Gata2b is a restricted early regulator of hemogenic endothelium in the zebrafish embryo

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ABSTRACT
The adult blood system is established by hematopoietic stem cells (HSCs), which arise during development from an endothelial-to-hematopoietic transition of cells comprising the floor of the dorsal aorta. Expression of aortic runx1 has served as an early marker of HSC commitment in the zebrafish embryo, but recent studies have suggested that HSC specification begins during the convergence of posterior lateral plate mesoderm (PLM), well before aorta formation and runx1 transcription. Further understanding of the earliest stages of HSC specification necessitates an earlier marker of hemogenic endothelium. Studies in mice have suggested that GATA2 might function at early stages within hemogenic endothelium. Two orthologs of Gata2 exist in zebrafish: gata2a and gata2b. Here, we report that gata2b expression initiates during the convergence of PLM, becoming restricted to emerging HSCs. We observe Notch-dependent gata2b expression within the hemogenic subcompartment of the dorsal aorta that is in turn required to initiate runx1 expression. Our results indicate that Gata2b functions within hemogenic endothelium from an early stage, whereas Gata2a functions more broadly throughout the vascular system.

KEY WORDS: Hematopoietic stem cell, Hemogenic endothelium, Subfunctionalization, Gata2, Notch

INTRODUCTION
Hematopoietic stem cells (HSCs) are tissue-specific stem cells that give rise to, and ultimately maintain, the adult blood system over a lifetime. During embryogenesis, HSCs arise from a population of hemogenic endothelium, primarily within the ventral wall of the dorsal aorta (DA) (Ciau-Uitz et al., 2014; Clements and Traver, 2013; Dzierzak and Speck, 2008; Swiers et al., 2013; Taoudi and Medvinsky, 2007). Aortic endothelium transdifferentiates into HSCs via an endothelial-to-hematopoietic transition (EHT) (Chen et al., 2009; Jaffredo et al., 1998; Zovein et al., 2008), a process that is conserved across vertebrates (Bertrand et al., 2010; Boisset et al., 2014, 2010; Chen et al., 2009; Jaffredo et al., 2000, 1998; Kissa and Herbomel, 2010; Lam et al., 2010). EHT produces hematopoietic stem and progenitor cells (HSPCs) that rapidly enter circulation in zebrafish (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010; Lam et al., 2010) or proliferate and differentiate locally to form hematopoietic clusters in the chick and mammalian embryo (Boisset et al., 2014; Jaffredo et al., 2000; North et al., 2002; Tavian et al., 1999; Yokomizo and Dzierzak, 2010). Nascent HSPCs home to the caudal hematopoietic tissue (CHT) of zebrafish and to the placenta and fetal liver in mammals, where they undergo proliferation and maturation. Finally, HSCs colonize the kidney in zebrafish and the bone marrow in mammals, where they establish residence for the remainder of life.

The zebrafish model has proven valuable to our understanding of HSPC development, including the first direct in vivo visualization of their emergence (Bertrand et al., 2010; Kissa and Herbomel, 2010; Lam et al., 2010). The transcription factor Runx1 is required for EHT in both mice and zebrafish (Chen et al., 2009; Kissa and Herbomel, 2010; Lancrin et al., 2009). Within the zebrafish embryo, runx1 marks the subpopulation of cells within the DA with hemogenic potential from as early as 23 h post-fertilization (hf) (Wilkinson et al., 2009), providing one of the earliest reported markers of HSC commitment. However, there is evidence that hemogenic identity is established earlier than the onset of runx1 expression. We have recently reported that, during the convergence of the posterior lateral plate mesoderm (PLM) prior to the formation of the DA or its rudiment, the vascular cord, contact between the ventral somite and the PLM is necessary to transmit requisite Notch signals into HSC precursors (Kobayashi et al., 2014). That somite-to-PLM signaling occurs between 14 and 18 hpf indicates that cells acquire hemogenic endothelial identity earlier than previously appreciated. Therefore, determining additional markers that distinguish HSCs from vascular cells is essential to investigate early events in HSC specification. In the present study, we identified a novel early hemogenic endothelial marker, gata2b, in zebrafish.

Expression of Gata2 is driven by activation of the NOTCH1 receptor and its transcriptional partner RBPjκ in the DA of mice (Robert-Moreno et al., 2005). Mice deficient in GATA2 die at embryonic day (E) 10.5 with defects in primitive and definitive hematopoiesis (Tsai et al., 1994). Targeted deletion of Gata2 within the endothelium results in edema, hemorrhage and loss of functional HSCs (de Pater et al., 2013; Johnson et al., 2012; Lim et al., 2012). In addition, GATA2 serves iterative roles in early vascular cells and their hemogenic progeny (de Pater et al., 2013). GATA2 is required within the endothelium for the expression of Runx1 (Gao et al., 2013) that is essential for EHT (Chen et al., 2009; Kissa and Herbomel, 2010; Lam et al., 2010).
Herbomel, 2010; Lancrin et al., 2009), and this is likely to be through direct regulation of a hemogenic endothelial-specific Runx1 enhancer (Nottingham et al., 2007). Together, these studies suggest that GATA2 functions downstream of Notch signaling within the endothelium to activate Runx1 expression.

A genome duplication event in early teleosts generated two gata2 paralogs in the zebrafish genome (Gillis et al., 2009). The gata2a paralog is expressed throughout the PLM and trunk vasculature (Brown et al., 2000; Detrich et al., 1995) but is not affected by the loss of Notch signaling downstream of Wnt16, which affects HSC but not arterial specification (Clements et al., 2011). Additionally, the loss of Gata2a in gata2aum27 mutants results in defects in vascular morphogenesis and circulation (Zhu et al., 2011), making it difficult to address possible hematopoietic functions of Gata2a. In this study, we focus on the gata2b paralog in the context of HSC specification. We show that gata2b is expressed specifically within hemogenic endothelium prior to runx1. We generated gata2b:Gal4 transgenic animals, which specifically label hemogenic endothelium and nascent HSCs. Moreover, gata2b is required genetically upstream of runx1. Whereas gata2a is required for vascular development and circulation (Zhu et al., 2011), gata2b is not required for these processes. Our findings suggest that the duplication event resulting in gata2a and gata2b has segregated the endothelial and hematopoietic functions of Gata2 in zebrafish, allowing for future studies of the hemogenic function of Gata2 without disruption of the vasculature or circulation.

RESULTS
gata2b is expressed in hematopoietic precursor cells
A chromosomal duplication event in the teleost lineage resulted in two zebrafish paralogs of Gata2, gata2a and gata2b (Gillis et al., 2009), which share only 57% sequence identity and 67% similarity (Fig. 1). Although gata2b has been identified in the genome, little is known about its expression or function. To better understand gata2b, we explored its expression during embryogenesis by quantitative PCR (qPCR) and whole-mount in situ hybridization (WISH). gata2b is maternally expressed, and embryonic transcription initiated at 16 hpf (Fig. 2A). By WISH, no expression of gata2b was observed in the developing early PLM, in contrast to gata2a, which is expressed in the PLM from approximately the 3-somite stage, at ∼10.3 hpf (Li et al., 2009) (supplementary material Fig. S1). By 18 hpf, expression of gata2b is detectable in a small number of cells at and near the midline.
(Fig. 2C, arrowheads). No expression was detected in this region at 16 hpf, indicating that expression of gata2b at the midline initiated between 16 and 18 hpf (Fig. 2B,C). By 20 hpf, gata2b is expressed in the DA (Fig. 2D, yellow arrowheads), as well as in branchiomotor neurons (Fig. 2D, blue arrowheads). At 50 and 72 hpf, gata2b expression can be observed in hematopoietic cells within the CHT region (Fig. 2F,G, pink arrowheads).

To investigate whether gata2b-expressing cells at the midline at 18 hpf are from the PLM, we sorted cells positive for fli1a:EGFP, which marks the PLM and the hematopoietic cells that it gives rise to (Lawson and Weinstein, 2002; Thompson et al., 1998). In line with our gata2b WISH analysis, we did not detect gata2b by qPCR at 16 hpf in fli1a+ cells, but detected expression in this tissue from 18 to 26 hpf (Fig. 2H). To determine gata2b expression with greater spatial precision, we transversely sectioned the embryonic trunk region. In 18-hpf embryos, gata2b expression was observed within the vascular cord, as well as in rare cells outside the midline, consistent with initiation near the end of PLM convergence (Fig. 2I). At 25 hpf, transverse trunk sections showed gata2b expression in an endothelial subpopulation polarized to the ventral wall of the aorta, similarly to runx1 (Fig. 2J,K), whereas gata2a was detected throughout the DA and posterior cardinal vein (PCV), similarly to the vascular marker kdrl, as well as in primitive erythrocytes (Fig. 2L,M). In conclusion, although both gata2a and gata2b are expressed in the DA, they have distinctively different expression patterns within this tissue.

Next, we examined gata2b expression within hemogenic endothelium. By combining fluorescent reporter animals that mark vasculature (kdrl:mCherry) or hematopoietic cells (cmyb:GFP), nascent HSPCs (kdrl:mCherry; cmyb:GFP) can be distinguished from vascular, non-hemogenic endothelium (kdrl:mCherry; cmyb:GFP) at 36 hpf (Bertrand et al., 2010). At 36 hpf, gata2a is enriched within vascular endothelium as compared with nascent
HSPCs (Fig. 2N). Consistent with expression observed by WISH, gata2b expression is enriched within hematopoietic cells generated by the aortic endothelium (Fig. 2N). The specific expression and early initiation of gata2b make it of particular interest for studying mechanisms governing HSC specification prior to runx1 initiation.

**A gata2b:Gal4 transgene marks hemogenic endothelium**

To further characterize gata2b as a possible marker of hemogenic endothelium and nascent HSCs, we generated transgenic zebrafish reporting gata2b expression. As gata2b is expressed specifically but at a low level within hemogenic endothelium, we utilized the Gal4-UAS system to amplify expression. This system also creates flexibility, allowing a single transgenic Gal4 driver line to be used in conjunction with a variety of UAS-driven effector lines. Using BAC recombineering (Bussmann and Schulte-Merker, 2011) and Tol2-mediated transgenesis, we created transgenic animals driving optimized Gal4 (KalTA4) (Distel et al., 2009) under the control of gata2b regulatory elements. By crossing gata2b:Gal4 to Gal4-responsive fluorescent reporter lines, including UAS:GFP and UAS: lifeactGFP, we observed GFP fluorescence at 24 hpf in cells with a flattened morphology in the aortic floor region (Fig. 3A). At 30 hpf, GFP expression was consistent with the hematopoietic expression of gata2b as assessed by WISH, although no fluorescence was observed in branchiomotor neurons (Fig. 3B). By 3 days post-fertilization (dpf), gata2b:Gal4;UAS:lifeactGFP fluorescence remained restricted to hematopoietic populations in the CHT, thymus and kidney, with round hematopoietic cells also observed in association with the heart lumen (Fig. 3C; supplementary material Movie 1). This indicates that gata2b:Gal4 drives expression specifically in cells at sites of HSC emergence and colonization (CHT, thymus and kidney). By contrast, gata2a:GFP shows GFP expression in the DA, PCV, primitive erythrocytes and spinal cord neurons, consistent with endogenous gata2a expression observed by WISH (supplementary material Fig. S1).

Our data suggested that gata2b:Gal4 drives expression in hemogenic endothelial cells. Hemogenic endothelial cells are integral to the aortic wall (Bertrand et al., 2010; Chen et al., 2011; de Bruijn et al., 2002; Jaffredo et al., 1998; North et al., 1999) and undergo a characteristic morphological change during EHT (Kissa and Herbomel, 2010). To determine whether gata2b+ cells in gata2b:Gal4 animals behave in a similar way, we analyzed gata2b:Gal4 in the context of the pan-vascular marker kdrl. In gata2b:Gal4;UAS:GFP; kdrl:mCherryembryos, GFP+ cells co-express the vascular marker kdrl:mCherry, indicating that they are indeed within the aortic endothelium (Fig. 3D). Furthermore, gata2b:Gal4-driven expression marked cells undergoing the stereotypical budding characteristic of EHT (Kissa and Herbomel, 2010) (Fig. 3E). Using time-lapse imaging, we visualized the emergence of GFP+ cells from the endothelium (supplementary material Movie 2).

Following EHT, HSPCs migrate to the CHT for amplification and differentiation, before homing to the thymus, which is the primary site of T-cell development (Bertrand et al., 2008; Kissa et al., 2008; Murayama et al., 2006; Zhang et al., 2011). In gata2b:Gal4;UAS:lifeactGFP fish, we observed gata2b+ cells first in the aortic floor and later in the thymus (Fig. 3B,C). By photoconverting gata2b+ cells in gata2b:Gal4;UAS:Kaede fish, we traced cells from the aorta at 46 hpf to the thymus at 4 dpf, confirming that GFP+ thymic cells derive from gata2b+ hemogenic endothelial cells (Fig. 3F,G). Moreover, thymic gata2b+ cells coexpressed the T-cell marker lck: nls-mCherry at 4 dpf (supplementary material Fig. S2), indicating that gata2b+ cells have lymphoid potential.

Together, these data indicate that gata2b-expressing cells first reside within the DA, later emerging through EHT to migrate to the CHT, thymus and embryonic kidney. Furthermore, gata2b+ cells...
have lymphoid potential, suggesting that they might be early HSPCs.

**gata2b**+ cells give rise to adult hematopoietic cells

Nascent HSCs seed the kidney, where they amplify and are maintained during adult homeostasis, and differentiate into erythroid, lymphoid, myeloid and hematopoietic precursor cells within the adult whole kidney marrow (WKM) can be separated by flow cytometry based upon light scatter characteristics (Traver et al., 2003). In adult zebrafish, gata2a:GFP is expressed in the kidney marrow, where it primarily labels eosinophils (Balla et al., 2010; Traver et al., 2003). By contrast, gata2b:Gal4 does not drive fluorescence in the adult kidney marrow when combined with either UAS:GFP or UAS:lifeactGFP (Fig. 4A,B).

Permanent genetic labeling of endothelial cells and their derivatives with endothelial-specific kdrl:Cre in combination with the lineage marker actB2:LoxP-STOP-LoxP-DsRedEx has indicated that embryonic hemogenic endothelium is the origin of zebrafish adult blood cells (Bertrand et al., 2010). In this lineage-tracing
approach, actB2:LoxP-STOP-LoxP-DsRedEx (also known as βactin:Switch-DsRed) undergoes excision of the STOP cassette in Cre-expressing cells, resulting in a permanent ‘switch’ from non-fluorescence to DsRed+ for these cells and their progeny. To investigate whether gata2b−/− cells have the potential to give rise to the adult hematopoietic system, we performed lineage tracing in gata2b−/−;Gal4;UAS:Cre;βactin:Switch-DsRed zebrafish. As expected, DsRed fluorescence marked cells in the CHT, thymus and kidney in 3 dpf embryos (Fig. 4C-E), closely resembling gata2b−/−;Gal4;UAS:lactateGFP embryos at this time (Fig. 3C). Whereas gata2b−/−;Gal4 initially drives expression in the DA, we did not detect gata2b−/− cells in the endothelium at 3 dpf (Fig. 3A,C).

To determine the contribution of gata2b−/− cells to adult blood, we analyzed DsRed+ cells in the WKM by flow cytometry. Lineage tracing of the endothelium in kdr1:Cre;βactin:Switch-DsRed fish labeled the vast majority of hematopoietic cells within the WKM, with no DsRed+ cells in the WKM of βactin:Switch-DsRed zebrafish alone (Fig. 4F,G). In comparison, within the WKM of 2-month post-fertilization gata2b−/−;Gal4;UAS:Cre;βactin:Switch-DsRed fish, 87.8±1.8% of lymphoid cells, 84.8±7.0% of myeloid cells and 85.2±6.6% of precursor cells were DsRed+ (n=3). Although we observed some leakiness of the UAS:Cre transgene within a small number of DsRed+ muscle and neural cells in 3-dpf embryos, adult UAS:Cre;βactin:Switch-DsRed fish had less than 0.1% DsRed+ cells in the lymphoid, myeloid and precursor fractions of the WKM (n=2) (Fig. 4H), indicating that gata2b−/− cells give rise to the majority of blood cells in the adult WKM in a multilinenage manner. Similarly, switched DsRed+ cells comprised the majority of the erythroid and lymphoid cells within the spleens of kdr1:Cre;βactin:Switch-DsRed (Fig. 4J) and gata2b−/−;Gal4;UAS:Cre;βactin:Switch-DsRed (Fig. 4K) fish, whereas no labeling was observed in the spleens of βactin:Switch-DsRed or UAS:Cre;βactin:Switch-DsRed control animals. Using the gata2b−/−;Gal4 transgenic line, we have demonstrated that the majority of hematopoietic cells are derived from gata2b−/− cells.

Taken together, our data indicate that gata2b is expressed specifically within aortic hemogenic endothelium, which gives rise to the vast majority of adult hematopoietic cells.

gata2b is required for HSC formation

To establish whether gata2b is required for HSC development, we performed targeted knockdown of gata2b using a splice-blocking morpholino oligonucleotide (MO). This MO results in intron retention leading to missense sequence and a premature STOP codon before the zinc-finger domains (supplementary material Fig. S3), which is predicted to inhibit the DNA binding of Gata2b. Initially, gata2b morphants express normal levels of cmyb, which is required for the formation of HSCs. To confirm the hemogenic endothelial defect in gata2b morphants, we assessed expression of the transcription factor cmyb, which is required for the formation of HSCs. Consistent with the loss of cmyb expression in gata2b morphants at 4 dpf (Fig. 5C,D), thymocytes were severely reduced in gata2b morphants at 4 dpf (Fig. 5E,F), further indicating that gata2b is essential for the production of functional HSCs.

Owing to the remarkable differences in endothelial gata2a and gata2b expression, we hypothesized that gata2b might have taken on a specialized role within the hemogenic endothelial compartment while gata2a has remained essential to vascular morphogenesis. Strengthening this hypothesis, depletion of gata2b did not affect the expression of kdr or cfnb2α, indicating normal vascular development and arterial specification. Importantly, circulation was normal in gata2b morphants (supplementary material Movies 3 and 4). By contrast, gata2adum77 mutants lack trunk circulation (Zhu et al., 2011) and experience hemorrhages (supplementary material Fig. S1), consistent with the consequences of vascular endothelial deletion of Gata2 in mice (de Pater et al., 2013; Johnson et al., 2012; Lim et al., 2012).

We next tested whether the Gata2a and Gata2b proteins are functionally redundant by attempting to rescue gata2b morphants with ectopic expression of gata2a or gata2b. We found that gata2b successfully rescued gata2b splice morphants, whereas gata2a did not (supplementary material Fig. S3I-M), consistent with our hypothesis that Gata2a and Gata2b fulfill distinct requirements. Together, our results suggest that the requirement of GATA2 for endothelial integrity appears to be maintained by the zebrafish...
Gata2a paralog, whereas Gata2b serves a specialized role within hemogenic endothelium.

Notch signaling is required for hematopoiesis through its upstream regulation of Runx1 (Nakagawa et al., 2006). In zebrafish, Notch signaling is required for aortic cmyb expression, and cmyb loss as a result of Notch inhibition can be rescued by runx1 mRNA injection (Burns et al., 2005). To determine where Gata2b functions in this pathway, we tested whether ectopic runx1 could rescue cmyb expression in gata2b morphants. Co-injection of gata2a MO with runx1 mRNA was sufficient to rescue the loss of cmyb in hemogenic endothelium (supplementary material Fig. S3N-Q), indicating that gata2b is required upstream of runx1 in hemogenic endothelium.

**gata2b is regulated by Notch signaling**

Both cell-autonomous and non-cell-autonomous Notch signaling is required for the formation of vertebrate HSCs (Hadland et al., 2004; Kim et al., 2014). Notch signaling is required for both arterial specification and HSC formation from arterial vasculature. Gata2 is a direct Notch target in the mouse DA (Robert-Moreno et al., 2005) and is regulated by hematopoietic, rather than arterial, Notch signaling (Robert-Moreno et al., 2008). Somitic Wnt16 is required upstream of both somite-intrinsic and somite-to-PLM Notch signaling without affecting arterial specification (Clements et al., 2011; Kim et al., 2014; Kobayashi et al., 2014), demonstrating that the Notch requirements of arterial and HSC development are separable in zebrafish. Surprisingly, gata2a expression in the aortic endothelium is unaffected by knockdown of wnt16 (Clements et al., 2011), which led us to investigate whether Notch signaling is required for the expression of gata2b in hemogenic endothelium.

We first performed inhibition of Notch signaling in the whole embryo. The E3 ubiquitin ligase Mindbomb (Mib) is required for functionality of the Notch ligand on the signal-emitting cell (Itoh et al., 2003). Knockdown of Notch signaling using a mib MO resulted in downregulation of both runx1 and gata2b (Fig. 6A-D).

This finding was recapitulated by chemical inhibition of γ-Secretase, which prevents release of the transcriptionally active intracellular domain from the membrane-bound Notch receptor (Mumm et al., 2000). Treatment with the γ-Secretase inhibitor dibenzazepine (DBZ) (Milano et al., 2004; van Es et al., 2005) from 10 hpf resulted in downregulation of both runx1 and gata2b in the DA (Fig. 6E,H). Furthermore, knockdown of wnt16, which functions upstream of the Notch signaling required for HSC formation, resulted in the reduction of both runx1 and gata2b in the DA (Fig. 6I-L), suggesting that Notch regulates zebrafish gata2b in a similar manner to murine Gata2.

Three of the four Notch receptors are necessary for runx1 expression in zebrafish but function in different tissues (Kim et al., 2014). The NOTCH1 homologs Notch1a and Notch1b act directly within endothelial cells, whereas Notch3 is dispensable within the endothelium but required in the somite via an unknown mechanism (Kim et al., 2014). Because Gata2 is a direct NOTCH1 target in the murine DA (Robert-Moreno et al., 2005), we examined whether the receptors required within the endothelium function upstream of gata2b. Individual knockdowns of notch1a and notch1b resulted in decreased runx1 and gata2b expression (Fig. 7A-D,G-J). By contrast, expression of gata2a was unaffected by loss of either receptor (Fig. 7E, F,K,L). The endothelial Notch signaling that is required for the hemogenic capacity of the DA thus regulates gata2b but not gata2a.

Together, our results demonstrate that gata2b is expressed in early hemogenic endothelium, where it is required upstream of runx1 to...
determine HSC fate. In addition, gata2b is regulated by the Notch receptors that function specifically within the endothelium to promote hematopoiesis, whereas gata2a is not. These data suggest that, following duplication of the ancestral gata2 gene, subfunctionalization of each paralog has resulted in gata2b regulating HSC emergence.

DISCUSSION

Subfunctionalization of Gata2a and Gata2b in zebrafish

The division of labor between duplicated genes to recapitulate the functions of the ancestral gene is known as subfunctionalization (Force et al., 1999). Genome duplication within the teleost lineage has yielded two Gata2 paralogons that have been maintained in these jawed, bony, ray-finned fish, including medaka, fugu (pufferfish), Tetraodon, stickleback and zebrafish (Gillis et al., 2009). We demonstrate a divergence in the expression patterns of gata2a and gata2b in zebrafish, with gata2a expressed throughout the endothelium and gata2b restricted to the hemogenic subpopulation of the DA. Despite both being expressed in the endothelium, gata2a and gata2b have a high level of sequence divergence and are regulated differently, suggesting that they might have divergent functions within the embryonic endothelium. Accordingly, gata2a is required for endothelial integrity and vascular morphogenesis (Zhu et al., 2011), whereas gata2b is required in an HSC-specific manner. During mouse embryogenesis, GATA2 is required in the endothelium for both the maintenance of endothelial integrity and HSC formation (de Pater et al., 2013; Johnson et al., 2012; Lim et al., 2012). Together, Gata2a and Gata2b appear to additively fulfill the endothelial roles of GATA2, suggesting that duplication of the gata2 locus has led to an evolutionary separation of its endothelial and hematopoietic functions.

The zinc-finger domains of GATA2 that mediate DNA binding and interaction with transcriptional partners (Vicente et al., 2012) are largely conserved between zebrafish Gata2a and Gata2b and the human and mouse GATA2 proteins. Outside this region the proteins are largely conserved between zebrafish Gata2a and Gata2b and the evolutionary separation of its endothelial and hematopoietic functions.

Expression of gata2b marks early hemogenic endothelium

The process by which endothelial cells acquire hemogenic capacity has not been fully resolved. Understanding when and how this occurs during embryogenesis can inform future efforts to derive these cells in vitro. HSCs emerge from ventral endothelium of the DA (Bertrand et al., 2010; Boisset et al., 2010; Chen et al., 2009; Kissi and Herbomel, 2010; Lam et al., 2010; Taoudi and Medvinsky, 2007). Several signaling pathways, including Hedgehog, VEGF and multiple Notch inputs, are important for the formation of hemogenic endothelium upstream of runx1 expression (Gering and Patient, 2005; Kim et al., 2014). Owing to a paucity of markers for the early detection of hematopoietic specification within endothelium, we lack a full understanding of how these cues drive the establishment of HSC fate.

The markers that distinguish hemogenic endothelium from arterial endothelium, including runx1, cmyb and cd41 (itga2b – ZFIN), have only been observed in the endothelium following formation of the DA. It is now evident that specification of both arterial and hemogenic endothelium begins earlier than previously postulated. In particular, migrating PLM cells appear to adopt arterial or venous fate well before formation of the vascular cord (Hong et al., 2006; Kohli et al., 2013; Quillien et al., 2014), with arterial specification beginning within the PLM as early as 11 hpf, at the 5-somite stage (Quillien et al., 2014). Somatic Notch ligands DeltaC and DeltaD downstream of Wnt16 are required for the formation of hemogenic endothelium (Clements et al., 2011), and these ligands activate Notch signaling in the PLM during migration to the midline between 14 and 18 hpf (Kobayashi et al., 2014). When this signal is missing, arterial specification is unaffected but hemogenic endothelium is lost (Kobayashi et al., 2014). As we observe that the expression of gata2b initiates in a small population of flh1a+ cells just before and during formation of the vascular cord at 18 hpf, it is plausible that Notch signaling from the somite initiates gata2b expression to prime the HSC program.

Regulation of gata2b expression

Notch signaling regulates HSC formation through both direct and indirect mechanisms (Clements et al., 2011; Hadland et al., 2004; Kim et al., 2014; Kobayashi et al., 2014). Our data demonstrate that Notch signaling is required for the expression of gata2b in hemogenic endothelium. In mouse, both GATA2 and NOTCH1 are required cell-autonomously for formation of HSCs (Hadland et al., 2004; Tsai et al., 1994). The transcriptionally active NOTCH1 intracellular domain (NICD1) associates with the Gata2 promoter in the mouse embryo at E9.5, just before HSC emergence, and is required for Gata2 expression in the DA (Guu et al., 2013; Robert- Moreno et al., 2005). These findings suggest that Gata2 is a direct target of a cell-autonomous Notch signal within hemogenic endothelium. In zebrafish, the Notch1a and Notch1b receptors are required specifically within the endothelium for HSC formation (Kim et al., 2014). Our data demonstrate that Notch1a and Notch1b are required for gata2b, but not gata2a, in the DA, suggesting that there might be a conserved role of endothelial Notch signaling in the regulation of gata2b in zebrafish.

RUNX1 is required cell-intrinsically within hemogenic endothelium for HSC formation (Chen et al., 2009). Expression of runx1 in the DA is dependent upon Notch signaling in both mice and zebrafish (Burns et al., 2005; Robert- Moreno et al., 2005). The hematopoietic defect in the absence of Notch signaling can be rescued by artificial induction of runx1 in cell culture (Nakagawa et al., 2006). Similarly, the hematopoietic defect resulting from Notch deficiency can be partially rescued through provision of exogenous runx1 mRNA in zebrafish (Burns et al., 2005), indicating that Notch is required for HSC formation upstream of runx1 activation in hemogenic endothelium. In our study, the hematopoietic defect of gata2b morphants is partially rescued by provision of runx1 mRNA, suggesting that loss of runx1 accounts for the hematopoietic phenotype observed with gata2b knockdown. Hematopoietic endothelial expression of runx1 is governed by an intronic enhancer element that contains consensus sites for Runx, Cmyb, Gata, ETS and Box transcription factors (Bee et al., 2009; Nottingham et al., 2007), but not for the Notch partner RBPjκ, suggesting that Notch signaling might be required through the induction of intermediate transcription factors to promote runx1 expression in hemogenic endothelium. The GATA binding site is crucial for the activity of this enhancer (Nottingham et al., 2007). Dysregulation of Gata2 in the endothelium results in a reduction in runx1 expression in the AGM region (Gao et al., 2013). In this study we report a similar reduction in runx1 expression in the DA with gata2b knockdown, indicating a conserved requirement for Gata2 within hemogenic endothelium.
The *gata2b*:*Gal4* transgene as a tool for future hematopoiesis studies

The *gata2b*:*Gal4* driver marks hemogenic endothelium and nascent HSCs from the DA to the CHT, thymus, pronephros and adult kidney. To our knowledge, *gata2b*:*Gal4* is the earliest, most specific marker of aortic hemogenic endothelium, allowing the direct visualization of hemogenic endothelium from as early as 24 hpf. In the future, this transgenic driver will prove useful in the unambiguous detection and tracking of hemogenic endothelial cells and HSCs. Because this line drives *Gal4* rather than a fluorescent protein, it can be used in conjunction with the growing number of UAS-driven transgenes.

Furthermore, we have shown that *gata2b*:*Gal4*;**UAS::Cre**;**βactin::Switch-DSRed** effectively labels endothelial-derived blood until adulthood in a multilineage manner, demonstrating that *gata2b* is expressed in HSCs. With the increasing ease of genetic manipulation using the CRISPR/Cas9 system in zebrafish (Auer et al., 2014), hematopoiesis-specific disruption of genes, such as *gata2a*, whose mutation results in developmental defects and embryonic lethality, but might serve unappreciated roles in the developing hematopoietic system.

**MATERIALS AND METHODS**

**Zebrafish husbandry and maintenance**

Zebrafish (*Danio rerio*) were maintained according to the guidelines of the UCSD Institutional Animal Care and Use Committee. The following zebrafish lines have been described previously: *Tg(fli1a::GFP)* (Lawson and Weinstein, 2002), *Tg(kdrl::Has.HRAS-mCherry)* (Chi et al., 2008), *Tg(mcbv:EGFP)* (North et al., 2007), *Tg(q7.0;gata2a::EGFP)I* (Traver et al., 2003), *Tg(flk1a:GFP)I* (Distel et al., 2009), *Tg(UAS::lifeactGFP)* (Chen et al., 2013), *Tg(UAS:kaede)* (Hatta et al., 2006), *Tg(actb2:loxP-STOP-loxP-DSRed)* (Bertrand et al., 2010), *gata2a-m27* (Zhu et al., 2011), *Tg(lmo2::GFP)* (Zhu et al., 2005), *Tg(gata1:DsRed)* (Traver et al., 2003) and *Tg(rag2-GFP)* (Langenau et al., 2003). For clarity, throughout the text *Tg(kdrl::Has.HRAS-mCherry)* is referred to as *kdrl::mCherry*, *Tg(q7.0;gata2a::EGFP)* is referred to as *gata2a-GFP*, *Tg(flk1a:GFP)* is referred to as *UAS::GFP*, *Tg(actb2:loxP-STOP-loxP-DSRed)* is referred to as *βactin::Switch-DSRed*, *Tg(BAC::gata2a::KaetAA4)* is referred to as *gata2b:*Gal4* and *Tg(UAS::Cre,CY)* is referred to as *UAS::Cre*.

**Whole-mount in situ hybridization (WISH)**

Embryos were treated with 1% phenyl-2-thiourea (PTU) and fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight at 4°C, then dehydrated and stored in methanol prior to staining. WISH was performed as described (Thisse et al., 1993). In situ hybridization probes were synthesized using the DIG RNA Labeling Kit (Roche). Hybridization probes were prepared as described for *et al.*, 1993). In situ hybridization probes were synthesized using the DIG RNA Labeling Kit (Roche). Hybridization probes were prepared as described for *et al.*, 1993). In situ hybridization probes were synthesized using the DIG RNA Labeling Kit (Roche). Hybridization probes were prepared as described for *et al.*, 1993).

**Fluorescence-activated cell sorting (FACS) and quantitative real-time PCR**

For each sample, ~50-100 embryos were stored on ice in 500 µl PBS with 2% FBS and were dissociated by pipetting. Cell suspensions were filtered using a 40-µm mesh and stained with SYTOX Red Cell Death Stain (Molecular Probes). Samples were sorted using a FACSAria Ilu cell sorter (BD Biosciences). mRNA was isolated using the RNeasy Mini Kit (Qiagen). During mRNA extraction, 500 ng polyinosinic acid potassium salt (Sigma) was added to the RLT buffer for each sample. cDNA was synthesized using the Quantitect cDNA Synthesis Kit (Qiagen) according to the manufacturer’s instructions. Quantitative PCR (qPCR) was performed using the BioRad CFX96 real-time system according to the manufacturer’s instructions, with the following primers (5′-3′): *gata2a*-Q-F, TCTTGG-AATCACCAGTGGCTC; *gata2a*-Q-R, GGACTGCTGATAGGTGTTG; *gata2b*-Q-F, ACCACCACATCTCGGAGAC; *gata2b*-Q-R, CTGTTGGCTG- GTCTGAACT-ACC; ef1a (ef1a-1a – ZFIN) primers have been previously described (Bertrand et al., 2007). Relative expression was calculated as 2^(-Ct gene of interest-Ct ef1a). For quantification of *gata2a* and *gata2b* transcripts, an *in vitro* standard curve was generated by transfecting HEK cells with pCS2-“gata2a” or pCS2-“gata2b” plasmid.

**Flow cytometry**

Adult WKM samples were prepared as described (Traver et al., 2003), stained using SYTOX Red Cell Death Stain and analyzed using an LSR II flow cytometer (BD Biosciences).

**gata2b MO design and validation**

Antisense MOs were synthesized by Gene Tools. 1 nl MO solution was injected into single-cell embryos at the following concentrations: 30 mg/ml MO1- *gata2b* (20 ng/gate acceptor MO) 5′- TTCACTGCTCAGGTGCTCAGC-3′; 30 mg/ml *gata2b* mismatch 5′- TTCACTGCTTACCTACCC-3′. For MO validation, wild-type and morphant embryos were collected and mRNA was prepared using the RNeasy Mini Kit (Qiagen), and cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen). RT-PCR was performed using primers (5′-3′): G2bEx3-F, CTGTCGAGA-CATGACGAC; G2bEx5-R, GTATAGACCAGCAGGCAGTG. RT-PCR products were isolated using the QIAquick Gel Extraction Kit (Qiagen). Purified PCR products were sequenced by Genewiz using primers G2bEx3-F (above) and G2bEx4-R (CTGGGTGTCGTGATGAGGT). Sequences were analyzed using ApE software and sequence chromatograms were prepared using CodonCode Aligner.

**Generation of expression constructs**

To generate the pCMV6-*gata2b* construct, *gata2b* was amplified from 1- to 2-cell stage zebrafish embryo cDNA using Phusion polymerase (New England Biolabs) and primers (5′-3′): *gata2b*-F, TCGGAGATCATCTCTG- TACTG; *gata2b*-R, GTTCCTCAAGCCATACGTCAGT. The PCR product was ligated into pCRII-Blunt-ToPO using the Zero Blunt Topo Cloning Kit (Life Technologies). The *gata2b* cDNA fragment was subcloned into pCMV6-Ac-Myc-His (Origene) using BamHI and XhoI (New England Biolabs). The pCS2-*gata2a*-*2A*-tdTomato construct was made using MultiSite Gateway Cloning (Life Technologies). *gata2a*, excluding the STOP codon, was amplified by PCR from 2-dpf embryo cDNA using the primers (5′-3′): Aatb1*-gata2a-F, GGGGACACAGGGTGTGGTCCACACATGGCTCAGGATGCA- G; Aatb2*-gata2a-R (no-stop), GGGGACACACTTTGTTACAAAGAGGAGTGGTAAC- TGCAGTGCATGTTGCTGGCCAGCCGCGG. The resulting fragment was cloned into pDONR221 by BP reaction, generating pME-*gata2a* (no stop). *2A*-tdTomato was amplified from pCS2-TAG (Addgene, 26772) using primers (5′-3′): Aatb2-2A-tomato-F, GGGGACACACTTTGTTAGTCAAAATGACAGGCGAGCCATTCCGAGTGCCTGAGTGAAG; Aatb2-2A-tomato-R, GGGGACACACTTTGTTACAAAGAGGAGTGGTAAC-TGCAGTGCATGTTGCTGGCCAGCCGCGG. The resulting fragment was cloned into pDONR221 by BP reaction, generating pME-*gata2a* (no stop). p3E-2A*-tdTomato and pCS2-TAG were cut to create the final assembly of pCS2-*gata2a*-*2A*-tdTomato.

**MO, plasmid and mRNA microinjection**

For all MO, plasmid and mRNA microinjections, 1 nl was injected into 1- to 2-cell-stage zebrafish embryos. For previously published MOs, the
following concentrations were used: 5 mg/ml MO1-Mib (Itoh et al., 2003), 5 mg/ml Wnt16 MO (Clements et al., 2011), 10 mg/ml Notch1a-sp MO (Ma and Jiang, 2007) and 10 mg/ml Notch1b MO (Kim et al., 2014). For runx1 mRNA rescue, runx1 mRNA was synthesized from pCS2-runx1 using the mMessage mMACHINE Kit (Life Technologies) and co-injected at 100 ng/µl. For plasmid rescue experiments, pCMV6-gata2b and pCSDest-gata2a-2A-tetTomato were injected at 20 ng/µl.

Generation of TgBAC(gata2b:KaiTA4)3422 zebrafish
The improved Gal4 variant KaiTA4 (Distel et al., 2009) was inserted at the start codon of gata2b on the BAC CH211-157B11 (BACPAC Resources, Children’s Hospital Oakland Research Institute, Oakland, CA, USA) using BAC recombining as previously described (Bussmann and Schulte-Mmerker, 2011). The modified BAC was then injected together with Tg(4xUAS:GFP) plasmid and screening offspring for GFP expression.

Generation of Tg(lck:nls-mCherry)437 zebrafish
The lck:nls-mCherry-CG2 transgenesis construct was created by Multisite Gateway recombination of p5’Entry-lck-7.4 kb (a kind gift from J. Yoder, North Carolina State University, Raleigh, NC, USA; Addgene, 58891), PcmE:nls-mCherry (Tol2kit #233), p3A-polyA4 (Tol2kit #302) and pDestTol2CG2 (Tol2kit #395). pDestTol2-lck:nls-mCherry-CG2 was injected with Tol2 mRNA into Tg(4xUAS:GFP) zygotes (Distel et al., 2009). Transgenic fish were identified by mating to Tg(4xUAS:GFP) plasmid and screening offspring for GFP expression.

Generation of Tg(UAS:Cre,CY)zd17 zebrafish
pDestTol2CY-UAS-Cre (pCM339) features a UAS-controlled Cre recombinease ORF in a Tol2 transgenesis vector harboring alpha-crystallin:YFP as a transgenesis marker. The vector was assembled using Multisite Gateway cloning with the Tol2kit vector #327 (p5E-fellowship [11PRE7580185 to E.B.]; EMBO fellowships (to M.D. and B.W.); a Children’s Hospital Oakland Research Institute and the University of North Carolina at Chapel Hill) and pCM339 (2011). Rapid BAC selection for tol2- mediated transgenic lines; E.B., M.D., C.P., B.W., I.K., D.L.S., K.N., N.D. and N.D.L. generated transgenic lines; E.B., M.D., C.M., F.E.P., A.M. and R.D. designed experiments; E.B., K.N., C.-W.N. and K.T. generated expression constructs; M.D., E.B., C.M., F.E.P., A.M. and R.D. analyzed transgenic lines; E.B. and D.T. wrote the paper with minor contributions from the remaining authors.

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Supplementary material
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References


