

## *Gata2* as a Crucial Regulator of Stem Cells in Adult Hematopoiesis and Acute Myeloid Leukemia

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### SUMMARY

Subversion of transcription factor (TF) activity in hematopoietic stem/progenitor cells (HSPCs) leads to the development of therapy-resistant leukemic stem cells (LSCs) that drive fulminant acute myeloid leukemia (AML). Using a conditional mouse model where zinc-finger TF *Gata2* was deleted specifically in hematopoietic cells, we show that knockout of *Gata2* leads to rapid and complete cell-autonomous loss of adult hematopoietic stem cells. By using short hairpin RNAi to target *GATA2*, we also identify a requirement for *GATA2* in human HSPCs. In *Meis1a/Hoxa9*-driven AML, deletion of *Gata2* impedes maintenance and self-renewal of LSCs. Ablation of *Gata2* enforces an LSC-specific program of enhanced apoptosis, exemplified by attenuation of anti-apoptotic factor *BCL2*, and re-institution of myeloid differentiation—which is characteristically blocked in AML. Thus, *GATA2* acts as a critical regulator of normal and leukemic stem cells and mediates transcriptional networks that may be exploited therapeutically to target key facets of LSC behavior in AML.

### INTRODUCTION

Hematopoiesis is contingent on a rare pool of self-renewing hematopoietic stem cells (HSCs) and progenitors that are regulated by a combination of a cell-extrinsic program, imparted by the bone marrow (BM) niche in which they reside, and a cell-autonomous program driven by transcription factors (TFs), which regulate gene expression (Beck et al., 2013). Through loss-of-function genetic mouse models, the differentiation-stage requirement for specific TFs in myeloid and lymphoid committed progenitors has been characterized comprehensively (Orkin, 2000); yet, with few exceptions (Hock et al., 2004; Kranc et al., 2009; Pajeroski et al., 2010), the transcriptional program critical to adult HSC survival remains unclear.

Perturbed TF activity in the form of TF chromosomal translocations and loss- or gain-of-function genetic and epigenetic mutations in TFs has been implicated in the pathogenesis of hematologic malignancies, including acute myeloid leukemia (AML). AML is driven by a subset of leukemia cells—namely leukemic stem cells (LSCs) (Bonnet and Dick, 1997)—which are acknowledged to underpin leukemic growth and maintenance and, notably, are refractory to standard AML chemotherapeutics (Craddock et al., 2013; Ishikawa et al., 2007; Saito et al., 2010). Like their

normal HSC counterparts, however, the requirement for TFs in LSC function is poorly defined; thus a deeper understanding of transcriptional regulation in this setting will afford insights toward specific targeting of LSCs, which will be necessary for improving the clinical armamentarium in AML.

*Gata2*, part of the zinc finger family of GATA family of TFs (Nagai et al., 1994), has been identified as a critical regulator of HSCs during ontogenesis of the hematopoietic system (Tsai et al., 1994). Constitutive lack of *Gata2* (*Gata2*<sup>-/-</sup>) leads to embryonic fatality at embryonic day 10.5 (E10.5) due to anemia (Tsai et al., 1994). Chimera experiments demonstrated that *Gata2*<sup>-/-</sup> embryonic stem cells were unable to contribute to the formation of myeloid and lymphoid cells at the fetal liver (FL) developmental stage and in adult mice, suggesting that *Gata2* acts as a crucial regulator of HSCs (Tsai et al., 1994). To formally assess the requirement for *Gata2* in HSCs during embryonic development, using conditional knockout (KO) genetic models, *Gata2* has been deleted in hemogenic endothelium cells, before the generation of HSCs using *Vec-Cre* (de Pater et al., 2013; Lim et al., 2012), and immediately following HSC formation using *Vav-Cre* (de Pater et al., 2013). Through functional analysis of *Gata2*-deficient cells from temporally restricted hematopoietic organs





(aorta-gonad-mesonephros, FL) in these embryonic settings, *Gata2* has been revealed as a pivotal regulator of cell-autonomous HSC generation and, thereafter, for HSC survival in the embryo. By analysis of haploinsufficient *Gata2*<sup>+/-</sup> mice that express reduced *Gata2*, we and other laboratories have examined the impact of *Gata2* gene dosage in adult hematopoiesis (Guo et al., 2013; Ling et al., 2004; Rodrigues et al., 2005). BM from *Gata2*<sup>+/-</sup> mice displayed a reduction in abundance of immunophenotypically defined HSCs and in functionality during transplantation assays (Guo et al., 2013; Rodrigues et al., 2005). This was associated with decreased HSC proliferation, enhanced HSC quiescence and apoptosis, and attendant downregulation of anti-apoptotic BCL-XL in *Gata2*<sup>+/-</sup> mice (Ling et al., 2004; Rodrigues et al., 2005). *Gata2* haploinsufficiency also reduces granulocyte-macrophage progenitor (GMP) cell function while leaving other myeloid committed progenitors intact (Rodrigues et al., 2008). Conversely, forced expression of GATA2 in adult HSCs has similarly implicated *Gata2* as a critical level-dependent regulator of adult HSC proliferation and differentiation (Persons and Allay, 1999).

Emphasizing the biological and clinical imperative to maintain normal GATA2 expression in adult hematopoiesis, deregulated GATA2 expression has been linked to the pathogenesis of pre-leukemic myelodysplastic syndrome (MDS) and AML. In its capacity as a tumor suppressor gene, loss of GATA2 expression in the context of sporadic or hereditary GATA2 haploinsufficiency mutations in coding or enhancer regions leads to immunodeficiency syndromes that predispose to MDS/AML when secondary mutations are attained (Hahn et al., 2011; Hsu et al., 2013; Soukup et al., 2019). Far more frequently, however, GATA2 overexpression has been implicated as a harbinger of poor prognosis in adult and pediatric AML, suggesting an oncogenic role for GATA2 in AML (Luesink et al., 2012; Vicente et al., 2012). Yet the requirement for GATA2 in AML cell fate decisions, including in the AML-propagating LSC compartment, remains poorly defined.

Using a genetic mouse model harboring conditional alleles of *Gata2* and an inducible *Mx1-Cre* (*Gata2*<sup>fl/fl</sup>;*Mx1-Cre*<sup>+</sup>), in which *Gata2* expression can be deleted acutely in hematopoietic cells on administration of the interferon- $\alpha$  mimic, polyinosinic-polycytidylic acid (pIpC), we investigated the hitherto unknown requirement for *Gata2* in adult HSCs and LSCs. We identify *Gata2* as an essential TF for adult HSC survival in BM and a unique hematopoietic TF required throughout HSC ontogeny. Using a gene knockdown (KD) approach, we independently demonstrate a conserved regulatory role for GATA2 in human cord blood (CB) hematopoietic stem/progenitor cells (HSPCs). Finally, we show that *Gata2* regulates LSC

capacity to propagate AML through modulation of self-renewal, apoptosis and myeloid differentiation fates.

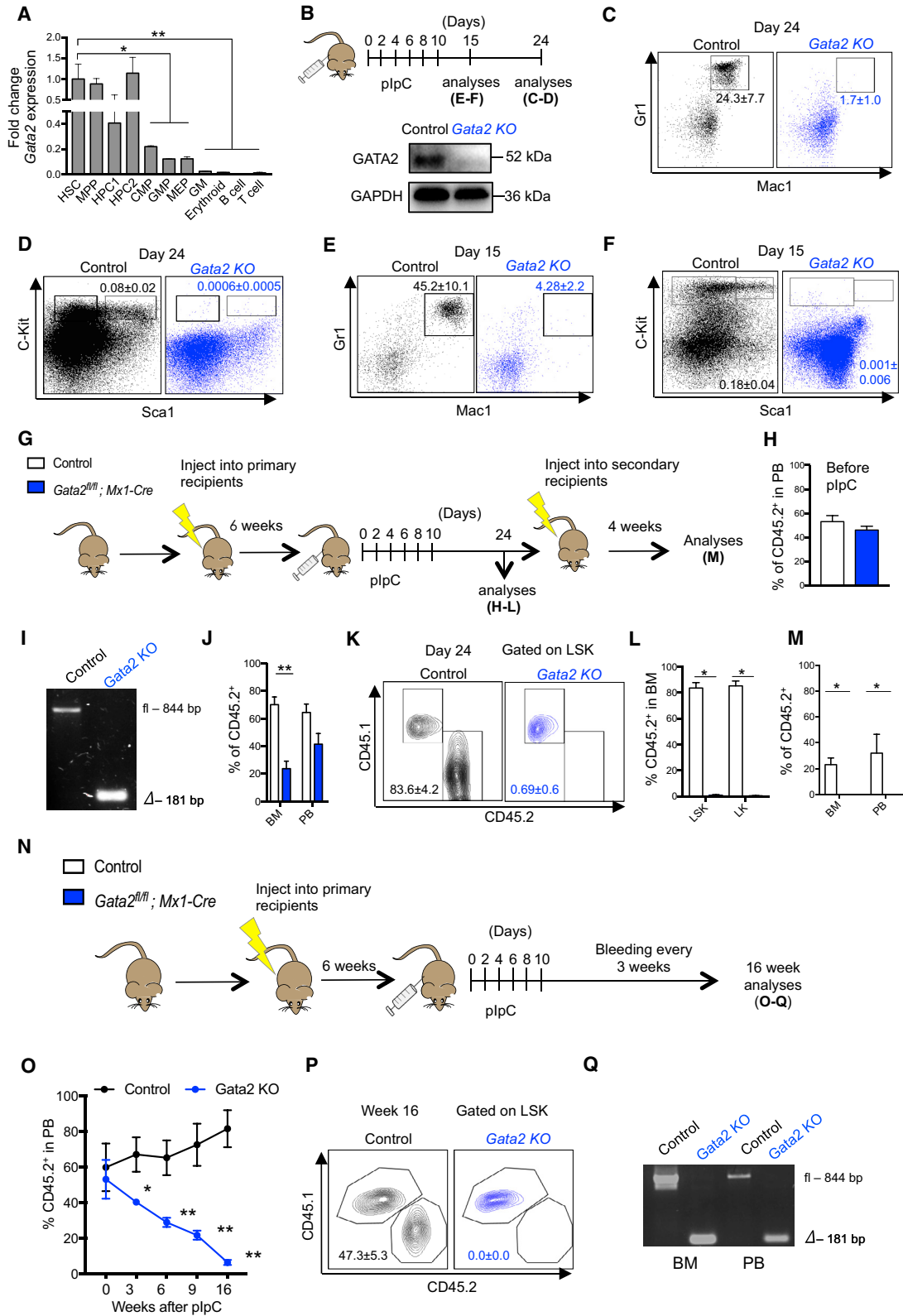
## RESULTS

### Gata2 Is Essential for Cell-Autonomous Adult HSC Maintenance

We first assessed *Gata2* expression in prospectively isolated HSPCs from adult BM. *Gata2* expression was highest in HSCs, multipotent progenitors and hematopoietic progenitor cells-1/-2, reduced in committed myeloid progenitor cells, and nearly extinguished in terminally differentiated blood cell populations (Figure 1A).

Next, to assess the requirement for *Gata2* in adult HSC maintenance, we bred *Gata2* harboring conditional alleles of *Gata2* (*Gata2*<sup>fl/fl</sup> mice) with *Mx1-Cre* to obtain a cohort of *Gata2*<sup>fl/fl</sup>;*Mx1-Cre*<sup>+</sup> or control mice (*Gata2*<sup>fl/fl</sup>;*Mx1-Cre*<sup>-</sup>) and administered pIpC on alternate days for 10 days to achieve acute deletion of *Gata2* (*Gata2* KO). The impact of *Gata2* on hematopoiesis was assessed 14 days after the last injection of pIpC (Figure 1B). Near complete loss of GATA2 protein and *Gata2* transcript was confirmed in BM by western blot and qPCR analysis, respectively (Figures 1B and S1A). BM cellularity was significantly attenuated in *Gata2* KO mice in comparison with control mice (Figure S1B). Immunophenotypic analysis revealed that, while lymphoid lineages remained intact, a striking reduction of myeloid and erythroid cells was observed in the BM and/or peripheral blood (PB) of *Gata2* KO mice (Figures 1C, S1C, and S1D). To identify the origin of myeloid and erythroid cell loss in the context of *Gata2* deficiency, we assessed HSPCs from BM of *Gata2* KO mice and found a near complete loss of cells in the LSK compartment (which contain HSCs and primitive progenitor cells) and LK compartment (comprising committed myeloid and erythroid progenitors) (Figure 1D). To investigate the kinetics of HSC loss after *Gata2* deletion, we assessed the BM of *Gata2* KO mice 5 days after the last pIpC dose (Figure 1B). Remarkably, we observed a near complete depletion of myeloid cells and LSK and LK cells by day 5 (Figures 1E and 1F), indicating a rapid loss of HSCs immediately following *Gata2* deletion, consistent with a profound HSC survival defect.

*Mx1-Cre* mediates gene deletion in hematopoietic cells, extramedullary tissues, and in the BM niche (Kühn et al., 1995). To stringently evaluate whether *Gata2* functions to regulate HSC maintenance cell autonomously, we transplanted CD45.2<sup>+</sup> BM cells from *Gata2*<sup>fl/fl</sup>;*Mx1-Cre*<sup>+</sup> or control mice, which have not received pIpC, together with unfractionated CD45.1<sup>+</sup> BM cells in a competitive transplant (Figure 1G). Similar engraftment was observed in the PB of recipients transplanted with *Gata2*<sup>fl/fl</sup>;*Mx1-Cre*<sup>+</sup>



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and control cells before *Gata2* deletion (Figure 1H). Six weeks after transplantation, *Gata2* deletion was induced by pIpC administration, and efficient *Gata2* deletion in CD45.2<sup>+</sup> BM cells of recipients transplanted with *Gata2<sup>fl/fl</sup>;Mx1-Cre<sup>+</sup>* cells was confirmed 14 days after the last pIpC dose (Figure 1I). We observed a decrease in the frequency of CD45.2<sup>+</sup> *Gata2* KO cells in BM, and to a lesser extent in PB (Figure 1J). Further immunophenotypic analysis revealed a complete loss of myeloid cells in BM and PB and a significant reduction of erythroid cells in BM (Figures S1E and S1F). Strikingly, only CD45.1<sup>+</sup> BM support cells were able to contribute to HSPC maintenance; no CD45.2<sup>+</sup> *Gata2* KO cells were detectable within the LSK and LK compartments (Figures 1K and 1L). To test that HSPC loss was not merely due to an attenuation of cell surface markers that define HSCs, such as Sca-1 and c-kit, we isolated vestigial *Gata2* KO CD45.2<sup>+</sup> BM cells from primary recipients and transplanted them together with unfractionated CD45.1<sup>+</sup> competitor cells into secondary recipients (Figure 1G). Four weeks after transplantation we found no *Gata2* KO cells in the BM or PB of the mice transplanted with *Gata2* KO BM cells (Figure 1M).

Having shown entire loss of the myeloid lineage while lymphoid cells were preserved at 14 days after the last injection of pIpC in the BM of *Gata2* KO mice, we next investigated whether the lack of multi-lineage failure after *Gata2* deletion was due to the relatively longer lifespan of

lymphoid cells compared with myeloid cells. To test this directly, we performed a long-term transplantation assay to abolish *Gata2* expression in a cell-autonomous manner and monitored mice for 16 weeks to study the impact of *Gata2* deletion on long-term hematopoiesis (Figure 1N). Analysis of PB revealed a continual decline in contribution of *Gata2* KO cells to blood lineages of recipient mice, with less than 5% engraftment of *Gata2* KO cells detectable at 16 weeks after *Gata2* deletion, indicating a multi-lineage defect in *Gata2* KO cells (Figure 1O). In addition, BM analysis showed an absence of CD45.2<sup>+</sup> *Gata2* KO BM cells in the LSK compartment at 16 weeks (Figure 1P). Full deletion of the *Gata2* gene was achieved in residual PB and BM *Gata2* KO cells at 16 weeks (Figure 1Q).

To eliminate the possibility of *Gata2*-dependent extrinsic HSC regulation through the BM niche, we transplanted unfractionated CD45.1<sup>+</sup> BM cells into lethally irradiated CD45.2<sup>+</sup> *Gata2<sup>fl/fl</sup>;Mx1-Cre<sup>+</sup>* or control mice. Six weeks after transplantation, we administered pIpC to obtain *Gata2*-deficient (*Gata2* KO) or control BM niche recipient mice and monitored the engraftment for up to 12 weeks (Figure S1G). The frequency of CD45.1<sup>+</sup> cells in the PB of *Gata2* KO recipients remained comparable with those transplanted in control mice (Figures S1H and S1I). In concert, these data demonstrate that *Gata2* is critically required for cell-autonomous maintenance of HSCs and multi-lineage hematopoiesis.

### Figure 1. *Gata2* Is Essential for Cell-Autonomous HSC Maintenance

(A) *Gata2* expression in hematopoietic cells compared with *Gata2* expression in HSCs (n = 3 independent experiments). Statistical analyses: one-way ANOVA.

(B) Top: control and *Gata2<sup>fl/fl</sup>;Mx1-Cre<sup>+</sup>* mice received six injections of pIpC on alternate days and were analyzed 15 or 24 days after the first injection. Bottom: GATA2 protein level in BM cells from control and *Gata2* KO at day 24 after pIpC administration against GAPDH.

(C and D) Fluorescence-activated cell sorting (FACS) plots of (C) myeloid (Gr1<sup>+</sup>Mac1<sup>+</sup>) and (D) hematopoietic stem and progenitor cells (HSPCs) from control and *Gata2* KO mice at day 24 (n = 4).

(E and F) FACS plots showing (E) myeloid cells and (F) HSPCs from control and *Gata2* KO mice at day 15 (n = 3).

(G) CD45.2<sup>+</sup> BM cells from untreated control or *Gata2<sup>fl/fl</sup>;Mx1-Cre<sup>+</sup>* mice were transplanted with CD45.1<sup>+</sup> wild-type (WT) BM cells into irradiated recipients. Six weeks later, mice received six doses of pIpC. Fourteen days after last pIpC dose (day 24) mice were analyzed and CD45.2<sup>+</sup> BM cells transplanted into secondary irradiated recipients.

(H) Frequency of CD45.2<sup>+</sup> cells in PB at week 6, before pIpC injection (n = 4).

(I) Representative gel showing genotyping of donor-derived CD45.2<sup>+</sup> fraction BM from pIpC-treated recipient mice. Δ, excised allele; fl, undeleted conditional allele.

(J) Frequency of CD45.2<sup>+</sup> cells in BM and PB at day 24 (n = 4).

(K) FACS plots of CD45.1<sup>+</sup> versus CD45.2<sup>+</sup> chimerism in LSKs from transplanted mice at day 24 (n = 4).

(L) Frequency of CD45.2<sup>+</sup> cells in LSK and LK cells at day 24 (n = 4).

(M) Frequency of CD45.2<sup>+</sup> cells in BM and PB 4 weeks after secondary transplant (n = 4).

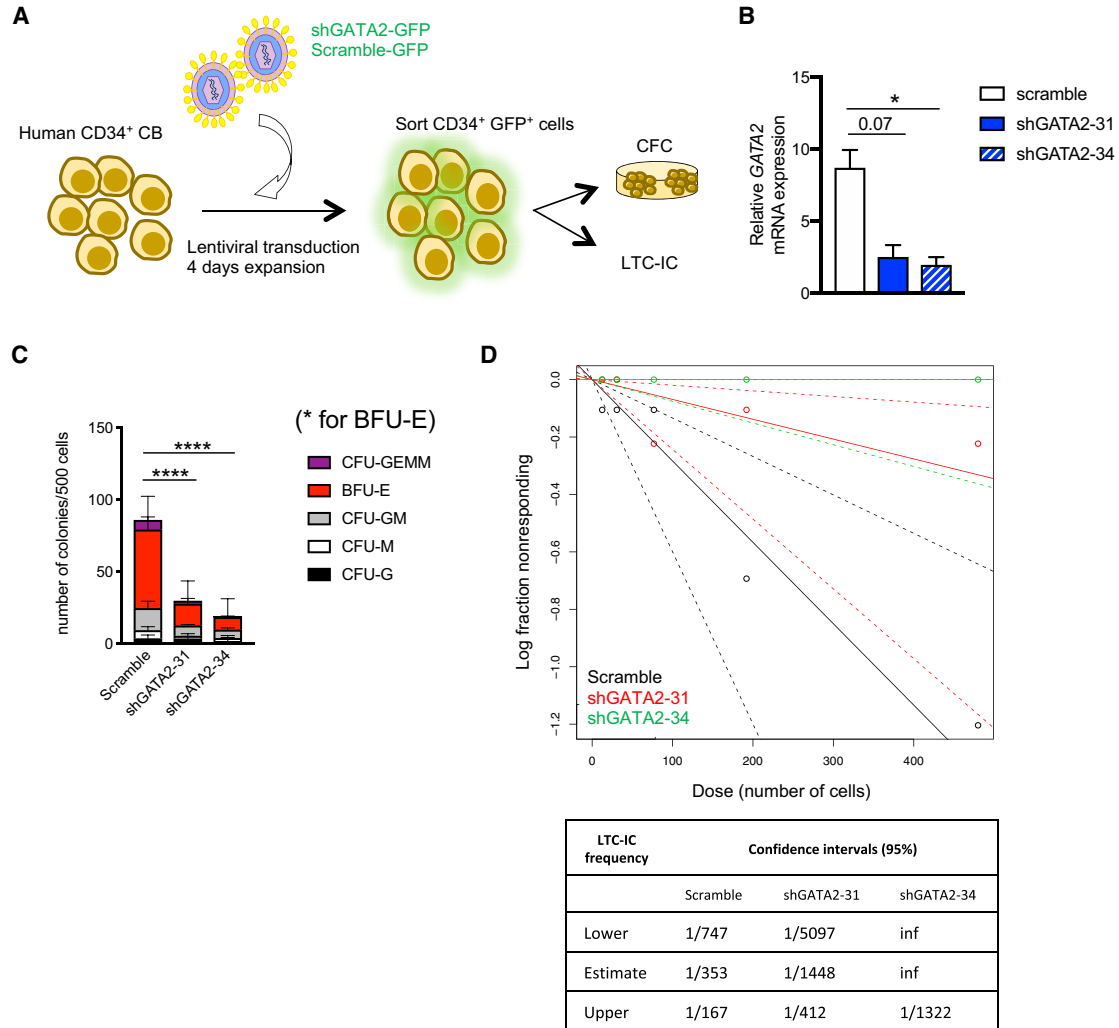
(N) CD45.2<sup>+</sup> BM cells from untreated control and *Gata2<sup>fl/fl</sup>;Mx1-Cre<sup>+</sup>* mice were transplanted with CD45.1<sup>+</sup> WT BM cells into irradiated recipients. Six weeks later, mice received six doses of pIpC and were analyzed for reconstitution for up to 16 weeks after last pIpC dose.

(O) Frequency of CD45.2<sup>+</sup> cells in PB after pIpC injection (n = 4). Error bars depict SD. Statistical analyses: one-way ANOVA.

(P) FACS plots of CD45.1<sup>+</sup> versus CD45.2<sup>+</sup> chimerism in LSK cells at week 16 (n = 3).

(Q) Representative gel showing genotyping of donor CD45.2<sup>+</sup> fraction of the BM and PB of pIpC-treated recipients at week 16. Δ, excised allele; fl, undeleted conditional allele.

Data are mean ± SEM and n denotes number of mice used per genotype unless otherwise stated. Statistical analyses: Mann-Whitney U test unless otherwise indicated. \*p < 0.05, \*\*p < 0.01.



## Figure 2. *GATA2* Is a Crucial Regulator of Human CB HSPC Function

(A) CB CD34<sup>+</sup> cells were transduced with lentiviruses encoding a short hairpin against human *GATA2* (or scramble control) and a GFP reporter. GFP<sup>+</sup> cells were FACS sorted and plated into CFC or LTC-IC.

(B) Relative *GATA2* mRNA expression in CB CD34<sup>+</sup> cells compared with *GAPDH* ( $n = 3$  independent experiments).

(C) Colony-forming units (CFU) per 500 CD34<sup>+</sup> GFP<sup>+</sup> cells plated ( $n = 3$  independent experiments). Statistical analyses: one-way ANOVA. Data are mean  $\pm$  SEM.

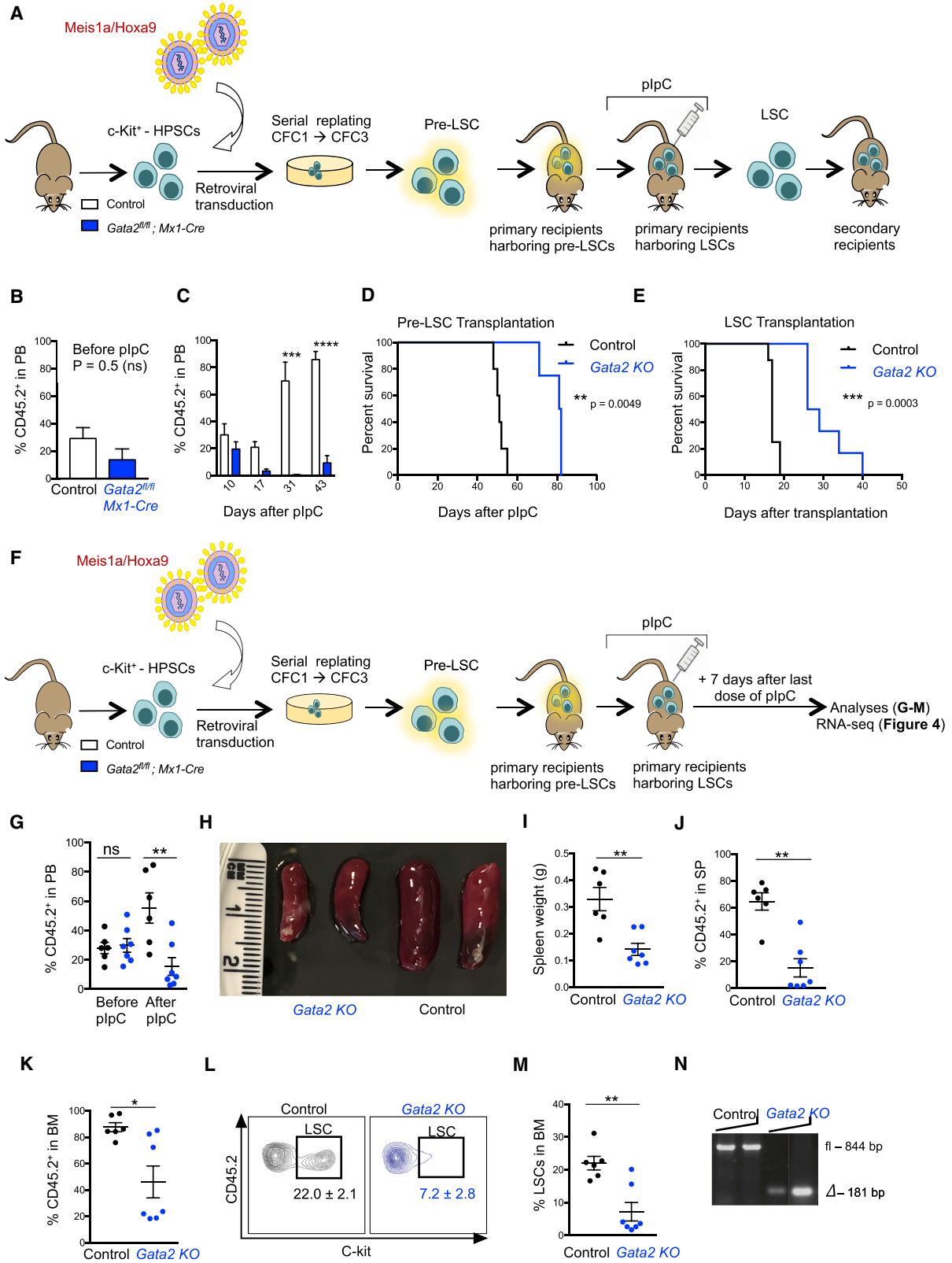
(D) Top: plot showing representative log-fraction of the limiting dilution model for the LTC-IC assay. The dotted lines give the 95% confidence interval. Bottom: LTC-IC frequency calculated using ELDA software ( $n = 2$  independent experiments).

\* $p < 0.05$ , \*\*\*\* $p < 0.0001$ .

## *GATA2* Regulates Human CB HSPC Function

Having shown the requirement for *Gata2* in mouse adult HSC function, we next sought to assess whether this role was conserved in human HSPCs. Using a bicistronic lentiviral vector system carrying a GFP reporter and short hairpin (shRNA) targeted to *GATA2* expression in CB CD34<sup>+</sup> HSPCs, we conducted functional analysis in colony-forming cells (CFCs) and long-term culture-initiating cell (LTC-IC) assays following *GATA2* KD (Figure 2A). Reduction in *GATA2* expression was confirmed

in CB CD34<sup>+</sup> cells transduced with two independent KD constructs (Figure 2B). Overall CFC growth capacity and lineage-specific CFCs (e.g., BFU-E) were markedly reduced using either *GATA2* KD constructs (Figure 2C). Limiting dilution LTC-IC analysis revealed a marked reduction in HSPC frequency using both KD constructs, in keeping with the lack of multipotent progenitors (CFC-GEMM) observed in CFC assays following *GATA2* KD (Figures 2C and 2D). Together these data demonstrate that *GATA2* functions as a critical regulator of human HSPC function.



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### Acute Deletion of *Gata2* Impairs LSC Maintenance and Self-Renewal in *Meis1a/Hoxa9*-Driven AML

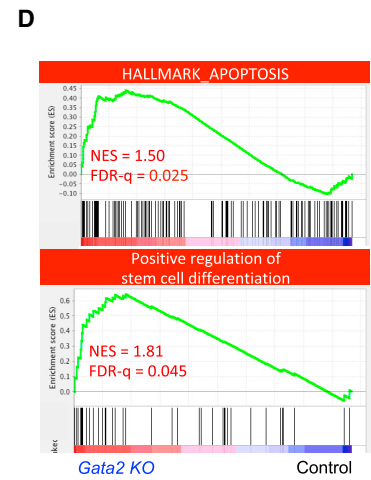
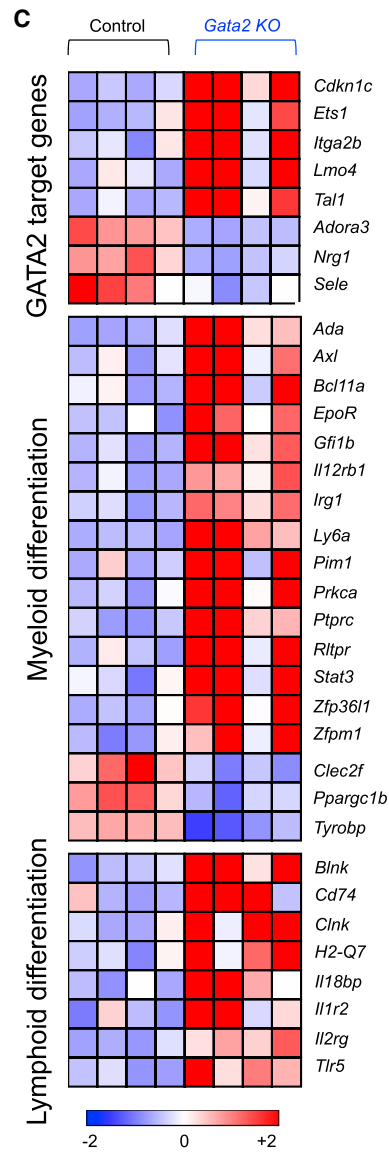
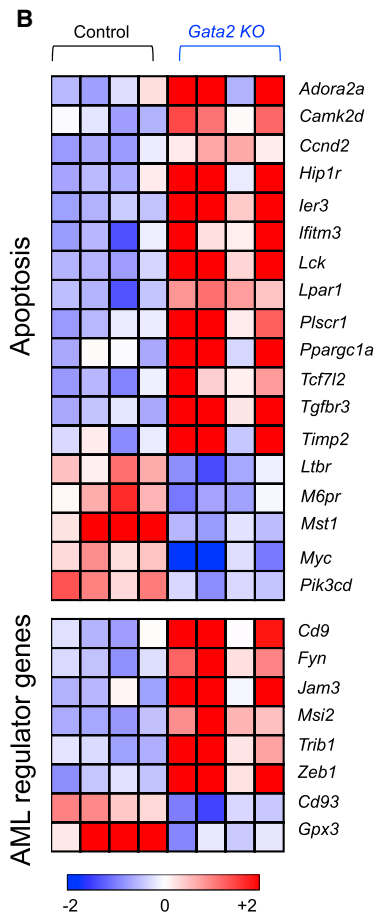
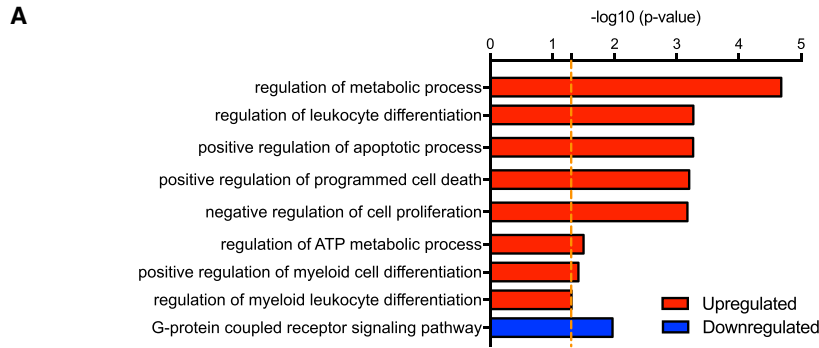
To investigate the requirement of *Gata2* in LSCs, we employed a tractable retroviral leukemogenic assay in which leukemic transformation of murine C-KIT<sup>+</sup> HSPCs is driven by overexpression of *Meis1a/Hoxa9*, which is observed in 40% of AML patients (Dorsam et al., 2004; Lawrence et al., 1993). *Meis1a/Hoxa9*-transduced cells were serially replated for three rounds in a CFC assay to generate pre-LSCs, which, when injected into primary lethally irradiated mice, become LSCs and cause AML (Figure 3A). Given that *Gata2*<sup>fl/fl</sup>; *Mx1-Cre*<sup>+</sup> mice lack HSPCs by day 15 after pIpC administration (Figure 1F), we instead co-transduced HSPCs from non-induced *Gata2*<sup>fl/fl</sup>; *Mx1-Cre*<sup>+</sup> or control mice with retroviruses expressing *Meis1a* and *Hoxa9*, and after three rounds of CFC we harvested *Meis1a/Hoxa9* pre-LSC and transplanted them into primary recipients together with unfractionated BM support cells (Figure 3A). We monitored leukemia development by episodic bleedings (Figure 3B) and induced *Gata2* deletion with pIpC once the percentage of leukemic cells in the PB reached 20%–25%, a threshold for the diagnosis of AML. Leukemia cells in the PB of recipients transplanted with *Gata2*<sup>fl/fl</sup>; *Mx1-Cre*<sup>+</sup> pre-LSCs were dramatically reduced at weeks 4 and 6 after pIpC treatment (Figure 3C). Consistent with this, recipients of *Gata2* KO LSCs succumbed to AML with a significantly delayed latency compared with recipients of control LSCs, indicating that *Gata2* deletion impairs LSC maintenance (Figure 3D). Notably, these results were obtained with 33% recipients harboring *Gata2* KO leukemic cells with complete *Gata2* deletion.

To investigate whether *Gata2* deletion also compromises LSC self-renewal, we prospectively isolated LSCs from BM of mice exhibiting complete deletion of *Gata2* or controls by fluorescence-activated cell sorting and transplanted them into secondary recipients together with unfractionated BM support cells (Figure 3A). Recipients transplanted with *Gata2* KO LSCs developed AML more slowly than recipients transplanted with control LSCs (Figure 3E). Taken together, these data demonstrate that *Gata2* is required for LSC maintenance, self-renewal, and leukemia propagation in a *Meis1a/Hoxa9*-driven AML mouse model.

That recipients of *Gata2* KO LSCs eventually succumb to AML demonstrates that, in striking contrast to their normal HSC counterparts (Figure 1), LSCs survive *Gata2* loss, suggesting differential requirements for *Gata2* in subsets of C-KIT<sup>+</sup> HSPCs during transformation. The cell of origin for *Meis1a/Hoxa9*-driven AML in C-KIT<sup>+</sup> HSPCs is either LSK or common myeloid progenitor (CMP) but not GMP (Wang et al., 2010). We investigated which of these HSPC subsets was dependent on *Gata2* during transformation in *Meis1a/Hoxa9*-driven AML. *Meis1a/Hoxa9* LSK and CMP-derived pre-LSCs from *Gata2*<sup>fl/fl</sup> or *Gata2*<sup>+/+</sup> mice were transduced with lentiviruses encoding Cre and *iVenus* reporter. Transduced *iVenus*<sup>+</sup> cells were serially plated in CFC assays for three rounds, and were immunophenotypically assessed at each stage for expression of C-KIT<sup>+</sup>, as a marker of LSCs, and GR1 and MAC1 myeloid differentiation markers, which are blocked in AML development. While LSC and MAC1 expression was similar between genotypes in LSK and CMP compartments throughout replating (Figures S2A–S2D), LSK cells from *Gata2* KO

### Figure 3. Acute Deletion of *Gata2* Impairs LSC Development and Self-Renewal

- (A) C-kit<sup>+</sup> cells from control or *Gata2*<sup>fl/fl</sup>; *Mx1-Cre*<sup>+</sup> mice were transduced with retroviruses expressing *Meis1a/Hoxa9* and plated into CFC assays. After CFC3, pre-LSCs (CD45.2<sup>+</sup> c-kit<sup>+</sup>) were sorted and transplanted into lethally irradiated recipients with CD45.1<sup>+</sup> unfractionated BM cells. Three weeks later, mice were injected with pIpC. LSCs (CD45.2<sup>+</sup> c-kit<sup>+</sup>) from primary recipients were transplanted into secondary recipients.
- (B) Frequency of leukemic (CD45.2<sup>+</sup>) cells in PB before pIpC (n = 6).
- (C) Frequency of leukemic cells in PB at the indicated days after pIpC injection (n = 6). Statistical analyses: one-way ANOVA.
- (D) Kaplan-Meier survival curve of recipients transplanted with pre-LSCs (n = 6). Statistical analyses: Mantel-Cox test.
- (E) Kaplan-Meier survival curve of recipients transplanted with LSCs (n = 6–8). Statistical analyses: Mantel-Cox test.
- (F) Pre-LSCs (CD45.2<sup>+</sup> c-kit<sup>+</sup>) from the two genotypes were sorted and transplanted into lethally irradiated recipients together with CD45.1<sup>+</sup> unfractionated BM cells. Three weeks later, mice were injected with pIpC, and were analyzed or used for RNA-seq 7 days after the last dose of pIpC.
- (G) Frequency of leukemic cells in PB before and after pIpC (n = 6–7).
- (H) Representative images of reduced splenomegaly in *Gata2* KO mice.
- (I) Spleen weight at day 7 (n = 6–7).
- (J and K) Frequency of leukemic cells in (J) SP and in (K) BM at day 7 (n = 6–7).
- (L) Representative FACS plot of LSCs in BM.
- (M) Frequency of LSCs (CD45.2<sup>+</sup> c-kit<sup>+</sup>) in BM at day 7 (n = 6–7).
- (N) Representative gel showing genotyping from donor-derived LSC fraction of the BM of pIpC-treated recipient mice at day 7. Δ, excised allele; fl, undeleted conditional allele.
- Data are mean ± SEM and n denotes number of mice used per genotype. Statistical analyses: Mann-Whitney U test unless otherwise stated. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.



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had enhanced GR1 expression in CFC3 (Figure S2E). In contrast, a relative blockade in GR1 expression was observed from *Gata2* KO in *Meis1a/Hoxa9*-transformed CMP at CFC3 (Figure S2F). These data suggest that HSC-enriched LSKs mediate GATA2 regulation of transformation in *Meis1/Hoxa9* AML, whereas CMPs regulate transformation in this setting independently of GATA2.

### Acute Deletion of *Gata2* Promotes an LSC-Specific Program of Apoptosis and Myeloid Differentiation in *Meis1a/Hoxa9*-Driven AML

To examine the molecular signature governing LSC maintenance and self-renewal driven by GATA2, we performed global gene expression analysis by RNA sequencing (RNA-seq) of *Gata2* KO and control LSCs. MEIS1a and HOXA9 expressing pre-LSCs (CD45.2<sup>+</sup> c-kit<sup>+</sup>) were transplanted into lethally irradiated recipient mice together with unfractionated CD45.1<sup>+</sup> BM support cells (Figure 3F). When the levels of AML in PB reached 25%, we administered pIpC to delete *Gata2* in LSCs and analyzed mice 7 days after the last dose of pIpC (Figure 3F). The frequency of AML cells in PB was comparable between both genotypes before pIpC administration (Figure 3G), yet in mice harboring *Gata2* KO LSCs there was a 3.5-fold reduction in the PB at 7 days after administration of the last dose of pIpC (Figure 3G). Splenomegaly, a hallmark of AML, was markedly reduced in recipients with *Gata2* KO LSCs 7 days after the last pIpC injection (Figures 3H and 3I). This corresponded to a reduced population of engrafting AML cells in the spleen of *Gata2* KO LSC recipients (Figure 3J). Immunophenotypic analysis was also conducted on BM, which revealed a decrease in bulk AML cells and a 3-fold reduction in the abundance of LSC (CD45.2<sup>+</sup> c-kit<sup>+</sup>) in recipients harboring *Gata2* KO LSCs (Figures 3K–3M). Full deletion of *Gata2* was achieved in *Gata2* KO LSCs (Figure 3N). Using pathway analysis of RNA-seq using the Gene Ontology Consortium and gene set enrichment analysis, we observed differential upregulation of pathways modulating metabolic processes and pathways that curtail cell proliferation and downregulation of G-coupled receptor signaling, oxidative phosphorylation, and C-MYC target genes in *Gata2* KO LSCs (Figures 4A and S3A). Furthermore, *Gata2* KO LSCs exhibited dampening of lysosome signaling, which targets AML LSCs (Sukhai et al., 2013), and the

nuclear factor  $\kappa$ B signaling pathway, the activation of which is associated with a pro-inflammatory environment permissive for the development and maintenance of AML (Figures S3A and S3B) (Wang et al., 2014; Zambetti et al., 2016).

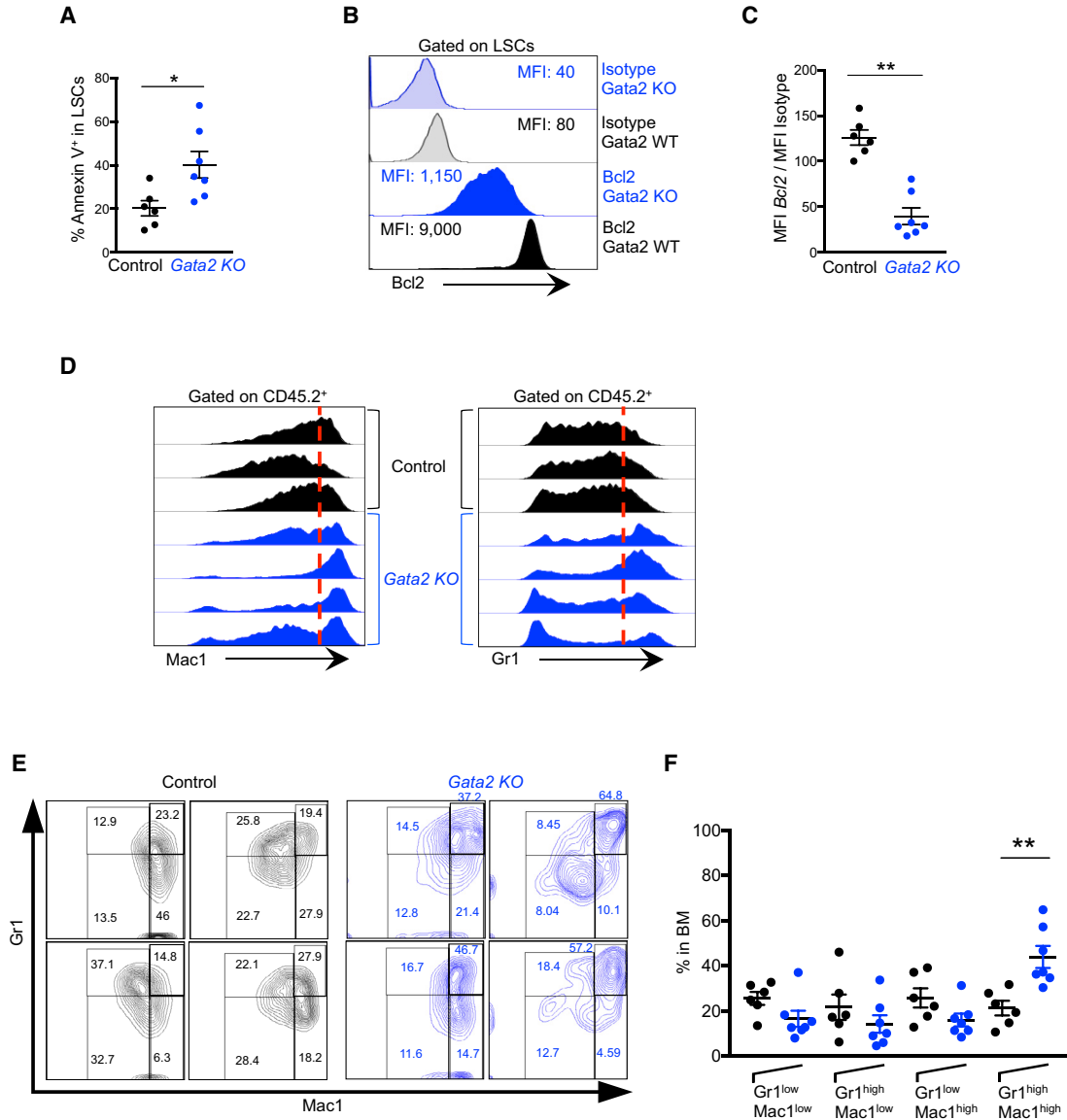
To identify GATA2 target genes specific to LSCs rather than HSCs, we compared RNA-seq data from *Gata2* KO LSCs with GATA2 target genes identified from HSCs. As genetic deletion of *Gata2* entirely abolishes immunophenotypically defined HSCs (Figure 1), we instead conducted RNA-seq on HSCs from *Gata2* haploinsufficient mice bearing a single floxed allele deleted by *Vav-iCre* (*Gata2*<sup>+/fl</sup>; *Vav-iCre*<sup>+</sup>) *in vivo*, where adult HSCs remain isolatable but are reduced in abundance (de Pater et al., 2013) (our unpublished observations). Using Ingenuity Pathway Analysis of differentially expressed genes in HSCs and LSCs, we found that several GATA2-mediated biological pathways, including ERK/MAPK and interleukin-4 signaling, were shared between HSCs and LSCs (Figure S3C). Seventy genes were differentially expressed in *Gata2*<sup>+/fl</sup>; *Vav-iCre*<sup>+</sup> HSCs and 689 differentially expressed in *Gata2* KO LSCs, yet a mere 4 GATA2 target genes (*Lphn2*, *Mllt3*, *Gimap4*, and *Zhx2*) were common to both HSCs and LSCs. Thus, the vast majority of GATA2 target genes identified here were exclusive to LSCs. In agreement with the notion that lack of *Gata2* engenders an anti-leukemic effect in LSCs, established AML tumor suppressors or oncogenes were up- or downregulated, respectively, in *Gata2* KO LSCs (Figure 4B). As expected, previously identified GATA2 target genes were deregulated in *Gata2* KO LSCs (Figure 4C). We observed gene signatures in *Gata2* KO LSCs consistent with re-activation of both lymphoid and erythroid differentiation potential (e.g., *Epo-R*) (Figure 4C). Most notably, *Gata2* KO LSCs registered a robust pro-apoptotic and myeloid cell differentiation gene signature (Figures 4A–4D). These data suggest that ablation of *Gata2* could alleviate the myeloid differentiation and apoptotic block in AML LSCs—cell fates that are characteristic and indispensable for facilitating AML maintenance and propagation (Ishikawa et al., 2007; Sykes et al., 2015).

Next, to directly assess whether apoptosis was augmented in *Gata2* KO LSCs, we conducted an annexin V assay on *Gata2* KO LSCs at 7 days after the last pIpC injection (Figure 3F), which indeed confirmed an increase

### Figure 4. Transcriptional Signature of *Gata2* KO LSCs Shows Deregulated Apoptotic and Myeloid Differentiation Pathways

Gene expression analyses were performed in sorted control or *Gata2* KO LSCs using the same experimental design as Figure 3F (n = 4 mice per genotype).

(A) Biological processes enriched in up- and downregulated genes in *Gata2* KO LSCs compared with control LSCs. Analyses was performed using the Gene Ontology Consortium database. Data are shown as  $-\log_{10}$  (p value), and the dotted orange line indicates p value = 0.05. (B and C) Heat maps reflecting expression levels of (B) apoptotic genes and regulators of AML leukemogenesis, and (C) *Gata2* target genes, and myeloid and lymphoid differentiation-associated genes that are significantly differentially expressed between control or *Gata2* KO LSCs. (D) GSEA plots showing apoptosis and stem cell differentiation signature gene sets.



### Figure 5. Deletion of *Gata2* in LSCs Leads to an Increase in Apoptosis and Enhanced Myeloid Differentiation

Analyses were performed in control or *Gata2* KO LSCs using the experimental design in Figure 3F.

(A) Frequency of annexin V<sup>+</sup> cells in LSCs (n = 6–7).

(B) Representative histogram plot showing the levels of BCL2 protein in control and *Gata2* KO LSCs.

(C) MFI of BCL2 in LSCs in relation to the MFI of isotype control (n = 6–7).

(D) Representative histogram plot showing the levels of Mac1 and Gr1 protein in leukemic cells. Red dotted line indicates the highest expression of each marker.

(E) Gating strategy to distinguish AML blast cells with differentially expressed Gr1 and Mac1 levels: Gr1<sup>high</sup>Mac1<sup>high</sup>, Gr1<sup>low</sup>Mac1<sup>high</sup>, Gr1<sup>high</sup>Mac1<sup>low</sup>, and Gr1<sup>low</sup>Mac1<sup>low</sup>. Representative FACS plots showing an increase in Gr1<sup>high</sup>Mac1<sup>high</sup> population after deletion of *Gata2* in LSCs.

(F) Frequency of the indicated populations in BM of leukemic mice (n = 6–7). Statistical analyses: one-way ANOVA.

Data are mean ± SEM and n denotes number of mice used per genotype. Statistical analyses: Mann-Whitney U test unless otherwise indicated. \*p < 0.05, \*\*p < 0.01.

in apoptosis (Figure 5A). Using intracellular flow cytometry, we measured protein levels of crucial apoptotic regulators in *Gata2* KO LSCs or control LSCs. Between *Gata2* KO

LSCs and their LSC control counterparts, no changes were discernible with respect to the mean fluorescence intensity (MFI) of P53, or the activation of the survival-promoting



phosphatidylinositol 3-kinase-AKT (Figures S3D and S3E). Given that BCL-XL regulates cell survival in the context of GATA factor-dependent erythroid cell differentiation (Gregory et al., 1999), we assessed BCL-XL expression in *Gata2* KO LSCs and *Gata2* haploinsufficient HSCs from both *Mx1-Cre* or *Vav-iCre* systems, but found that expression was unperturbed in all settings (Figures S3F, S3H, and S3J). In stark contrast, a significant reduction in the MFI of anti-apoptotic protein BCL2 was found only in *Gata2* KO LSCs (Figures 5B, 5C, S3G, and S3I). Thus, acute deletion of *Gata2* produces a rapid loss of LSCs, consistent with perturbed cell survival and mediated partly by attenuation of BCL2 expression. Furthermore, GATA2-mediated regulation of *Bcl2* in LSCs represents a novel GATA-dependent cell survival mechanism specific to LSCs rather than HSCs.

Having identified an enhanced myeloid differentiation gene signature in *Gata2* KO LSCs, we next asked whether this reflected GATA2-mediated alterations in myeloid differentiation of AML blasts *in vivo*. Using immunophenotyping, we analyzed the differentiation status of total BM from mice harboring *Gata2* KO LSCs at 7 days after the last pIpC injection (Figure 3F). A trend toward increased GR1 or MAC1 expression was observed in bulk AML cells derived from *Gata2* KO LSCs (Figure 5D), which mapped specifically to a significant increase in the abundance of GR1<sup>high</sup>MAC1<sup>high</sup> myeloid cells (Figures 5E–5F). Together with gene expression profiling data of *Gata2* KO LSCs, these data support the contention that ablation of *Gata2* in LSCs increases their differentiation capacity *in vivo*, raising the possibility that targeting the GATA2 differentiation affiliated transcriptional network in LSCs could pave the way toward novel therapeutics in AML.

## DISCUSSION

In this report, to explore the genetic requirement for *Gata2* in adult hematopoiesis, we employed a conditional gene-targeting approach using the *Mx1-Cre* system to specifically delete *Gata2* in hematopoietic cells from adult mice. Consistent with data using *ER-Cre*-mediated deletion of the *Gata2* C-terminal zinc-finger domain (Li et al., 2016), we demonstrate that acute genetic ablation of *Gata2* leads to a rapid and complete cell-autonomous loss of adult HSCs and multi-lineage potential. Therefore, *Gata2* joins a select list of TFs that are indispensable for adult HSC maintenance and survival (Hock et al., 2004; Kranc et al., 2009; Pajerowski et al., 2010). Loss-of-function studies in mice have identified various TFs required for distinct temporal and developmental stages of hematopoiesis, as typified by *Runx1*, which is required for HSC generation

from hemogenic endothelium, but expendable for HSC maintenance for the remainder of gestation and in adult HSCs in the BM (Chen et al., 2009). *Cited2*, a reported upstream regulator of *Gata2* in hematopoiesis (Saito et al., 2015), is a pivotal functional regulator of embryonic and adult HSCs, yet genetic KO models also highlight key stage-specific requirements during HSC development. For example, conditional genetic KO of *Cited2* in adult hematopoiesis using *Mx1-Cre* demonstrated the requirement for *Cited2* in adult HSC survival via *Ink4a/Arf* and *p53* (Kranc et al., 2009). In contrast, conventional germline deletion of *Cited2* produced isolatable HSCs at the FL stage, although *Cited2*<sup>-/-</sup> HSCs were impaired both in abundance and functionality (Chen et al., 2007). Here, in concert with data from de Pater et al. (2013), we importantly define *Gata2* as a unique TF that is necessary throughout HSC ontogenesis—from cell-autonomous HSC generation (using the *Vec-Cre* system) and HSC survival (using the *Vav-Cre* model) in the nascent blood system of the embryo to adult HSC maintenance in BM. *Evi1*, another upstream regulator of *Gata2* in hematopoiesis, mirrors the requirement of *Gata2* for HSC survival during development and in the adult (Goyama et al., 2008; Sato et al., 2008; Yuasa et al., 2005), although the explicit requirement for *Evi1* in HSC generation from hemogenic endothelium in the embryo remains untested and merits further investigation.

Using a lentiviral-based shRNA KD approach, we extended this study to examine the currently ill-defined requirement for GATA2 in human HSPCs. Mirroring data from the *Mx1-Cre* genetic mouse model, we observed that reducing GATA2 expression in human CB CD34<sup>+</sup> cells profoundly altered HSPC function, as assessed by CFC and LTC-IC assays. Together, these data identify GATA2 as a regulator of human HSPCs, which may bear relevance for understanding clinical GATA2 haploinsufficiency syndromes (Dickinson et al., 2011; Hahn et al., 2011; Katsumura et al., 2018; Soukup et al., 2019), in which the impact of GATA2 mutations in HSPCs remains unclear. Identifying and characterizing the genetic and epigenetic dependencies of specific HSPC population(s) transformed by loss-of-function GATA2 mutations and associated secondary mutations, such as *ASXL1*, may offer mechanistic insights into the wide range of complex pathologies and lineage-specific hematopoietic effects eventually leading to MDS/AML in GATA2 haploinsufficiency syndromes.

Considering that accumulating evidence also suggests an oncogenic function for GATA2 when it is overexpressed in pediatric and adult AML (Luesink et al., 2012; Vicente et al., 2012), we elected to interrogate the requirement for *Gata2* in LSCs by using an *in vivo* model of AML driven by *Meis1a/Hoxa9*. Genetic deletion of *Gata2* delayed *in vivo* AML development by impairing the maintenance and self-renewal of LSCs. Of note, gene expression patterns in



*Gata2* KO LSCs diametrically opposed those observed in the setting of either *Gata2* overexpression- or in *Gata2* haploinsufficiency-related models (Katayama et al., 2017; Nandakumar et al., 2015; Tipping et al., 2009). For example, lymphoid differentiation blockade and *c-Myc* expression activation has been observed in the context of *Gata2* overexpression studies (Nandakumar et al., 2015; Tipping et al., 2009), whereas *Gata2* KO LSCs display a gene signature consistent with reactivation of lymphoid differentiation and downregulation of MYC target genes. Similarly, in leukemic cells from *Gata2* haploinsufficiency-related models (Katayama et al., 2017), in which *Gata2* acts as a tumor suppressor rather than an oncogene, enrichment for oxidative phosphorylation and MYC target genes was observed, yet *Gata2* KO LSCs from *Meis1a/Hoxa9* AML demonstrate reduction in oxidative phosphorylation pathways. Thus, ablation of *Gata2* in LSCs pares down pro-oncogenic stimuli indicating the potential for therapeutic intervention in AML.

To this end, deletion of *Gata2* enhanced LSC apoptosis, enriched for a pro-apoptotic and myeloid differentiation signature in LSCs, and caused AML blast cell differentiation, suggesting that characteristic biological traits of AML—enhanced leukemic cell survival and a myeloid differentiation block—can be ameliorated through inhibition of GATA2 or targeting the GATA2 transcriptional network in LSCs. Whether direct, transient inhibition of GATA2 in LSCs is a possibility remains to be tested and is questionable given the pivotal role identified here for *Gata2* in adult hematopoiesis and the known tumor-promoting role of GATA2 in the context of haploinsufficiency. However, in favor of this proposition, the proteasome inhibitor K7174, which inhibits GATA2, has been found to be beneficial in the treatment of multiple myeloma (Imagawa et al., 2003; Kikuchi et al., 2013; Majik et al., 2012). We speculate that, alternatively, it may be feasible to selectively target the GATA2 transcriptional network in LSCs as GATA2 LSC target genes identified here were specific to LSCs and not HSCs (Iwasaki et al., 2015; Kikushige et al., 2010), which likely reflects differential transcriptional dependencies for *Gata2* in different cellular contexts (Rodrigues et al., 2005, 2008).

Our study suggests that the GATA2 pro-apoptotic transcriptional network in LSCs, exemplified by *Bcl2*, may be particularly susceptible to therapeutic targeting in AML, while sparing normal HSPCs (Lagadinou et al., 2013; Pollyea et al., 2018). Auspiciously, responses of BCL2 pharmacological inhibitor, venetoclax, in combination with standard AML chemotherapy agents, cytarabine, or hypomethylating agents, azacitidine and decitabine, are favorable compared with conventional induction therapy (Konopleva and Letai, 2018). Furthermore, venetoclax resistance in AML may be circumvented by using an inhibitor toward another BCL2 family member, MCL1 (Ramsey et al., 2018).

The characteristic myeloid differentiation block observed in AML has been overcome successfully in a subtype of AML, acute promyelocytic leukemia (APL), where a combination of all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO) has now become the mainstay of treatment for APL patients (Lallemand-Breitenbach and De Thé, 2013). Evidence suggests that the combination of ATRA and ATO cures APL by invoking myeloid differentiation in LSCs and their progeny, AML blasts, thereby precipitating LSC exhaustion (Werner et al., 2014; Zheng et al., 2007). However, attempts to replicate induction of myeloid differentiation in a similar manner across the genetic and clinically diverse AML subtypes have met with limited success (Sykes et al., 2016). We show here that ablation of *Gata2* in a non-APL AML model of LSCs overrides the myeloid differentiation block coupled to a depletion of LSC survival and self-renewal ability, presumably by promoting myeloid differentiation and apoptosis at the expense of self-renewing LSCs. Because LSCs are central to therapy resistance and relapse in AML (Craddock et al., 2013; Ishikawa et al., 2007; Saito et al., 2010), further investigation into how these LSC cell fates are coordinated and transcriptionally regulated by GATA2 should lead to an understanding of whether combinatorial therapies are needed to effectively exhaust the functionality of LSCs in AML.

Finally, the data presented here should also have direct relevance to cancer stem cell (CSC) biology beyond AML. Bearing in mind that LSCs serve as a paradigm for CSCs in other tissues, this study may hold significant importance for understanding GATA2-mediated transcriptional programming and developing clinical therapies against CSCs in prostate and lung cancer, in which GATA2 is overexpressed and linked to aggressiveness (Kumar et al., 2012; Vidal et al., 2015).

## EXPERIMENTAL PROCEDURES

### Mice

Gene targeting of *Gata2*<sup>f/f</sup> mice has been described previously (Charles et al., 2006), and mice were provided by Prof. Julian Downward. *Gata2*<sup>f/f</sup> mice were bred with *Mx1-Cre* and *Vav-iCre* mice (Vukovic et al., 2016). *Gata2* conditional gene deletion in the *Mx1-Cre* model was achieved by administration of six to eight intraperitoneal injections of pIpC every other day (0.25 mg per dose; GE Healthcare). All animals used for experiments were 8- to 12 weeks old in a C57BL/6 genetic background in accordance with UK Home Office regulations.

### *Meis1a/Hoxa9* Leukemic Transformation Assay

*Meis1a-puro* and *Hoxa9-neo* MSCV plasmids were used as described previously (Vukovic et al., 2015). Further information provided in Supplemental Experimental Procedures.



## Transplantation Assays

C57BL/6 SJL (CD45.1), or C57BL/6 (CD45.2) for niche experiment, adult recipient mice were irradiated with a split dose of 1,000 cGy ( $^{137}\text{Cs}$  source) 24 h before transplantation. Further information provided in [Supplemental Experimental Procedures](#).

## Flow Cytometry

Samples were analyzed with a BD LSRFortessa and sorting experiments were performed with a FACSAria Fusion (BD Biosciences). Antibodies used are listed in [Table S1](#). Further information provided in [Supplemental Experimental Procedures](#).

## CB CD34<sup>+</sup> HSPCs Isolation and Culture

Umbilical CB was obtained from full-term healthy pregnancies at the Maternity Unit of the University Hospital of Wales, Cardiff, following informed consent and approval from the South East Wales Research Ethics Committee in accordance with the 1964 Declaration of Helsinki. Further detailed information provided in [Supplemental Experimental Procedures](#).

## qRT-PCR and Western Blot

Further information provided in [Supplemental Experimental Procedures](#).

## RNA-Seq and Bioinformatics

RNA-seq was performed on CD45.2<sup>+</sup>c-kit<sup>+</sup> LSCs. Further information provided in [Supplemental Experimental Procedures](#).

## Statistical Analyses

Further information provided in [Supplemental Experimental Procedures](#).

## ACCESSION NUMBERS

The accession number for the *Gata2* LSC RNA-seq reported in this paper is GEO: GSE133245. The accession number for the *Gata2* HSC RNA-seq reported in this paper is GEO: GSE133248.

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.stemcr.2019.07.005>.

## AUTHOR CONTRIBUTIONS

J.B.M.-G. designed and performed the experiments, analyzed/interpreted the data, prepared figures, and contributed to the manuscript preparation. M.V. designed the experiments, analyzed/interpreted the data, and reviewed the manuscript. A.Abdelfattah performed the experiments and analyzed/interpreted the data. L.S., A.Almotiri, L.-A.T., A.A.-L., A.Azevedo, A.C.M., G.T., and S.E. performed the experiments. K.K. and P.G. performed bioinformatics analysis, F.A.-A. and A.T. contributed to the experimental design/analysis and reviewed the manuscript. A.S.B. and K.R.K. contributed significantly to experimental design, data analysis/interpretation and contributed to the manuscript preparation. K.R.K. funded M.V. and K.K. and contributed vital reagents. N.P.R. conceived and supervised the project, designed

the experiments, analyzed/interpreted the data, and wrote the manuscript.

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