autonomous GATA-2 activity sustains adult blood cell generation. We found GATA-2 regulates hematopoietic progenitor survival and contributes to maintenance of *Kit* expression in multipotent progenitor cells. Moreover, enforced c-Kit expression partially recovered myeloid colony-forming activity of *Gata2 C-ZnF*-deficient progenitors. Collectively, our results suggest GATA-2 sustains hematopoiesis in the bone marrow via a cell intrinsic role involving maintenance of c-Kit expression.

MATERIAL AND METHODS

Animals and animal procedures

Gata2 C-ZnF^{fllox/flox} (*Gata2*^{fl/f}) mice have LoxP sites flanking the *Gata2* 5th exon, which encodes the C-terminal ZnF domain (13). *Gata2*^{fl/f} mice were obtained from the University of Missouri Mutant Mouse Regional Resource Center (MMRRC) supported by NIH. *Gata2*^{fl/f} mice were backcrossed to the C57Bl6 strain (CD45.2⁺) and bred with ER-Cre⁺ animals (from the Jackson Laboratory, on the C57Bl6 background) to generate ER-Cre⁺ *Gata2*^{fl/f} mice. Age-matched ER-Cre⁻ *Gata2*^{fl/f} mice were used as controls (Supplemental Table 1).

Bone marrow chimeras were generated by transferring 2×10^6 bone marrow cells from ER-Cre⁺ *Gata2*^{fl/f} or ER-Cre⁻ *Gata2*^{fl/f} mice (CD45.2⁺) into lethally irradiated CD45.1⁺ CD45.2⁺ *Gata2*^{+/+} recipients (F1 generation from CD45.2⁺ C57Bl6 $\times$ congenic CD45.1⁺ cross) (Supplemental Table 1). Six to eight weeks after transplantation, reconstitution of peripheral blood was assessed by flow cytometry; mice with reconstitution >90% were used for analyses. Mixed bone marrow chimeras were generated by transferring a 1:1 ratio of bone marrow cells from congenic CD45.1⁺ *Gata2*^{+/+} and CD45.2⁺ ER-Cre⁺ *Gata2*^{fl/f} mice (2×10^6 cells/genotype) into lethally-irradiated CD45.1⁺ CD45.2⁺ *Gata2*^{+/+} recipients. Deletion of *Gata2* C-ZnF coding sequences was stimulated by tamoxifen treatment (5 mg/50 μ l corn oil/mouse every other day, for a total of 3 treatments). In some experiments, *Tek-cre*⁺ mice (from the Jackson Laboratory, on C57Bl6 background) were bred to *Gata2*^{fl/f} mice to generate *Tek-cre*⁻ *Gata2*^{+/+} and *Tek-cre*⁺ *Gata2*^{+/+} animals (Supplemental Table 1).

All mice were housed in a specific pathogen-free (SPF) barrier facility. Animals between 6–12 weeks old were used in experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Texas MD Anderson Cancer Center.

Peripheral blood and organ analysis

Peripheral blood was collected by retro-orbital puncture; complete blood counts (CBCs) were determined by automated counting (Scil Animal Care Company, IL). Hematopoietic lineages in bone marrow and spleen were measured by flow cytometry. Single cell suspensions were treated to remove RBCs, cells were washed and stained with fluorescently-labeled antibodies to CD45 isoforms and lineage markers, including CD45.1, CD45.2, Gr1, CD11c, CD4, CD8, Fc γ RII/III (BD Bioscience), and CD3, CD19, CD11b, Ter119, B220, Sca-1, c-kit, IL-7R, CD115, CD135, CD34 (eBioscience) antibodies prior to analysis. For cell cycle assays, cells were stained with antibodies to lineage and CD45 markers, fixed with 75% ethanol overnight and incubated in PBS containing 50 mg/ml propidium iodide and 25 mg/ml RNase for 15 min at room temperature. Samples were analyzed on LSRII or Fortessa flow cytometers (BD Bioscience); FlowJo software was used for data analysis.

Bone marrow side population (SP) analysis, colony forming assays and retroviral transduction

For side population analyses, total bone marrow cells (10^6 /ml, excluding RBCs) were stained with Hoechst 33342 (5 μ g/ml at 37°C for 90 min) in a circulating water bath. Samples were analyzed on a BD Influx machine (BD Bioscience) as described (14). For colony forming assays, bone marrow cells were plated in 6-well dishes (2×10^4 /well) containing Methocult 3434 medium (Stem Cell Technologies, VC), incubated 9 d at 37°C and colonies were enumerated. In some experiments, bone marrow cells were infected with retrovirus encoding c-Kit or control vector (15) for 6–8 h at 37°C in the presence of IL-3, IL-7, TPO and SCF (50 ng/ml each), prior to plating in methylcellulose cultures.

Total RNA isolation and quantitative PCR (qPCR)

Total RNA was purified using Trizol. cDNA synthesis was performed with iScript reverse transcriptase (Bio-Rad). Real-time qPCR was done in triplicate with SYBR Green PCR master mix (Bio-Rad) with an iCycler Sequence Detection System (Biorad). The expression of individual genes was calculated and normalized to RPL13A via the C_T method.

Bone decalcification and hematoxylin and eosin (H&E) staining

Mouse femurs and tibias were collected and fixed in 10% neutral buffered formalin for 1–2 d, followed by washing and decalcification (RDFTM rapid decalcifier; StatLab). The tissues were then dehydrated by a series of ethanol washes, embedded in paraffin, step sectioned, mounted on glass slides, and stained with H&E in the Department of Immunology Histology Core (MD Anderson Cancer Center). Slides were visualized using an Olympus BX 41 microscope; representative pictures were taken from each section.

Reporter assays and chromatin immunoprecipitations (ChIPs)

For reporter assays, 32D cells were transfected with a pGL3 reporter construct (Promega) containing the murine *Kit* promoter (–622/+22 kb), along with plasmids encoding GATA-2, GATA-2 C-ZnF mutant (C-ZnF), or C/EBP α (WT) by electroporation (Nucleofector Kit V and Nucleofector Device; Lonza). Cells were cultured in IL-3-containing medium for 36 h, and luciferase activity was measured with a Dual Luciferase assay kit (Promega) using a Sirius luminometer (Berthold Detection Systems).

For ChIPs, 32D cells were transfected with plasmids encoding GATA-2 and C/EBP α as described above. ChIPs were performed with antibodies against GATA-2, C/EBP α or IgG control (Santa Cruz Biotechnology) using a ChIP assay kit (Millipore). The ChIP samples were analyzed with qPCR using primers amplifying the distal or proximal region of the *Kit* promoter (Supplemental Fig. 4A). ChIP results were normalized to input controls, and then to IgG controls.

Statistical analysis

Data are presented as mean $\pm$ SEM. The statistical significance between groups was calculated by a two-tailed *t*-test using Graphpad Prism 5 software. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

RESULTS

Induced *Gata2* C-ZnF deletion in adult hematopoiesis

To mimic *GATA2* loss-of-function in human disease, we generated animals with tamoxifen-inducible *Gata2* C-ZnF deletion by breeding *Gata2* C-ZnF floxed mice (*Gata2* C-ZnF^{flox/flox}, referred to as *Gata2*^{f/f}) with ER-Cre⁺ animals (Fig. 1A). We confirmed *Gata2* C-ZnF deletion in bone marrow cells upon tamoxifen treatment, using qPCR (Fig. 1B) (tamoxifen-treated ER-Cre⁺ *Gata2*^{f/f} mice are termed *Gata2* C-ZnF; see Supplemental Table 1). *Gata2* C-ZnF mice became moribund 1–2 weeks following tamoxifen administration, while *Gata2*^{f/f} mice tolerated this treatment (Fig. 1C). CBC analysis revealed a substantial reduction in total white blood cell (WBC), granulocyte, monocyte and red blood cell (RBC) amounts as well as reduced hemoglobin (HGB) and hematocrit (HCT). Circulating lymphocyte numbers were also decreased in *Gata2* C-ZnF animals vs. *Gata2*^{f/f} controls, albeit more modestly than myeloid populations (Fig. 1D).

Analysis of bone marrow hematopoiesis revealed substantial reduction in myeloid populations, including fewer mature (Gr1^{hi}) and immature (Gr1^{lo}) granulocytes as well as less conventional (cDC) and plasmacytoid (pDC) DCs (Fig. 1E; subset analysis shown in Supplemental Fig. 1). Histological examination confirmed a severe loss of nucleated cells in the bone marrow of *Gata2* C-ZnF mice 10 d following tamoxifen treatment (Fig. 1F), indicative of massive hematopoietic failure. In addition, splenic B and T lymphocytes, including CD4⁺ and CD8⁺ T cells, were decreased significantly in *Gata2* C-ZnF mice (Fig. 1E). Moreover, *Gata2* C-ZnF mice exhibited an almost complete loss of thymic cellularity (Fig. 1G). Taken together, these data indicate *Gata2* C-ZnF depletion across all organs in adult mice causes severe bone marrow failure, reduced lymphoid organ cellularity and rapid lethality.

To examine cell autonomous function, we generated bone marrow chimeric mice with ER-Cre⁻ *Gata2*^{f/f} or ER-Cre⁺ *Gata2*^{f/f} donor cells. After 6–8 weeks, both transplant groups displayed similar reconstitution (> 90%), as well as myeloid and lymphoid lineage representation in peripheral blood, bone marrow and spleen (Fig. 2A, 2B; Supplemental Fig. 2A). Following tamoxifen treatment, however, peripheral blood populations including WBCs, RBCs, granulocytes, monocytes, lymphocytes and platelets were substantially reduced in *Gata2* C-ZnF chimeras (Fig. 2A). HGB and HCT were also reduced significantly upon bone marrow *Gata2* C-ZnF depletion (Fig. 2A). Consistently, *Gata2* C-ZnF chimeras showed decreased bone marrow and splenic cellularity, as well as fewer myeloid, DC and lymphoid subsets within these tissues vs. tamoxifen-treated *Gata2*^{f/f} controls (Fig. 2B). These results parallel organ-wide *Gata2* C-ZnF depletion (Fig. 1). By contrast, NK1.1⁺ NK cell amounts were similar in *Gata2* C-ZnF and *Gata2*^{f/f} chimeras (data not shown). Circulating platelets were reduced in *Gata2* C-ZnF chimeras, unlike organ-wide *Gata2* C-ZnF depletion (Fig. 2A, 1D), suggesting blood platelet amounts may be regulated by GATA-2 function in non-hematopoietic (e.g., lymphatics) as well as hematopoietic cells. Tamoxifen also reduced circulating lymphocytes and splenic CD4⁺ T cells in ER-Cre⁻ *Gata2*^{f/f} chimeras, potentially due to general toxicity (Fig. 2A, 2B). Nonetheless, *Gata2* C-ZnF chimeras show rapid hematopoietic failure and significantly depleted innate and adaptive

immune populations, indicating a cell autonomous requirement for GATA-2 C-ZnF in bone marrow hematopoiesis.

We next utilized mixed bone marrow chimeras to confirm the importance of the GATA-2 C-ZnF and gauge the extent of potential compensatory mechanisms upon its deletion. For these studies, we transplanted a 1:1 ratio of bone marrow cells from congenic CD45.1⁺ *Gata2*^{+/+} and CD45.2⁺ ER-Cre⁺ *Gata2*^{f/f} mice into lethally-irradiated CD45.1⁺ CD45.2⁺ *Gata2*^{+/+} recipients. The *Gata2*^{+/+} and ER-Cre⁺ *Gata2*^{f/f} bone marrow cells exhibited similar repopulation capabilities, as determined by analysis of CD45.1⁺ and CD45.2⁺ fractions (Fig. 2C; Supplemental Fig. 2B). Upon tamoxifen treatment, we observed specific depletion of bone marrow and spleen CD45.2⁺ *Gata2*^{C-ZnF} cells in mixed chimeras, with *Gata2*^{C-ZnF} neutrophils and cDCs within bone marrow affected particularly (Fig. 2C). While the absolute number of bone marrow CD45.2⁺ pDC and splenic CD45.2⁺ lymphoid populations also decreased with tamoxifen, we did not see a major shift in the proportion of CD45.2⁺ vs. CD45.1⁺ pDCs or lymphocytes (Fig. 2C; Supplemental Fig. 2B). This may be due to a modest loss of CD45.1⁺ *Gata2*^{+/+} cells in response to tamoxifen. Collectively, these results indicate GATA-2 C-ZnF has an intrinsic role in bone marrow hematopoiesis, and suggest myeloid/cDC populations may be uniquely sensitive to *Gata2*^{C-ZnF} depletion.

As a complementary approach, we also investigated hematopoiesis in *Tek-cre* *Gata2*^{f/f} mice, which induce *Gata2* deletion in hematopoietic and endothelial lineages (16). We failed to detect *Tek-cre*⁺ *Gata2*^{f/f} animals at birth, while heterozygous and littermate control pups were found at expected Mendelian ratios (data not shown), suggesting biallelic *Gata2*^{C-ZnF} deletion via *Tek-cre* leads to embryonic lethality. Heterozygous *Tek-cre*⁺ *Gata2*^{+/f} animals had fewer bone marrow cell numbers as well as less bone marrow granulocytes, cDCs and pDCs vs. *Tek-cre*⁻ *Gata2*^{+/f} controls (Supplemental Fig. 3), resembling myelodysplasia in individuals with MonoMAC/DCML (1, 3). However, *Gata2*^{C-ZnF} depletion by LysM cre, which induces gene deletion in neutrophils and macrophages (17), did not affect hematopoiesis (data not shown). These results implicate GATA-2 C-ZnF function at progenitor stages of bone marrow hematopoiesis, and suggest a stringent requirement in myeloid lineage progenitors.

Regulation of hematopoietic progenitors by GATA-2

The intrinsic requirement for the GATA-2 C-ZnF domain in multiple hematopoietic lineages suggested a function in bone marrow HSC/HSPC populations, consistent with roles for GATA-2 in embryonic and fetal hematopoiesis (8, 9, 11, 12). *Gata2* mRNA expression was restricted to bone marrow lin⁻ Sca-1⁺ c-Kit⁺ (LSK) and common myeloid progenitor (CMP) subsets; however, we were unable to detect bone marrow progenitor populations that require LSK (or c-Kit⁺) delineation (e.g., LSK CD150⁺ CD48⁻ HSCs, CMPs, granulocyte-macrophage progenitors/GMPs) in *Gata2*^{C-ZnF} bone marrow (data not shown). Moreover, c-Kit-expressing cells were absent from the lin⁻ fraction of CD45.2⁺ *Gata2*^{C-ZnF} bone marrow in mixed chimeras (Fig. 3A). To examine whether this was due to loss of HSPCs from the CD45.2⁺ *Gata2*^{C-ZnF} subset, we employed SP analysis, which measures HSPCs but does not rely on c-Kit detection. We found a dramatic reduction in the frequency of SP-HSPCs within the CD45.2⁺ *Gata2*^{C-ZnF} bone marrow fraction, as well as reduced overall

cellularity, compared to CD45.1⁺ *Gata2*^{+/+} controls (Fig. 3B). Propidium iodide staining showed the majority of CD45.2⁺ lin⁻ *Gata2*^{C-ZnF} cells were subdiploid (>90%), while CD45.1⁺ lin⁻ *Gata2*^{+/+} cells were primarily in G0/G1 (>95%) (Fig. 3C), suggesting enhanced progenitor apoptosis upon *Gata2*^{C-ZnF} depletion. Moreover, CD45.2⁺ *Gata2*^{C-ZnF} bone marrow cells from mixed chimeras gave rise to significantly fewer colonies vs. CD45.1⁺ *Gata2*^{+/+} controls (Fig. 3D), consistent with the inability of *Gata2*^{C-ZnF} cells to sustain hematopoiesis *in vivo*. These data indicate the GATA-2 C-ZnF is required for HSPC survival and function within bone marrow.

Transcriptional control of c-Kit expression by GATA-2

In the hematopoietic system, the c-Kit tyrosine kinase receptor is expressed on HSPCs, where it signals to regulate blood cell growth and differentiation. Loss of c-Kit⁺ cells from the lin⁻ bone marrow fraction upon *Gata2*^{C-ZnF} removal (Fig. 3A) raised the possibility that GATA-2 regulates *Kit* expression. We found specific reduction of *Kit* mRNA in CD45.2⁺ lin⁻ *Gata2*^{C-ZnF} cells from tamoxifen-treated mixed bone marrow chimeras, relative to CD45.1⁺ lin⁻ *Gata2*^{+/+} cells (Fig. 4A). Moreover, *Kit* mRNA decreased upon treatment of ER-Cre⁺ *Gata2*^{fl/fl} LSKs with the estrogen analog 4-hydroxytamoxifen (4-TOH) *ex vivo* (Fig. 4B). The reduction in *Kit* mRNA was specific as evidenced by similar amounts of *Cebpa* and housekeeping gene mRNAs in *Gata2*^{C-ZnF} and *Gata2*-sufficient progenitors (Fig. 4A, 4B; data not shown), suggesting GATA-2 involvement in *Kit* regulation.

The *Kit* promoter contains multiple consensus sites for GATA-2 and C/EBPα, which coordinately regulate transcription in hematopoietic cells (Supplemental Fig. 4A) (18). Reporter assays showed GATA-2 enhanced *Kit* promoter activity ~25% (Fig. 4C). By contrast, a GATA-2 mutant lacking the C-ZnF domain (*C-ZnF*; Supplemental Fig. 4B) failed to stimulate the *Kit* reporter (Fig. 4C), suggesting GATA-2 contributes to *Kit* transcriptional activation via its C-ZnF domain. Strikingly, C/EBPα inhibited *Kit* promoter activity, while addition of GATA-2 with C/EBPα alleviated C/EBPα-mediated repression through a mechanism dependent on the C-ZnF (Fig. 4C). In agreement with transcriptional roles, GATA-2 and C/EBPα associated with the *Kit* promoter *in vivo*, as judged by chromatin immunoprecipitation assays from the hematopoietic progenitor cell line 32D (Fig. 4D). Collectively, these data suggest GATA-2 regulates *Kit* transcriptional activation via the C-ZnF domain.

To evaluate whether c-Kit loss accounts for the defective function of *Gata2*^{C-ZnF} HSPCs, we examined effects of restoring c-Kit expression. We infected ER-Cre⁺ *Gata2*^{fl/fl} bone marrow cells with retrovirus encoding c-Kit or empty vector. We induced *Gata2*^{C-ZnF} deletion *ex vivo* with 4-TOH and assessed colony-forming activity of *Gata2*^{C-ZnF} progenitors. 4-TOH-treated ER-Cre⁺ *Gata2*^{fl/fl} progenitors (*Gata2*^{C-ZnF}) formed few (20–50 fold less) hypocellular colonies versus untreated controls with intact *Gata2*^{C-ZnF} sequences (Fig. 4E, 4F). Enforced c-Kit expression substantially enhanced colony-forming activity of *Gata2*^{C-ZnF} progenitors, as judged by comparison of 4-TOH-treated vector-infected and c-Kit overexpressing cells (Fig. 4E, 4F). All residual colonies in vector-infected ER-Cre⁺ *Gata2*^{fl/fl} cultures contained *Gata2*^{C-ZnF} coding sequences (data not shown), suggesting they arise from progenitors with incomplete *Gata2*^{C-ZnF} removal. We were unable to

assess infection frequency; however, prior experiments indicate an infection rate of ~20–30% in primary cell cultures (data not shown). Using 20% to approximate infection frequency, we estimate ~80–100% of c-Kit-expressing *Gata2*^{C-ZnF} progenitors formed colonies. Moreover, culture of non-infected ER-Cre⁻ *Gata2*^{f/f} bone marrow cells, or ER-Cre⁻ *Gata2*^{f/f} bone marrow cells infected with retrovirus encoding c-Kit or empty vector, in similar 4-TOH-containing conditions indicated little to no toxic effect of 4-TOH on colony-forming activity (Supplemental Fig. 5). Collectively, our data suggest enforced c-Kit expression overcomes defects in *Gata2*^{C-ZnF} progenitor colony-forming activity, implying GATA-2 regulation of c-Kit is a key response to maintain survival and differentiation activity of bone marrow HSPCs.

DISCUSSION

Our study provides new insight into mechanisms by which GATA-2 regulates hematopoiesis within the adult bone marrow, which may contribute to a better understanding of immunodeficiency and bone marrow failure associated with human *GATA2* mutation. We found GATA-2 C-ZnF is necessary for maintenance of adult HSC/HPSCs in the bone marrow microenvironment, as conditional loss of *Gata2*^{C-ZnF} from the hematopoietic compartment of adult mice led to rapid, multilineage cytopenia and reduction in bone marrow SP-HSPCs. This information indicates a conserved role for GATA-2 throughout the distinct developmental stages of murine hematopoiesis; GATA-2 function is also required for HSC/HPSC activity in the yolk sac and fetal liver, as well as the formation of HSCs from hemogenic endothelium (8–12). Moreover, we identified *Kit* as an important target of GATA-2 in multipotent hematopoietic progenitors, while add-back studies indicated that enforced c-Kit expression allows *Gata2*^{C-ZnF}-deficient progenitors to regain myeloid colony-forming activity. These data suggest GATA-2-mediated regulation of *Kit* expression is a key response needed to maintain HSC/HPSCs and proper development of the myeloid lineages.

Precise control of GATA-2 appears critical for hematopoiesis in humans, particularly myeloid lineage regulation. Aberrant GATA-2 expression (e.g., overexpression of *GATA2* mRNA) has been linked with MDS/AML (19, 20), while *GATA2* mutations associate with MonoMAC/DCML syndrome, MDS, AML or chronic neutropenia with risk of AML and/or MDS evolution (1, 2, 5, 6, 18, 21). Overexpression of GATA-2 in murine or human hematopoietic progenitors led to their increased quiescence, and human progenitors with high GATA-2 expression showed an inability to contribute to hematopoiesis in nonobese diabetic-severe combined immunodeficient (SCID) mice (22). These data are consistent with *GATA2* overexpression as a contributing factor in certain cases of MDS/AML (19, 20). By contrast, *GATA2* mutations linked with MonoMAC/DCML as well as MDS and AML are expected to render loss-of-function via different mechanisms including haploinsufficiency or dominant negative activity (1, 2, 5, 7, 18). These mutations include frameshifts, premature termination codons and single amino acid substitutions, and a majority (~2/3) localize in the C-ZnF domain, which mediates DNA binding and protein interactions (7, 23). Mutant *GATA2*-associated immunodeficiencies arise primarily in early adulthood, yet show variable penetrance and disease acquisition/evolution (7). The mouse models that we describe herein, which allow for conditional deletion of GATA-2 C-ZnF coding sequences, should enable

further investigation into the effects of hemizygous and/or timed *Gata2* C-ZnF-deficiency during adult hematopoiesis, as well as identification of important molecular regulatory circuits disrupted upon *Gata2* C-ZnF removal.

In mice, GATA-2 is prominently expressed in the embryo and multipotent hematopoietic progenitors in the yolk sac, fetal liver and adult bone marrow (24). *Gata2* mRNA initiating from the distal first exon (*Gata2* IS) is confined to hematopoietic progenitor (lin^- or c-Kit $^+$ Sca-1 $^+$ bone marrow fractions) or neural cells (25, 26). Using qPCR, we found *Gata2* IS is selectively enriched within bone marrow LSK and CMP populations, relative to other progenitor subsets (data not shown). Furthermore, by evaluating mRNA expression patterns through the Immunological Genome Project database (27), we found a strong correlation between abundant *Gata2* and *Kit* mRNA in long- and short-term repopulating progenitors, multipotent progenitors and CMPs, versus other stem/progenitor subsets (not shown). Interestingly, *Cebpa* mRNA expression is relatively low in these early progenitor subsets, and accumulates to high amounts only at the GMP stage, compared to other stem and progenitor subsets analyzed (not shown). These data are consistent with our observations that GATA-2 is a positive regulator of *Kit* promoter activity, while C/EBP α inhibits *Kit* promoter function in hematopoietic progenitor cells.

GATA-2 has been shown to regulate c-Kit expression in committed erythroid progenitors and mast cells, lineages that retain c-Kit expression past multipotent progenitor stages (28–30). Mast cells require c-Kit expression for terminal differentiation and tissue residence, and maintenance of c-Kit depends upon sustained GATA-2 expression throughout mast cell differentiation (30, 31). By contrast, c-Kit must be downregulated during erythropoiesis beyond the committed erythroid progenitor stage, to enable proliferation arrest and terminal erythroid differentiation (32, 33). c-Kit downregulation in committed erythroid progenitors is mediated by the erythroid lineage specifying factor, GATA-1, which is upregulated in developing erythroid cells, and which functions to suppress GATA-2 expression directly as well as GATA-2-mediated *Kit* transcription (28, 34). Our results provide new evidence indicating GATA-2 involvement in *Kit* regulation in lin^- hematopoietic progenitors. Moreover, our data suggests opposing activities for GATA-2 and C/EBP α in *Kit* transcription, which may parallel *Kit* regulation in erythropoiesis. Induction of C/EBP α during progression from lin^- hematopoietic progenitors to committed myeloid progenitors may suppress GATA-2-mediated *Kit* transcription and enable terminal myeloid differentiation. C/EBP α controls the transition from CMPs to GMPs, as well as the development of common dendritic cell progenitors (CDPs) from HSC/HPSCs (35, 36); interestingly, myeloid and DC subsets are profoundly affected by *GATA2* mutation in humans (7). Further work is needed to understand the molecular mechanisms by which GATA-2 and C/EBP α regulate *Kit* in primary myeloid/DC progenitors during normal hematopoiesis as well as the impact of *GATA2* loss-of-function upon *Kit* expression and myeloid/DC development.

In summary, we identified important roles for the GATA-2 C-ZnF in bone marrow hematopoiesis via control of c-Kit expression and HSC/HSPC survival. GATA-2 regulation of c-Kit may be particularly important to sustain myelopoiesis and DC development, potentially by antagonizing C/EBP α -mediated *Kit* suppression in developing myeloid/DC

progenitors. The phenotype we observed with loss of GATA-2 C-ZnF mimics aspects of bone marrow failure and immunodeficiency associated with human *GATA2* mutation, including severely reduced phagocytes and DCs (1–3, 6, 21). These results suggest mice with conditional *Gata2* C-ZnF depletion will be useful to model human immunodeficiency and further investigate GATA-2 targets in definitive hematopoiesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Generation and analysis of mice with inducible deletion of GATA-2 C-ZnF
(A) Schematic illustration of floxed (*Gata2^f*) and deleted (*Gata2^{C-ZnF}*) *Gata2* alleles. **(B–G)** ER-cre⁻ *Gata2^{f/f}* (*Gata2^{f/f}*) or ER-cre⁺ *Gata2^{f/f}* mice (*Gata2^{C-ZnF}*) were treated i.p. with 5 mg tamoxifen/50 μ l corn oil every other day for 3 times. Mice were sacrificed 7–10 d after initial treatment for analysis. **(B)** Total RNA was extracted from bone marrow cells and qPCR was used to determine the abundance of *Gata2* mRNA, using primers amplifying sequences encoding the C-ZnF. **(C)** Survival curve, 9–11 mice/group, 3 independent experiments. **(D)** Peripheral blood CBC analysis. **(E)** Lineage analysis of bone marrow (top) and spleen (bottom) by flow cytometry. Absolute numbers reflect bone marrow cells from 2 femurs and tibias/mouse and total splenocytes/mouse. **(F)** Representative photos of H&E stained femurs from tamoxifen-treated mice (magnification x20). **(G)** Total cell numbers in thymi from *Gata2^{f/f}* and *Gata2^{C-ZnF}* mice, determined by enumeration. **(B–G)** Results represent mean + SE. n=6–10, 3 independent experiments.

Figure 2. Cell-autonomous role for GATA-2 in adult hematopoiesis

(A–B) Bone marrow chimeric mice (ER-cre⁻ *Gata2*^{fl/fl} or ER-cre⁺ *Gata2*^{fl/fl} chimeras) were treated with tamoxifen every other day for a total of 3 treatments (+ TMX) or were left untreated (– TMX), as indicated. (A) Peripheral blood CBC analysis. (B) Lineage analysis of bone marrow (top) and spleen (bottom). Absolute numbers reflect bone marrow cells from 2 femurs and tibias/mouse and total splenocytes/mouse. (C) Mixed bone marrow chimeric mice (1:1 ratio CD45.1⁺ *Gata2*^{fl/fl} and CD45.2⁺ ER-cre⁺ *Gata2*^{fl/fl} donor cells) were treated with tamoxifen as described above (+ TMX) or left untreated (– TMX), as shown. CD45.1⁺ and CD45.2⁺ cells in bone marrow (top) and spleen (bottom) were analyzed for hematopoietic lineages by flow cytometry. (A–C) Results represent mean + SE. n=6–10, 3 independent experiments.

Figure 3. *Gata2* C-ZnF-deficiency impairs c-Kit expression and SP-HSCs

Mixed bone marrow chimeric mice were treated with tamoxifen as described in Figure 1; bone marrow cells were collected 7 d following initial tamoxifen dosing. (A) c-Kit⁺ cells in CD45.1⁺ lin⁻ *Gata2*^{+/+} and CD45.2⁺ lin⁻ *Gata2*^{C-ZnF} fractions. (B) SP HSPCs (gated area) in CD45.1⁺ *Gata2*^{+/+} and CD45.2⁺ *Gata2*^{C-ZnF} bone marrow fractions. (C) Cell cycle analysis of CD45.1⁺ lin⁻ *Gata2*^{+/+} and CD45.2⁺ lin⁻ *Gata2*^{C-ZnF} subsets. (D) Colony-forming activity of CD45.1⁺ *Gata2*^{+/+} and CD45.2⁺ *Gata2*^{C-ZnF} bone marrow cells (9 d culture). (A–C) Representative results shown. (D) Results represent mean + SE. (A–D) n = 6 each, 2–4 independent experiments.

Figure 4. GATA-2-regulated c-Kit mediates hematopoietic colony forming activity

(A) Mixed bone marrow chimeric mice were treated with tamoxifen as described in Figure 1. *Kit* and *Cebpa* were measured by qPCR in CD45.1⁺ lin⁻ *Gata2*^{+/+} and CD45.2⁺ lin⁻ *Gata2*^{C-ZnF} fractions, 7 d following initial tamoxifen dosing. (B) Purified LSKs from ER-cre⁺ *Gata2*^{f/f} mice were cultured in Methocult 3434 in the absence (-) or presence (+) of 4-TOH for 18 or 36 h *ex vivo* as shown; *Kit* and *Cebpa* were measured by qPCR. (C) 32D cells were transfected pGL3-*Kit* reporter and plasmids expressing wild type GATA-2 (WT), GATA-2 C-ZnF, C/EBPα or control vector (-) as indicated. Relative luciferase activity measured at 36 h is shown. (D) 32D cells were transfected with plasmids encoding GATA-2, C/EBPα or empty vector, as indicated. ChIPs were performed with GATA-2, C/EBPα or IgG control antibodies at 36 h. (E and F) ER-cre⁺ *Gata2*^{f/f} bone marrow cells were infected with retrovirus encoding c-Kit or empty vector; cells were cultured in Methocult 3434 in the absence (-) or presence (+) of 4-TOH for 9 d, as indicated. Colony numbers (E) and morphology (F, magnification × 4) are shown. (A-E) Results represent mean + SE. (A-F) Data represent 3 independent experiments.