

# *Gata2* is required for HSC generation and survival

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Knowledge of the key transcription factors that drive hematopoietic stem cell (HSC) generation is of particular importance for current hematopoietic regenerative approaches and reprogramming strategies. Whereas GATA2 has long been implicated as a hematopoietic transcription factor and its dysregulated expression is associated with human immunodeficiency syndromes and vascular integrity, it is as yet unknown how GATA2 functions in the generation of HSCs. HSCs are generated from endothelial cells of the major embryonic vasculature (aorta, vitelline, and umbilical arteries) and are found in intra-aortic hematopoietic clusters. In this study, we find that GATA2 function is essential for the generation of HSCs during the stage of endothelial-to-hematopoietic cell transition. Specific deletion of *Gata2* in *Vec* (*Vascular Endothelial Cadherin*)-expressing endothelial cells results in a deficiency of long-term repopulating HSCs and intra-aortic cluster cells. By specific deletion of *Gata2* in *Vav*-expressing hematopoietic cells (after HSC generation), we further show that GATA2 is essential for HSC survival. This is in contrast to the known activity of the RUNX1 transcription factor, which functions only in the generation of HSCs, and highlights the unique requirement for GATA2 function in HSCs throughout all developmental stages.

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Abbreviations used: AGM, aorta-gonad-mesonephros; CFU-C, colony-forming unit culture; DA, dorsal aorta; EHT, endothelial-to-hematopoietic transition; FL, fetal liver; HPC, hematopoietic progenitor cell; HSC, hematopoietic stem cell; sp, somite pair; YS, yolk sac.

The permanent adult hematopoietic system initiates with the formation of hematopoietic stem cells (HSCs) (Dzierzak and Speck, 2008). Expansion of HSCs in culture and reprogramming somatic cells into HSCs are as yet not possible and, thus, necessitate an understanding of the molecular programs directing the generation of HSCs. At the time of HSC generation in the mouse aorta-gonad-mesonephros (AGM) region (embryonic day [E] 10.5), clusters of hematopoietic cells are found closely associated with the ventral wall of the dorsal aorta and along the other major arteries (vitelline and umbilical; de Bruijn et al., 2000, 2002; North et al., 2002; Taoudi and Medvinsky, 2007; Zovein et al., 2008; Boisset et al., 2010; Yokomizo and Dzierzak, 2010). The *Gata2* transcription factor is expressed in the mouse embryo in a

pattern consistent with a role in hematopoietic cell development (Minegishi et al., 1999; Robert-Moreno et al., 2005). It is first expressed at E7.5 in the primitive streak and the endothelial cells of the paired dorsal aorta. Later, *Gata2* is expressed in endothelial cells lining the dorsal aorta, vitelline, and umbilical arteries and in the intra-arterial cluster cells at the time of definitive hematopoietic progenitor cell (HPC) and HSC formation. *Gata2* is also expressed in hematopoietic cells of the yolk sac (YS), fetal liver (FL), and placenta (PL) and in adult BM HSCs (Ng et al., 1994; Orlic et al., 1995; Minegishi et al., 1999; Nardelli et al., 1999; Robert-Moreno et al., 2005).

Germline *Gata2*<sup>-/-</sup> embryos suffer from FL anemia and die at E10, just before the appearance of the first HSCs, and *Gata2*<sup>-/-</sup> ES cells do not

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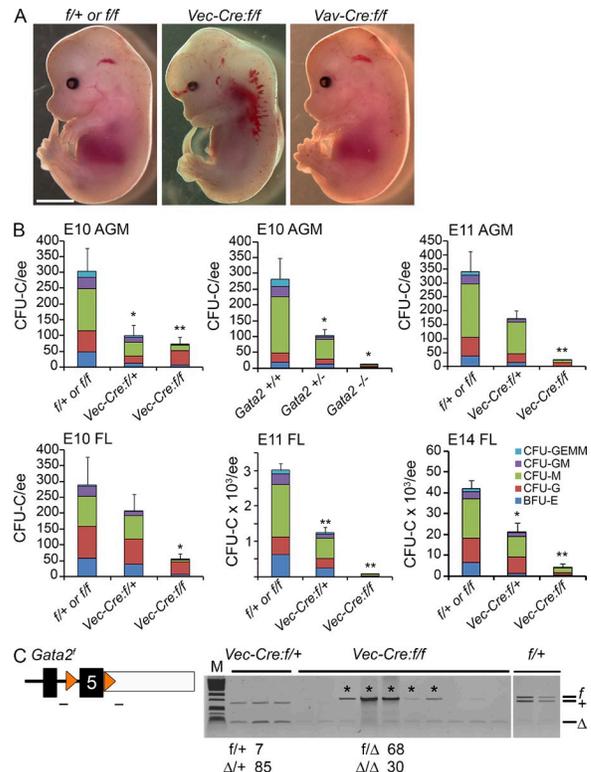
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contribute to definitive hematopoiesis in mouse chimeras (Ng et al., 1994; Tsai et al., 1994; Orlic et al., 1995; Tsai and Orkin, 1997; Minegishi et al., 1999; Nardelli et al., 1999; Robert-Moreno et al., 2005). Haploinsufficient *Gata2*<sup>+/-</sup> mice are severely affected in the production of early progenitors (Tsai et al., 1994), and *Gata2*<sup>+/-</sup> BM HSCs are qualitatively defective in serial transplantation assays (Ling et al., 2004; Rodrigues et al., 2008). When crossed with *Ly6A-GFP* mice as a marker for emerging hematopoietic cells, GFP<sup>+</sup> hematopoietic cells are decreased in the E11 AGM region of *Gata2*<sup>+/-</sup> embryos (Ling et al., 2004). Recent studies of the *Gata2* intron 4 enhancer show that FL HSCs are affected when *Gata2* is dysregulated (Johnson et al., 2012; Lim et al., 2012). Yet, it is unknown whether GATA2 is required in the endothelial compartment for the formation of intra-arterial clusters and AGM HSCs, and what the function of GATA2 is thereafter. Furthermore, GATA factors are described to act through combinatory interactions with other key regulators, including RUNX1 (Wilson et al., 2010; van Riel et al., 2012). Previously, RUNX1 was shown to be required for the generation of HSCs during the endothelial-to-hematopoietic transition (EHT). Although RUNX1 continues to be expressed, it is not required after HSCs are made (Chen et al., 2009).

Taking a conditional KO (cKO) approach to examine when GATA2 is required, we deleted a floxed allele of *Gata2* with *Vec-Cre* in *VE-Cadherin*-expressing endothelial cells before the generation of HSCs and with *Vav-Cre* in hematopoietic cells after the generation of HSCs (Stadtfeld and Graf, 2005; Charles et al., 2006; Chen et al., 2009). Using the same approach as was used for the conditional deletion of *Runx1*, we conclude that the developmental requirements for GATA2 and RUNX1 differ during the generation of HPCs, and although both transcription factors are required for the generation of HSCs, only GATA2 is further required for HSC survival.

## RESULTS AND DISCUSSION

Unlike germline *Gata2*<sup>-/-</sup> embryos, which die at E10, conditional *Vec-Cre:Gata2*<sup>fl/fl</sup> embryos survive until E14 and show FL anemia (Fig. 1 A). This phenotype is similar to embryos with *Gata2* deficiencies directed by the mutation/deletion of the *Gata2* intron 4 enhancer (Johnson et al., 2012; Lim et al., 2012). To examine whether HPC generation and/or function is affected, cells from the AGM and FL of *Vec* cKO embryos were tested in the colony-forming unit culture (CFU-C) assay. Already at E10, HPC numbers in the AGM (including vitelline and umbilical arteries) of *Vec-Cre:Gata2*<sup>fl/fl</sup> and *Vec-Cre:Gata2*<sup>fl/fl</sup> embryos were decreased by 3-fold ( $P < 0.05$ ) and 3.5-fold ( $P < 0.01$ ), respectively, as compared with WT. The decrease in HPCs in the heterozygous cKO AGM corresponds to the decrease observed in germline *Gata2*<sup>+/-</sup> AGMs (Fig. 1 B and Table 1). However, homozygous cKO HPC numbers were not decreased to the level observed in germline *Gata2*<sup>-/-</sup> AGMs (25-fold). Individual colonies were picked from methylcellulose plates and DNA was examined for recombination by PCR. Colonies showed a frequency of 92% recombination for one *Gata2* floxed allele. Surprisingly, 31% of colonies



**Figure 1. GATA2 is required in VE-Cadherin-expressing cells for hematopoietic progenitor formation.** (A) Bright field images of E14 WT, *Vec-Cre:Gata2*<sup>fl/fl</sup>, and *Vav-Cre:Gata2*<sup>fl/fl</sup> embryos. Bar, 2 mm. (B) CFU-C numbers per AGM and FL from *Gata2*-deleted embryos. Germline *Gata2*<sup>-/-</sup> AGMs at E10 (30–34 sp),  $n = 4$ . The number of embryos analyzed is 8 WT, 11 *Gata2*<sup>+/-</sup>, and 4 *Gata2*<sup>-/-</sup>. For *Vec-Cre*-mediated *Gata2* deletion: WT, *Vec-Cre:Gata2*<sup>fl/fl</sup>, and *Vec-Cre:Gata2*<sup>fl/fl</sup> AGMs and FLs at E10 (30–36 sp), E11 (43–48 sp), and FL E14,  $n = 2$ . The number of E10 embryos analyzed is 5 WT, 6 *Vec-Cre:Gata2*<sup>fl/fl</sup>, and 6 *Vec-Cre:Gata2*<sup>fl/fl</sup>;  $n = 3$ . The number of E11 embryos analyzed is 6 WT, 3 *Vec-Cre:Gata2*<sup>fl/fl</sup>, and 4 *Vec-Cre:Gata2*<sup>fl/fl</sup>; E14 FL  $n = 3$ . The number of embryos analyzed is 11 WT, 3 *Vec-Cre:Gata2*<sup>fl/fl</sup>, and 5 *Vec-Cre:Gata2*<sup>fl/fl</sup>. Error bars are mean  $\pm$  SEM of total colony numbers. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , significance determined by Student's *t* test. ee, embryo equivalent of specified tissue. (C) Schematic representation of the *Gata2* locus with *Loxp* sites and genotyping PCR primer location. *Loxp* sites are indicated by orange triangles. Gel showing the *Gata2* flox band of 844 bp (f) deleted ( $\Delta$ ) band of 181 bp and WT *Gata2* band (+) of 717 bp for individual CFU-C. The number of identified genotypes of individual colonies is shown beneath the gel.

deleted the second allele (Fig. 1 C), indicating that some progenitors are *Gata2* independent. This is underscored by the finding that the germline *Gata2*<sup>-/-</sup> AGMs still contain some CFU-C activity (Fig. 1 B and Table 1). At E11, *Vec-Cre:Gata2*<sup>fl/fl</sup> AGMs showed a more profound 15-fold ( $P < 0.01$ ) reduction of CFU-C compared with WT. Thus, most AGM HPCs require GATA2 in *VE-Cadherin*-expressing cells during the time of hematopoietic cluster generation (80% at E10 and 95% at E11).

Similar decreases in HPCs were found in the *Vec-Cre:Gata2*<sup>fl/fl</sup> FL. At E10, E11, and E14, FL HPCs were significantly reduced by a factor of 5, 45, and 10, respectively, compared

**Table 1.** CFU-C numbers in *Gata2*-deficient embryos

Tissue	Stage	Genotype		
		<i>Gata2</i> <sup>+/+</sup>	<i>Gata2</i> <sup>+/-</sup>	<i>Gata2</i> <sup>-/-</sup>
AGM	E10 (30-34sp)	282.2 ± 64.4	103.6 ± 19.1 (P < 0.05)	11.2 ± 2.7 (P < 0.05)
YS	E10 (30-34sp)	918.6 ± 147.9	584.5 ± 72.4	25.7 ± 5.3 (P < 0.01)
		<i>Gata2</i> <sup>fl/+</sup> or <i>Gata2</i> <sup>fl/fl</sup>	<i>Vec-Cre:Gata2</i> <sup>fl/+</sup>	<i>Vec-Cre:Gata2</i> <sup>fl/fl</sup>
AGM	E10 (30-36sp)	336.0 ± 72.3	99.0 ± 33.5 (P < 0.05)	72.8 ± 21.5 (P < 0.01)
FL	E10 (30-36sp)	1,158 ± 35.0	83.0 ± 20.8	22.5 ± 22.2 (P < 0.05)
YS	E10 (30-36sp)	1,132.0 ± 67.2	388.8 ± 93.0 (P < 0.01)	70.8 ± 16.0 (P < 0.01)
AGM	E11 (43-48sp)	340.0 ± 73.0	172.0 ± 28.9	22.5 ± 3.4 (P < 0.01)
FL	E11 (43-48sp)	3,015.0 ± 186.1	1,234.0 ± 156.1 (P < 0.01)	66.8 ± 12.6 (P < 0.01)
FL	E14	42,155.3 ± 3,813.5	21,320.8 ± 4,359.2 (P < 0.05)	4,068.1 ± 2,068.3 (P < 0.01)
		<i>Gata2</i> <sup>fl/+</sup> or <i>Gata2</i> <sup>fl/fl</sup>	<i>Vav-Cre:Gata2</i> <sup>fl/+</sup>	<i>Vav-Cre:Gata2</i> <sup>fl/fl</sup>
AGM	E11 (41-49sp)	456.0 ± 73.1	305.6 ± 35.3	170.5 ± 54.3 (P < 0.05)
FL	E11 (41-49sp)	2,950.0 ± 244.3	2,754.2 ± 173.9	1,249.1 ± 231.4 (P < 0.01)
FL	E14	43,320.2 ± 5,359.6	13,048.1 ± 3,647.0 (P < 0.01)	7,495.1 ± 3,847.1 (P < 0.01)

with WT. The FL of E14 *Vec-Cre:Gata2*<sup>fl/fl</sup> embryos contained 3.4× fewer cells ( $14.9 \pm 4.3 \times 10^6$  WT and  $4.3 \pm 1.6 \times 10^6$  *Vec-Cre:Gata2*<sup>fl/fl</sup> [P = 0.06]). Although all CFU-C types were decreased in number, the CFU-M and CFU-G colonies formed from *Vec-Cre:Gata2*<sup>fl/fl</sup> tissues were smaller and more compact than WT colonies (unpublished data), indicating a role for *Gata2* after the generation of macrophage and granulocyte progenitors, most likely affecting their differentiation and proliferation. These data are consistent with defects found in macrophage and granulocyte progenitors in germline haploinsufficient *Gata2*<sup>+/-</sup> adult BM (Rodrigues et al., 2008). The growth, size, and differentiation of the other hematopoietic colony types in *Vec-Cre:Gata2*<sup>fl/fl</sup> tissues was unaffected.

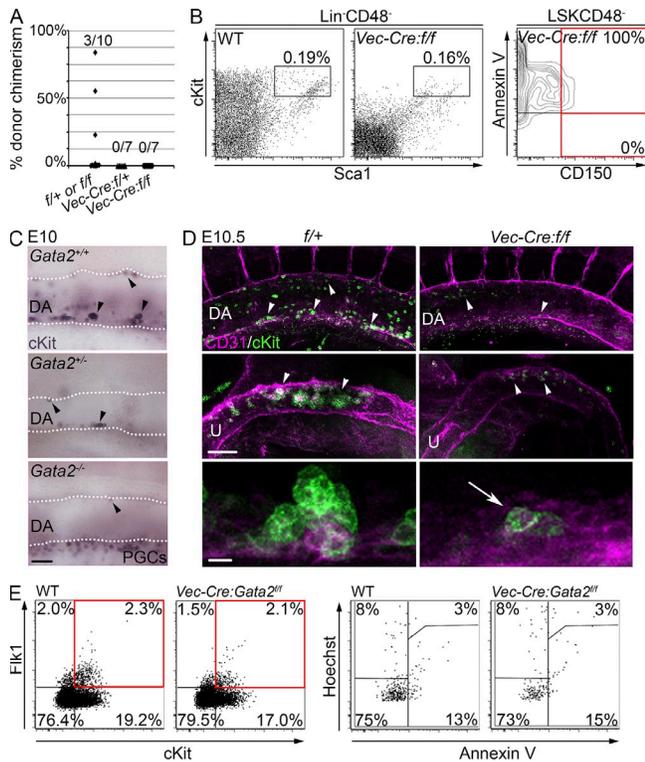
To test whether loss of *Gata2* in *VE-Cadherin*-expressing cells affects HSC generation, in vivo transplantation experiments were performed. E11 AGM cells were injected into irradiated adult recipients and assayed at 1, 2, 3, and 4 mo after transplantation. In contrast to WT controls, no recipients receiving *Vec-Cre:Gata2*<sup>fl/fl</sup> (or *Vec-Cre:Gata2*<sup>fl/+</sup>) cells showed donor repopulation in any hematopoietic tissue or lineage (Fig. 2 A), indicating that GATA2 is required in *VE-Cadherin*-expressing cells for the generation of functional adult repopulating HSCs.

Next, flow cytometric and immunohistochemical analyses were performed for the specific detection of HPC/HSCs in embryonic tissues and the vasculature; the presence of such phenotypic HSCs in the *Vec-Cre:Gata2*<sup>fl/fl</sup> embryos would indicate that GATA2 is essential for HPC/HSC function but not for their generation. It has been shown previously in WT embryos that E14 FL HSCs are Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup>CD48<sup>-</sup>CD150<sup>+</sup> (or LSK SLAM; Kim et al., 2006). Flow cytometric analysis of *Vec-Cre:Gata2*<sup>fl/fl</sup> E14 FL showed a severe reduction of LSK cells and a complete absence of viable LSK SLAM cells as compared with WT FL (Fig. 2 B; n = 4, P < 0.05). To visually examine whether vascular hematopoietic cluster cells were affected, embryo immunostaining was performed with anti-cKit antibody alone (Fig. 2 C) or with anti-cKit and anti-CD31 antibodies (Fig. 2 D). Imaging of an E10 *Gata2*<sup>-/-</sup> embryo

(30–somite pairs [sp] stage) revealed only 3 cKit<sup>+</sup> cells in the aorta, as compared with 25 in a *Gata2*<sup>+/-</sup> and 213 in a WT aorta. At the peak period of hematopoietic cluster formation in the embryo (E10.5), 38 ± 18 cKit<sup>+</sup> cluster cells in the *Vec-Cre:Gata2*<sup>fl/fl</sup> aorta were observed, as compared with 634 ± 43 cKit<sup>+</sup> cells in the WT aorta (Fig. 2 D, top right and left; n = 2, P < 0.01). Similar decreases in cluster numbers were found in the umbilical artery (Fig. 2 D, middle). Interestingly, cluster size was reduced to 1 or 2 cells, with the remaining cKit<sup>+</sup> cells of the *Vec-Cre:Gata2*<sup>fl/fl</sup> aorta showing a flat morphology, embedded within the endothelium (Fig. 2 D, bottom right). Also at E11, *Vec-Cre:Gata2*<sup>fl/fl</sup> AGMs contained far fewer cKit<sup>+</sup> cells in the dorsal aorta than WT embryos (58 in *Vec-Cre:Gata2*<sup>fl/fl</sup> versus 411 in WT; unpublished data). These data suggest that either hematopoietic cluster cells are not generated or they are apoptotic in *Gata2* cKO embryos.

To distinguish between these possibilities, we stained E11 AGMs with Annexin V to measure apoptotic status, and with anti-Flk1 and anti-cKit antibodies to identify endothelial cells and the cells at the base of emerging hematopoietic clusters (Yokomizo and Dzierzak, 2010; Fig. 2 E and Table 2). Flk1<sup>+</sup>cKit<sup>+</sup> cells showed no increase in Annexin V staining compared with WT. Normal numbers of endothelial cells (cKit<sup>-</sup>CD31<sup>+</sup> or cKit<sup>-</sup>Flk1<sup>+</sup>) were found in the AGM region of *Vec-Cre:Gata2*<sup>fl/fl</sup> embryos (not depicted and Table 2). Because we do not find a difference in viability of Flk1<sup>+</sup>cKit<sup>+</sup> cells in *Vec-Cre:Gata2*<sup>fl/fl</sup> aortas as compared with WT, we conclude that GATA2 is essential in the EHT for the generation of hematopoietic cluster cells and HSCs.

The fact that *Vav* is expressed in HSCs and their progeny (Ogilvy et al., 1999), but not in endothelial cells, allowed Chen et al. (2009) to demonstrate that the RUNX1 transcription factor is required during, but not after, the generation of HSCs. The effects of *Gata2* deletion on HPCs and HSCs after their generation were tested using this *Vav-Cre* model (Stadtfield and Graf, 2005). In contrast to *Vec-Cre:Gata2*<sup>fl/fl</sup> embryos, *Vav-Cre:Gata2*<sup>fl/fl</sup> embryos showed no FL anemia at E14 (Fig. 1 A;



**Figure 2. GATA2 is required in VE-Cadherin-expressing cells for the generation of HSCs and vascular hematopoietic cluster cells.**

(A) Graph showing the percentage of peripheral blood donor cell chimerism in adult recipients at 4 mo after transplantation. Recipients were injected with AGM cells from WT (1–2 ee; 3 of 10 recipients repopulated,  $n = 3$ ), *Vec-Cre:Gata2<sup>fl/fl</sup>* (1–2 ee; 0 of 7 recipients repopulated), or *Vec-Cre:Gata2<sup>fl/fl</sup>* (1–3 ee; 0 of 7 recipients repopulated) E11 embryos. Reconstitution kinetics showed similar outcomes at 1, 2, 3, and 4 mo after transplantation. (B) FACS analysis of Lin<sup>-</sup>CD48<sup>+</sup> gated, cKit<sup>-</sup> and Sca1<sup>-</sup> stained E14 FL cells from WT and *Vec-Cre:Gata2<sup>fl/fl</sup>* embryos. Percentage of cKit<sup>+</sup>Sca1<sup>+</sup> cells within the Lin<sup>-</sup>CD48<sup>+</sup> population is shown for WT ( $0.019 \pm 0.01\%$ ) and *Vec-Cre:Gata2<sup>fl/fl</sup>* ( $0.16 \pm 0.04\%$ ,  $P < 0.05$ ). The right panel shows FACS analysis of LSKCD48<sup>+</sup> gated, CD150<sup>-</sup> and Annexin V<sup>-</sup> stained *Vec-Cre:Gata2<sup>fl/fl</sup>* E14 FLs. Phenotypic HSCs are indicated within the red quadrants. 100% of LSK SLAM cells are Annexin V<sup>-</sup> positive ( $n = 2$ , number of embryos analyzed = 7 WT, 5 *Vec-Cre:Gata2<sup>fl/fl</sup>*; significance determined by Student's *t* test). (For WT, see Fig. 3 E.) (C) Immunostaining of E10 (30/31 sp) embryos showing cKit<sup>+</sup> hematopoietic cluster cells in germline *Gata2<sup>+/+</sup>*, *Gata2<sup>+/-</sup>*, and *Gata2<sup>-/-</sup>* aortae. Arrowheads indicate some of the hematopoietic cluster cells along the aortic wall. Bar, 50  $\mu$ m. DA = dorsal aorta, PGC = primordial germ cells ( $n = 1$ ). (D) Whole mount immunostaining of E10.5 (36/37 sp) *Gata2<sup>+/+</sup>* and *Vec-Cre:Gata2<sup>fl/fl</sup>* embryos showing CD31<sup>+</sup> vascular endothelial cells (magenta) and cKit<sup>+</sup> hematopoietic cluster cells (green). The top panels show the dorsal aorta (DA), middle panels the umbilical artery (U), and bottom panels a high-magnification image of a cKit<sup>+</sup> cluster and aortic endothelium. Arrowheads indicate a few cKit<sup>+</sup> hematopoietic cluster cells and arrow indicates a flat cKit<sup>+</sup> cell embedded in the endothelium ( $n = 2$ , number of embryos analyzed is 2 WT and 2 *Vec-Cre:Gata2<sup>fl/fl</sup>*; significance determined by Student's *t* test). Bars: (top and middle) 100  $\mu$ m; (bottom) 10  $\mu$ m. (E) FACS analysis showing Flk1<sup>-</sup> and cKit<sup>+</sup> stained WT and *Vec-Cre:Gata2<sup>fl/fl</sup>* E11 AGMs. Flk1<sup>+</sup> cKit<sup>+</sup> cells (red quadrant) and gating strategy for the viability (right) are shown. Hoechst<sup>-</sup>Annexin V<sup>-</sup> cells are viable, Hoechst<sup>-</sup>Annexin V<sup>+</sup> are early apoptotic cells, Hoechst<sup>+</sup>Annexin V<sup>+</sup> are late apoptotic cells, and Hoechst<sup>+</sup>Annexin V<sup>-</sup> cells are dead ( $n = 3$ ;

$15.8 \pm 3.5 \times 10^6$  WT and  $13.2 \pm 1.6 \times 10^6$  [ $P = 0.4$ ]) and survived past E16. Only 2 *Vav-Cre:Gata2<sup>fl/fl</sup>* offspring were born out of 36 pups in 6 litters (6%). Because both pups showed an incomplete deletion of the floxed *Gata2* alleles (unpublished data), these results suggest that *Vav-Cre*-mediated deletion of *Gata2* affects HPC/HSCs after their generation.

E11 *Vav-Cre:Gata2<sup>fl/fl</sup>* AGMs and FLs were significantly decreased in CFU-C numbers (2.7- and 2.4-fold), as compared with WT tissues (Fig. 3 A and Table 1). The observed decreases were not as severe as those in the *Vec-Cre* cKO tissues, and this is underscored by FACS analysis. The number of phenotypically defined CD31<sup>+</sup>cKit<sup>high</sup>CD41<sup>int</sup> HPC/HSCs in E11 *Vav-Cre:Gata2<sup>fl/fl</sup>* AGMs was not significantly altered from WT and corresponded to previously published numbers (Sánchez et al., 1996; Yokomizo and Dzierzak, 2010; Robin et al., 2011; Boisset et al., 2013). Moreover, cluster numbers in *Vav-Cre* cKO vessels were normal at E11, as compared with WT (unpublished data). Interestingly, whereas no significant differences in total cell numbers of E14 FLs were found, both E14 *Vav-Cre:Gata2<sup>fl/fl</sup>* and *Vav-Cre:Gata2<sup>fl/fl</sup>* FLs contained significantly fewer CFU-Cs than WT FLs (3.3- and 5.8-fold reduction, respectively; Fig. 3 A and Table 1). Indeed, flow cytometric analysis of *Vav-Cre:Gata2<sup>fl/fl</sup>* E14 FLs showed that phenotypic HSCs, defined by LSK SLAM staining, were reduced by a factor of two, as compared with WT (Fig. 3 B and Table 3). In most *Vav-Cre:Gata2<sup>fl/fl</sup>* E14 FLs, Lin<sup>-</sup>cKit<sup>high</sup> cell numbers are reduced (not depicted) and LSK SLAM cells were almost completely absent (Table 3). Additionally, whereas  $58.4 \pm 8.4\%$  of WT LSK SLAM cells were capable of forming a CFU-C ( $7.9 \pm 2.1\%$  CFU-GEMM), only  $31.6 \pm 6.7\%$  of *Vav-Cre:Gata2<sup>fl/fl</sup>* LSK SLAM cells were capable of forming CFU-C ( $2.3 \pm 1.5\%$  CFU-GEMM), and none of the *Vav-Cre:Gata2<sup>fl/fl</sup>* LSK SLAM cells resulted in CFU-Cs ( $P < 0.01$ ; Fig. 3 C). These results indicate that GATA2 is essential for the hematopoietic progenitor activity of phenotypic HSCs. Furthermore, E14 *Vav-Cre:Gata2<sup>fl/fl</sup>* FL cells did not long-term reconstitute the hematopoietic system of irradiated adult recipients (Fig. 3 D,  $P < 0.01$ ). Two recipients were found with *Vav-Cre:Gata2<sup>fl/fl</sup>* donor cell chimerism, but these donor cells retained at least one unrecombined floxed allele (as detected in the recipient peripheral blood and the donor FL cells; unpublished data). Thus, GATA2 is essential in HSCs after their formation.

Transplantation and CFU assays do not provide insight into the processes affected by deletion of *Gata2* after formation of HSCs because both require the cells to extensively proliferate and to be viable for 10 d to 4 mo. Therefore, we postulated that the reduction in the number and function of *Vav-Cre:Gata2<sup>fl/fl</sup>* HPC/HSCs in the FL may be due to defective cell amplification and/or lack of cell maintenance/survival. To check whether the loss of *Gata2* affects the cell cycle status of HPC/HSCs, Ki67 staining was performed on E14 FL (total and Lin<sup>-</sup>cKit<sup>high</sup>) cells. As expected, more cells in the S/G<sub>2</sub>/M

number of embryos analyzed = 7 WT and 6 *Vec-Cre:Gata2<sup>fl/fl</sup>*; significance determined by Student's *t* test).

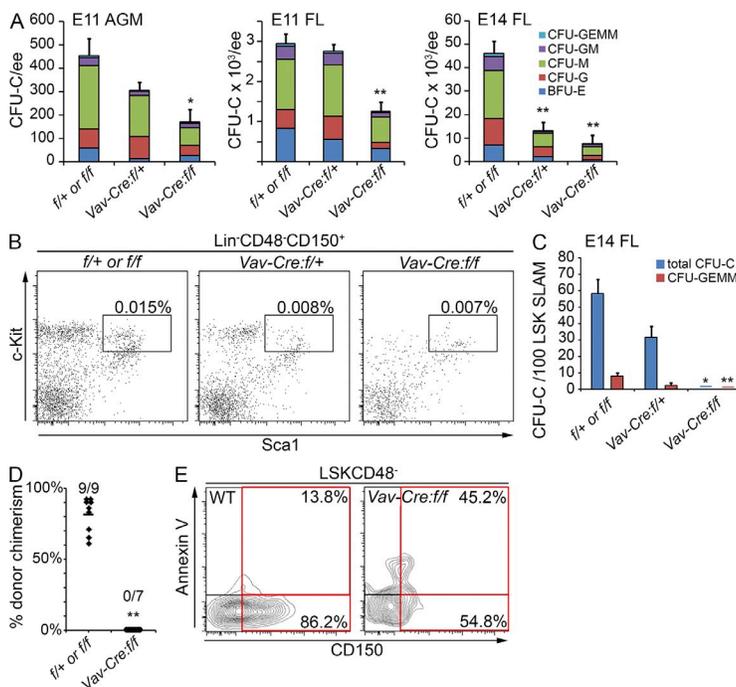
**Table 2.** Apoptosis analysis of *Gata2* E11 AGM and FL cells

Genotype/apoptotic status	AGM			FL	
	Flk1 <sup>+</sup> cKit <sup>-</sup> endothelial cells	Flk1 <sup>+</sup> cKit <sup>+</sup> cluster base cells	Flk1 <sup>-</sup> cKit <sup>+</sup> hematopoietic cells	CD31 <sup>+</sup> cKit <sup>+</sup> HPCs/HSCs	CD31 <sup>-</sup> cKit <sup>+</sup> hematopoietic cells
	%	%	%	%	%
<i>Gata2</i> <sup>f/+</sup> or <i>Gata2</i> <sup>f/f</sup> (WT)	Total: 2.0 ± 0.6	Total: 2.3 ± 0.6	Total: 19.2 ± 3.4	Total: 60.2 ± 4.2	Total: 32.4 ± 4.2
Viable	76	76	77	75	2
Early apoptotic	9	13	5	5	1
Late apoptotic	1	3	6	7	21
Dead	14	8	12	11	76
<i>Vec-Cre:Gata2</i> <sup>f/f</sup>	Total: 1.5 ± 0.3	Total: 2.1 ± 0.7	Total: 17.0 ± 2.3	Total: 22.1 ± 0.4**	Total: 68.6 ± 1.4**
Viable	76	74	74	30**	1
Early apoptotic	8	15	6	15	1
Late apoptotic	2	3	7	20	18**
Dead	14	8	12	35	80**

Results of flow cytometric analyses with various cell subset specific markers (Flk1, cKit, and CD31) show the percentage of cells within total populations of E11 AGM or E11 FL (Total). Apoptotic status of cells within these gated populations (cell subsets) was tested by Annexin V and Hoechst staining and is shown below the percentage of the total population. Viable cells are Annexin V<sup>-</sup>Hoechst<sup>-</sup>, early apoptotic are Annexin V<sup>+</sup>Hoechst<sup>-</sup>, late apoptotic are Annexin V<sup>+</sup>Hoechst<sup>+</sup>, and dead cells are Annexin V<sup>-</sup>Hoechst<sup>+</sup>. *n* = 3. Numbers of embryos analysed = 7 WT and 7 *Vec-Cre:Gata2*<sup>f/f</sup>. Significance was determined by Student's *t* test. \*\*, *P* < 0.01.

phase were found in the Lin<sup>-</sup>cKit<sup>high</sup> enriched FL HPC/HSC population than in the total FL population. However, no alteration in cell cycle could be detected between WT and *Vav-Cre:Gata2*<sup>f/f</sup> FL cells (Table 3). We also tested the survival of phenotypic HSCs in the E14 FL by Annexin V staining.

A significant threefold increase in apoptotic cells was found in *Vav-Cre:Gata2*<sup>f/f</sup> E14 FL LSK SLAM cells as compared with WT LSK SLAM FL (*P* < 0.05), demonstrating that GATA2 is required in HSCs during the FL stage for their survival (Fig. 3 E). This result is underscored by the finding of Linnemann et al.



**Figure 3. *Gata2* is required in HPCs and in HSCs for survival after their generation.** (A) Graphs showing the CFU-C numbers per WT, *Vav-Cre:Gata2*<sup>f/+</sup>, or *Vav-Cre:Gata2*<sup>f/f</sup> embryo equivalent (ee) of AGM and FL cells. Error bars indicate mean ± SEM. \*, *P* < 0.05; \*\*, *P* < 0.01. The number of E11 AGMs and FLs analyzed is 11 WT, 12 *f/+*, and 6 *f/f*; *n* = 4. For E11 AGM, *P* < 0.05, and E11 FL, *P* < 0.01. For E14 FL (*P* < 0.01; *n* = 3), the number of embryos analyzed = 10 WT, 5 *f/+*, and 20 *f/f*. Significance was determined by Student's *t* test. (B) FACS analysis of WT, *Vav-Cre:Gata2*<sup>f/+</sup>, and *Vav-Cre:Gata2*<sup>f/f</sup> E14 FL for LSK SLAM markers. Lin<sup>-</sup>CD48<sup>-</sup>CD150<sup>+</sup> gated cells were analyzed for cKit and Sca1. Percentages of LSK SLAM cells (gated region) are shown. (C) Graph of the percentage of total CFU-C and CFU-GEMM per 100 LSK SLAM cells of each genotype (*n* = 3). The number of embryos analyzed = 3 WT, 3 *f/+*, and 3 *f/f*. Error bars are mean ± SEM of total colony numbers and of CFU-GEMM only. \*, *P* < 0.05; \*\*, *P* < 0.01; significance determined by Student's *t* test. (D) Graph showing the percentage of peripheral blood donor cell chimerism in adult recipients injected with 10<sup>5</sup> WT (*n* = 2; 9 of 9 recipients repopulated) or 10<sup>5</sup> *Vav-Cre:Gata2*<sup>f/f</sup> E14 FL cells (0 of 7 recipients repopulated, *P* < 0.01). Diamonds represent individual recipients, with the mean represented by a horizontal bar. Significance was determined by Student's *t* test. (E) Annexin V FACS analysis of WT and *Vav-Cre:Gata2*<sup>f/f</sup> E16 FL LSK SLAM cells. LSKCD48<sup>-</sup> gated cells were analyzed for Annexin V and CD150 (*n* = 2). The number of embryos analyzed = 3 WT and 6 *f/f*. Phenotypic HSCs (red quadrant) and the percentages of apoptotic and viable cells are indicated. (Not depicted: for E14 FL *n* = 3; the number of embryos analyzed = 8 WT, 10 *f/+*, and 9 *f/f*.)

**Table 3.** Analysis of *Gata2* conditional knockout E14 FL

E14 FL cell subset/cell type	<i>Gata2</i> <sup>fl/+</sup> or <i>Gata2</i> <sup>fl/fl</sup>	<i>Vec-Cre:Gata2</i> <sup>fl/+</sup>	<i>Vec-Cre:Gata2</i> <sup>fl/fl</sup>
	%	%	%
<b>Differentiated cells</b>			
T cells	5.1 ± 1.6	4.5 ± 1.5	3.3 ± 1.1
B cells	6.5 ± 1.4	7.5 ± 2.5	5.9 ± 1.5
Erythroblasts	61.7 ± 3.0	62.6 ± 1.3	61.9 ± 3.1
Erythrocytes	28.7 ± 3.1	30.3 ± 1.4	33.2 ± 3.3
LSK-SLAM	0.0150 ± 0.0021	0.0080 ± 0.0011 (P < 0.05)	0.0066 ± 0.0017 (P < 0.01)
<b>Total</b>			
G <sub>0</sub>	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.3
G <sub>1</sub>	44.3 ± 1.8	50.8 ± 2.7	44.8 ± 2.0
S/G <sub>2</sub> /M	54.5 ± 3.2	48.1 ± 2.6	54.1 ± 2.1
<b>Lin<sup>-</sup>cKit<sup>high</sup></b>			
G <sub>0</sub>	0.1 ± 0.03	0.2 ± 0.1	0.2 ± 0.05
G <sub>1</sub>	31.5 ± 1.12	36.4 ± 3.9	35.5 ± 2.4
S/G <sub>2</sub> /M	67.9 ± 1.10	63.1 ± 3.8	63.9 ± 2.3
Annexin <sup>+</sup>	13.8 ± 5.2	19.1 ± 5.1	45.2 ± 16.0 (P < 0.05)

Values for differentiated cells and LSK-SLAM are percentage of viable cells. Values for Total and Lin<sup>-</sup>cKit<sup>high</sup> are percentage of single cells. Values for Annexin<sup>+</sup> are percentage of viable cells including pre-apoptotic. For the analysis of differentiated cells, *n* = 2 and number of embryos analyzed = 7, 4, and 8, respectively by genotype; LSK-SLAM, *n* = 5 and number of embryos analyzed = 12, 10, and 13, respectively; total and Lin<sup>-</sup>cKit<sup>high</sup>, *n* = 2 and number of embryos analyzed = 7, 3, and 6; and LSK-SLAM Annexin<sup>+</sup>, *n* = 3 and number of embryos analyzed = 8, 10, and 9. Significance was determined by Student's *t* test. ND = not done.

(2011) that the anti-apoptotic gene *Birc3* was down-regulated upon knockdown of *Gata2* in human endothelial cells.

Our results show for the first time that GATA2 is required for HSC generation in the AGM and continues to be required for HSC survival. GATA2 is thought to act in combination with other transcription factors, such as RUNX1, to promote/enhance transcription of genes relevant to hematopoietic cell development and growth (Wilson et al., 2010; van Riel et al., 2012). Because we used the *Vec-Cre* and *Vav-Cre* cKO approach used previously for *Runx1* deletion (Chen et al., 2009), the requirements for these two transcription factors can be directly compared. Whereas the RUNX1 transcription factor functions only during the EHT and not thereafter (Chen et al., 2009), our study shows that GATA2 functions in HSCs both during and after the EHT stage. At the EHT stage, we observed a severe reduction but not a complete absence of CFU-Cs in *Vec-Cre:Gata2*<sup>fl/fl</sup> AGMs. 31% of these HPCs have both alleles recombined, indicating that some HPCs are GATA2 independent. This is in contrast to *Vec-Cre:Runx1*<sup>fl/fl</sup> AGMs, in which no HPCs with both recombined alleles were found (Chen et al., 2009). In *Runx1* cKO embryos, no cKit<sup>+</sup> aortic hematopoietic clusters or cells are found, unlike the *Gata2* cKO. The difference between the phenotypes of the *Gata2* and the *Runx1 Vec-Cre* cKOs suggests that there are distinct subsets of HPCs. Collectively, our results are supportive of the concurrent function of GATA2 and RUNX1 in EHT, and they reveal important new information that GATA2 and RUNX1 also act separately to provide unique functions in HPCs and HSCs at different developmental stages.

## MATERIALS AND METHODS

**Conditional deletion by *Vec-Cre* or *Vav-Cre*.** WT, *Vec-Cre:Gata2*<sup>fl/+</sup>, and *Vec-Cre:Gata2*<sup>fl/fl</sup> conceptuses were generated by crossing *Vec-Cre:Gata2*<sup>fl/+</sup> males or females with *Gata2*<sup>fl/fl</sup> females or males. Similar breeding strategies were used for *Vav-Cre* deletion. WT and germline *Gata2*<sup>+/-</sup> and *Gata2*<sup>-/-</sup> conceptuses were generated by crossing *Gata2*<sup>+/-</sup> males and females. Genotyping was performed by PCR using the primers in Table S1. All experiments have been conducted according to Dutch law and have been approved by the animal experiments committee (Stichting DEC consult).

**Microscopic and histological analyses.** Conceptuses were suspended in phosphate-buffered saline with 10% FCS and visualized with a stereomicroscope. To analyze intra-aortic clusters, embryos were fixed in 2% PFA and stained with anti-cKit or anti-cKit and anti-Pecam1 (CD31) antibodies, and imaged in a 1:2 mix of benzyl alcohol and benzyl benzoate. Samples were analyzed with a laser-scanning confocal microscope (SP5; Leica), as previously described (Yokomizo et al., 2012), or analyzed with a brightfield microscope. Three-dimensional reconstructions were generated from Z-stacks (50–150 optical sections) using LasAF software.

**Hematopoietic assays.** Methylcellulose colony-forming assays were performed as described previously (Medvinsky et al., 2008). Embryo equivalents (ee; 1/10 and 9/10 ee of E10 and E11 AGMs and E10 FLs; 1/30, 1/10, and 26/30 ee of E11 FLs; and 1/1,000 and 1/10 ee or 10<sup>5</sup> cells of E14 FLs) were seeded per 1 ml M3434 medium and colonies counted after 10–12 d. Colonies were individually isolated from methylcellulose medium and genotyped to determine clonal recombination efficiency. AGMs and FL cells were transplanted as described previously (Medvinsky et al., 2008). After isolation of Ly5.2 cKO and WT conceptuses, YS material was genotyped using a fast genotyping kit (KAPA) and 1–3 AGMs of the same genotype were pooled or 10<sup>5</sup> FL cells were injected into lethally irradiated Ly5.1 recipients. Donor chimerism was determined through flow cytometric analysis of anti-Ly5.1 and anti-Ly5.2 antibody stained peripheral blood (PB) cells of recipients at 1, 2, 3, and 4 mo after transplantation. Multilineage organ chimerism analysis was similarly performed at 4 mo after transplantation. Transplanted recipients were scored positive if PB donor chimerism was at least 5% at 4 mo after transplantation

and multi-organ and myeloid and lymphoid reconstitution was at least 1% at the time of sacrifice (4 mo after transplantation).

**FACS analyses.** Single cell suspensions were made by 45-min collagenase type I treatment of dissected AGM + vitelline artery + umbilical artery and subsequent dissociation by pipetting or direct dissociation of FLs by pipetting. LSK SLAM analyses were performed according to Kim et al. (2006). Lineage cocktail included CD3e, Ly6C, CD45R, Ter119, and CD48. Annexin V staining was performed simultaneously with LSK SLAM staining in Annexin V binding buffer (BD) in PBS containing 10% FCS. Ki67/cell cycle analysis; Lin<sup>-</sup> CD48<sup>-</sup> sorted cells were stained with anti-Sca1, anti-cKit, and anti-CD150 antibodies, fixed with 2% paraformaldehyde, and permeabilized with Triton X-100. Ki67 staining was combined with Hoechst staining (BD). Other antibodies include anti-CD31 and anti-FLK1 (BD) and anti-CD41 (Santa Cruz Biotechnology, Inc.). Fluorochrome conjugates used were: FITC, phycoerythrin, phycoerythrin-Cy7, allophycocyanin-, or allophycocyanin-efluor 780. Stained cells were analyzed on a FACSaria III or SORP-FACSaria II flow cytometer (BD). Dead cells were excluded with Hoechst.

**Statistical analysis.** Statistical significance throughout this study was determined by Student's *t* test.

**Online supplemental material.** Table S1 shows genotyping primers and conditions. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20130751/DC1>.

The authors thank Drs. Catherine Robin, Derk ten Berge, Michael Chen, Gerben Schaaf, and all laboratory members for lively discussions and the Erasmus Dier (Animal) Center for animal care.

This work was supported by National Institutes of Health R03DK054077, FES Netherlands Institute for Regenerative Medicine, Erasmus Grant, Landsteiner Society for Blood Research 1109, and ZonMW 911-09-036.

The authors state that there are no competing financial interests.

Submitted: 11 April 2013

Accepted: 12 November 2013

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## SUPPLEMENTAL MATERIAL

de Pater et al., <http://www.jem.org/cgi/content/full/jem.20130751/DC1>**Table S1.** Genotyping primers and conditions

Primer	Primer sequence (5'-3')	Tm	Size PCR product	Strain:
Vec-F-219	CCCAGGCTGACCAAGCTGAG	58	300 bp	VE-Cadherin Cre
Vav-F23	GCGACAGTTACAGTCACAGAAGAGG	58	500 bp	Vav-Cre
Cre-R107	GCCTGGCGATCCCTGAACATG	58		VE-Cadherin Cre, Vav-Cre
Gata2 fl F	TCCGTGGGACCTGTTTCCTTAC	58	fl = 844bp, WT = 717bp,	Gata2 fl
Gata2 fl R	GCCTGCGTCTCCAACACCTCTAA	58	delta = 181bp	Gata2 fl
mGATA 39	GGAACGCCAACGGGGAC	60	mutant = 900bp, WT = 600bp	Gata2 KO
mGATA 208	GCTGGACATCTCCGATTCCGGGT	60		Gata2 KO
Neo 503	GATCTCCTGCATCTCACCTTGCT	60		Gata2 KO