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Teleost growth factor independence (gfi) genes differentially regulate successive waves of hematopoiesis

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Abstract

Growth Factor Independence (Gfi) transcription factors play essential roles in hematopoiesis, differentially activating and repressing transcriptional programs required for hematopoietic stem/progenitor cell (HSPC) development and lineage specification. In mammals, Gfi1a regulates hematopoietic stem cells (HSC), myeloid and lymphoid populations, while its paralog, Gfi1b, regulates HSC, megakaryocyte and erythroid development. In zebrafish, gfi1aa is essential for primitive hematopoiesis; however, little is known about the role of gfi1aa in definitive hematopoiesis or about additional gfi factors in zebrafish. Here, we report the isolation and characterization of an additional hematopoietic gfi factor, gfi1b. We show that gfi1aa and gfi1b are expressed in the primitive and definitive sites of hematopoiesis in zebrafish. Our functional
analyses demonstrate that Gfi1aa and Gfi1b have distinct roles in regulating primitive and definitive hematopoietic progenitors, respectively. Loss of Gfi1aa silences markers of early primitive progenitors, scl and gata1. Conversely, loss of Gfi1b silences runx-1, c-myb, ikaros and cd41, indicating that Gfi1b is required for definitive hematopoiesis. We determine the epistatic relationships between the gfi factors and key hematopoietic transcription factors, demonstrating that Gfi1aa and Gfi1b join lmo2, scl, runx-1 and c-myb as critical regulators of teleost HSPC. Our studies establish a comparative paradigm for the regulation of hematopoietic lineages by gfi transcription factors.

**Keywords**

primitive and definitive hematopoiesis; differentiation; lineage commitment

**INTRODUCTION**

Growth factor independence-1a (Gfi1a) is a proto-oncogene that was identified as a gene up-regulated by retroviral insertion of a Moloney murine leukemia virus in an interleukin 2 (IL-2) dependent T-cell lymphoma line (Gilks et al., 1993). A closely related gene, Growth factor independence-1b (Gfi1b) was subsequently cloned based on high nucleotide homology to Gfi1 (Grimes et al., 1996) Both Gfi genes are members of the highly conserved Snail superfamily of zinc-finger transcription factors that bind to consensus DNA sequences and act as transcriptional repressors by recruiting histone-modifying enzymes at target promoters. The Gfi1a and Gfi1b proteins share a highly homologous N-terminal 20 amino-acid SNAG (for Snail/Gfi1) domain, and a C-terminal domain that consists of six C2H2 type zinc fingers (van der Meer et al., 2010). The SNAG domain is critical to the function of Gfi1a as a position- and orientation-independent transcriptional repressor (Grimes et al., 1996).

In mammals, Gfi1a and Gfi1b function as hematopoietic transcription factors with distinct and overlapping roles as regulators of HSC and subsequent lineage commitment and differentiation (van der Meer et al., 2010). Both Gfi1a and Gfi1b are expressed in mouse HSC, where they are responsible for repressing HSC proliferation, maintaining stem cell quiescence and self-renewal capacity (Hock et al., 2004; Khandanpour et al., 2010). Interestingly, either Gfi1a or Gfi1b is dispensable for maintenance of mouse embryonic HSC, while loss of both Gfi genes in mouse is incompatible with HSC maintenance (Khandanpour et al., 2010).

Although Gfi1a and Gfi1b have overlapping roles in HSC, in downstream lineages, Gfi1a and Gfi1b have discrete roles as the respective regulators of myeloid and lymphoid lineages and erythroid and megakaryocyte lineages, respectively (van der Meer et al., 2010). Loss of Gfi1a in mouse results in neutropenia, a block in myeloid differentiation and impaired T-cell development (Schmidt et al., 1998; Karsunky et al., 2002). Additionally, human patients with dominant-negative mutations in GFI1A suffer from severe congenital neutropenia (Person et al., 2003), underscoring a conserved function of the mammalian Gfi genes. In addition to expression in hematopoietic tissues, Gfi1a is also expressed in the mouse lung and in precursor cells of the central and peripheral nervous systems including the sensory epithelia of the developing inner ear, where it is required for inner ear hair cell differentiation (Wallis et al., 2003).

In contrast to Gfi1a, loss of Gfi1b has no effect on myelopoiesis (Saleque et al., 2002). Rather, loss of Gfi1b specifically impairs erythrocyte and megakaryocyte development (Saleque et al., 2002). Gfi1b deficient mice have a block in megakaryocyte maturation and
die in utero during the transition from primitive to definitive erythropoiesis, suggesting that Gfi1b plays a critical role in facilitating the differentiation of bi-potential erythroid/megakaryocytic progenitors (Saleque et al., 2002). Due to the embryonic lethal phenotype of Gfi1b-knockout mice, investigators have turned to in vitro models to better characterize the role of Gfi1b in hematopoiesis. For example, recent studies in human HEK293 and K562 cells have demonstrated that Gfi1b repression of transforming growth factor-b receptor III is required for proper differentiation and expansion of erythroid progenitors (Randrianarison-Huetz et al., 2010), offering one mechanism by which Gfi1b regulates erythropoiesis.

To further characterize the function of the Gfi family genes in vivo, we utilized the genetics of Danio rerio. Notably, zebrafish reproduce oviparously and embryos can survive without blood cells for up to 10 days due to passive diffusion of oxygen from the water (Pelster and Burggren, 1996). These attributes make the zebrafish model particularly amenable to decipher the function of the Gfi transcription factors in hematopoiesis.

Zebrafish have three gfi family genes: gfi1ab (Dufourcq et al., 2004), gfi1aa (Wei et al., 2008) and gfi1b. Cloning and gene expression pattern studies in zebrafish have shown a non-hematopoietic role for gfi1ab, which is expressed in the ganglion cells of the neural retina and the hair cells of the inner ear (Dufourcq et al., 2004). We used the highly conserved zinc finger region of gfi1ab as a probe to screen a cDNA library of zebrafish HSC to identify two additional members of the zebrafish gfi family, gfi1aa and gfi1b. The cloning and functional analysis of gfi1aa has recently been described (Wei et al., 2008), advancing the understanding of its role as a regulator of primitive hematopoiesis (Wei et al., 2008). However, little is known about the role of gfi1aa in definitive hematopoiesis or about the role of gfi1b in blood development. Here, we used in vivo loss-of-function studies to analyze the roles of these two hematopoietic gfi factors in zebrafish HSPC biology. Our findings reveal that gfi1aa is a critical regulator of primitive hematopoietic progenitor populations, while gfi1b regulates definitive HSC. We further place these two gfi factors in a hierarchical, epistatic relationship relative to other transcription factor regulators of primitive and definitive hematopoiesis, thereby demonstrating that gfi1aa and gfi1b act in parallel with either primitive or definitive regulators of HSPC.

**MATERIALS AND METHODS**

**Nomenclature**

This work references orthologous Gfi-family genes using a newly standardized convention recommended by Zebrafish Information Network (ZFIN) and the HUGO Gene Nomenclature Committee (HGNC). The former names of the genes referenced in this work are included here in parentheses for reference: Hs GFI1A (GFI1), Mm Gfi1a (Gfi1), Dr gfi1ab (gfi1), and Dr gfi1aa (gfi1.1).

**Zebrafish Maintenance and Studies**

Wild-type (AB*, Tü) and transgenic zebrafish (Danio rerio) were maintained, bred and staged according to standard methods (Amigo et al., 2009). cloche (clo<sup>n39</sup>) (Stainier et al., 1995), scl (scl/tal1<sup>121384</sup>) (Bussmann et al., 2007), vlad tepes (vlt<sup>n651</sup>) (Lyons et al., 2002), frascati (frs<sup>tq223</sup>) (Shaw et al., 2006) and runx-1 (runx-1<sup>W845</sup>) (Sood et al., 2010) mutant lines have been described previously. The following transgenic zebrafish lines were used in this study: Tg(gata1:eGFP) (Long et al., 1997), Tg(pu.1:eGFP) (Hsu et al., 2004), Tg(globin-LCR:eGFP) (Ganis et al., 2012), and Tg(cd41:eGFP) (Lin et al., 2005). All zebrafish experiments were conducted with the guidance and approval of the Institutional Animal Care and Use Committee at Boston Children’s Hospital.
Isolation and Sequence Analysis of Zebrafish Hematopoietic *gfi* Genes

Embryonic and adult blood, and kidney marrow cells were collected from *Tg(cd41:GFP)* transgenic fish and subjected to fluorescence-activated cell sorting (FACS) using a Becton Dickenson FACS Vantage SE machine. After two rounds of sorting, cells expressing GFP$^{lo}$ and GFP$^{hi}$ were collected to generate a cDNA library in TriplEx2 (Clontech).

The cloning of the zebrafish *gfi1ab* has been described (Dufourcq et al., 2004). Using the published sequence, primers flanking zinc fingers 1 to 6 were designed to amplify a DNA fragment of zebrafish *gfi1ab*. The primer sequences are in Table S1. This fragment was used as a probe to screen $\sim 10^6$ plaque forming units of our cDNA library under a low stringency hybridization wash (2xSSC, 0.1% SDS, at 42°C). From this screen, we recovered three distinct *gfi* related family members. One was “*gfi1ab*”, including additional 5′ and 3′ untranslated region, was identical to a previously published “*gfi1*” clone (Dufourcq et al., 2004). The second clone was identified as “*gfi1aa*”, the putative zebrafish ortholog of *Gfi1a* and previously referred to as “*gfi1.1*” (Wei et al., 2008). The third clone was referred to as “*gfi1b*”, according to Zebrafish Information Network nomenclature standards (http://zfin.org/zf_info/nomen.html). The *gfi1b* cDNA sequence has been deposited in GenBank under accession code: HQ599192. *gfi1aa* and *gfi1b* were subcloned in the vector pCS2+.

Sequence Analysis and Bioinformatics

DNA and protein sequence analysis was performed using Lasergene software (DNASTAR, Madison, WI) and the protein prediction and analysis tools at the ExPASy home page (http://au.expasy.org/). Genomic DNA sequences data were produced by the Zebrafish Sequencing Group at the Sanger Institute and can be obtained from (http://www.ensembl.org/Danio_rerio/).

Whole Mount RNA *in situ* Hybridization, Hemoglobin and Cartilage Staining

Digoxigenin-labeled antisense RNA probes were generated according to the manufacture’s protocol using subcloned *gfi1aa* and *gfi1b* (Roche). Whole mount *in situ* hybridization (WISH) was performed as previously described (Amigo et al., 2009). Embryos were stained for hemoglobinized cells with o-dianisidine (Sigma) as previously described (Amigo et al., 2009). Embryo cartilage was stained with Alcian Blue as described previously (Amigo et al., 2009). For histological sectioning, embryos were processed by WISH and embedded in JB4 plastic resin (Polysciences Inc.); 8–10-μm sections were mounted onto glass slides and photographed as previously described (Burns et al., 2005).

Morpholino Injection

Custom antisense morpholino oligomers (MO) (Gene Tools) were designed against *gfi1aa* and *gfi1b*. Two different MO targeting distinct sequences (translational methionine initiator, ATG MO, and splice site blocking, splice MO) were used to knockdown each respective gene. Both *gfi1aa* MO have been used previously (Wei et al., 2008). A standard MO was used as a control. MO sequences can be found in Supplementary Table S1. Approximately 10–20 ng of *gfi1aa*, *gfi1b* or standard control MO was injected into 1–2 cell-stage embryos.

RNA Isolation, cDNA Synthesis, and Quantitative Reverse Transcription PCR (qRT-PCR)

Pools of 30 embryos were collected at 96 hpf; RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) and the First Strand cDNA Synthesis Kit (Roche) was used to generate cDNA as described previously (Nilsson et al., 2009). Real-time qRT-PCR was performed on an iQ5 Real-Time PCR Detection System (Bio-Rad). TaqMan Gene Expression Assays for *gfi1aa*, *gfi1b*, *itga2b* and *prf1dc1* were obtained from Applied Biosystems. Analysis was
performed by the $2^{-\Delta\Delta C(T)}$ method as described previously (Schmittgen and Livak, 2008; Nilsson et al., 2009).

**Fluorescence-Activated Cell Sorting of Transgenic Embryos**

Pools of 20–100 embryos were collected from MO-injected and control clutches. Disaggregated cells were sequentially passed through 70 μm and 40 μm cell strainers, washed in Hank’s Balanced Salt Solution (HBSS) (Sigma), and pelleted by low speed centrifugation. The cells were re-suspended in HBSS. Cells were sorted in a BD Biosciences FACS Vantage SE machine.

**DNA Isolation and Allele-specific Oligonucleotide Hybridization (ASO)**

DNA was isolated from paraformaldehyde-fixed embryos as described previously (Meeker et al., 2007). Primer sequences used to amplify a fragment of runx-1 DNA are in Table S1. For the ASO assay, approximately 10 ng of PCR products were dotted on a Hybond nylon membrane (Amersham) as described previously (Paw et al., 1990). ASO probe sequences for mutant and wild type runx-1 alleles are in Table S1. The ASO probes were 5′-kinased with [$\gamma$-32P]ATP (6000 Ci/mmol, Amersham). Membranes were washed with tetramethylammonium chloride solution (Sigma) as described previously (Farr et al., 1988) and subjected to autoradiography.

**Imaging and Analysis**

Stained embryos were photographed using a Leica MXFLIII microscope and a DC500 digital camera (8 bit). Transgenic embryos were photographed on a Nikon TE–200 microscope equipped with a 10X objective (numerical aperture, 1.4). Fluorescent images were acquired with an Orca IIER charge-coupled-device camera (Hamamatsu). The electronic shutters were controlled with Metamorph software (Universal Imaging Corporation of Molecular Devices).

**Statistical Analysis**

Data are displayed as relative mean ± standard error of the mean (SEM) with the number of experiments indicated. Pairwise comparisons were performed by the Student t test. Significance was set at $p < 0.05$.

**RESULTS**

**Isolation and Cloning of Zebrafish gfi Genes**

We performed a screen of a zebrafish cDNA library constructed from cd41 GFPlo expressing cells using a PCR amplified DNA fragment corresponding to the zinc finger domain of zebrafish gfi1ab as a probe (Dufourcq et al., 2004). cd41 GFPlo cells have been functionally characterized as zebrafish HSC (Ma et al., 2011). We isolated 9 full-length clones under a low-stringency hybridization wash. DNA sequencing analyses revealed that these clones encode polypeptides with high homology to mouse and human oncoprotein Gfi family members. Three clones encode full-length polypeptides containing a conserved 20 amino acid SNAG transcriptional repressor domain and 6 C2H2 zinc finger domains. One of the recovered clones was a full-length clone of the published zebrafish gfi1ab, including additional 5′ and 3′ un-translated region sequences to the published results (Dufourcq et al., 2004). The second gene was a full-length clone of the previously published zebrafish gfi1aa (Wei et al., 2008). Based on peptide homology and gene expression pattern, we termed the third gene gfi1b.
The genomic organization and exon-intron boundaries of zebrafish *gfi1aa* and *gfi1b* were assembled from available genomic DNA sequences using Genotrace and Spidey applications. The results revealed that both zebrafish genes consist of 6 coding exons and 5 introns with a genomic organization similar to the human *GFI1A* and *GFI1B* (data not shown). The first exon encodes the first 106 amino acids including the SNAG domain, which is conserved between human, mouse, and zebrafish *Gfi* genes (Figure 1A). The homologous C-terminal zinc fingers are coded by the 3’ end of exon 3 and continue to the exon 6. Our in silico data was verified by PCR amplification of zebrafish genomic DNA using intron-specific primers flanking the exon/intron boundaries (data not shown).

Phylogenetic analysis revealed that zebrafish *gfi1ab* and *gfi1aa* cluster most closely with mammalian *Gfi1a* factors (Figure 1B), suggesting that the functions of mammalian *Gfi1a* were split between the zebrafish *gfi1ab* and *gfi1aa* genes in a gene duplication event during teleost evolution (Postlethwait et al., 2000; Woods et al., 2000). Likewise, our phylogenetic analysis revealed that *gfi1b* clusters most closely with mammalian *Gfi1b* factors (Figure 1B). Since *gfi1ab* lacks expression in hematopoietic tissues (Dufourcq et al., 2004), we chose to focus our functional analyses on *gfi1aa* and *gfi1b*.

**Zebrafish gfi1aa and gfi1b are expressed in primitive and definitive hematopoietic tissues**

In zebrafish, hematopoietic progenitors are first observed in the lateral plate mesoderm (LPM), which subsequently gives rise to the intermediate cell mass (ICM), the functional equivalent of the yolk sac blood islands in mammals (Davidson and Zon, 2004). To compare the embryonic expression of *gfi1aa* and *gfi1b*, we performed WISH of wild-type zebrafish embryos at different developmental stages. Similar to the expression of *Friend of GATA1* (*FOG-1*), another hematopoietic transcription factor (Amigo et al., 2009), *gfi1aa* and *gfi1b* are expressed as maternal transcripts at the 2-cell stage (Figure 2A, a–c). The maternal expression pattern persists through the sphere stage, where *gfi1aa* and *gfi1b* are expressed along with *FOG-1* mRNA (Figure 2A, d–f). At the 5 and 10 somite stages (ss) *gfi1aa* and *FOG-1* zygotic expression is localized to the LPM, suggesting a role for *gfi1aa* in the earliest stages of hematopoiesis (Figure 2A, g–h, j–k). In contrast, *gfi1b* is abundantly expressed in the ectoderm (Figure 2A, i,l), analogous to *gata2* expression early in development (Thompson et al., 1998). As the LPM converges to the ICM at the 15 ss, *gfi1b* expression transitions from the ectoderm to the ICM (Figure 2A, o,r). Expression of *FOG-1* and *gfi1aa* persists in the LPM at the 15 ss (Figure 2A, m–n, p–q). By the 20 ss *gfi1aa* and *gfi1b* expression is localized to the ICM along with *FOG-1* (Figure 2A, s–u), after which point *gfi1aa* expression decreases at 24 hours post fertilization (hpf) (Figure 2A, w). In contrast, *gfi1b* and *FOG-1* are robustly expressed in the ICM at 24 hpf (Figure 2A, v,x). In zebrafish, definitive hematopoiesis occurs in the ventral wall of dorsal aorta, which is analogous to the aorta-gonad-mesonephros (AGM) region in mammals (Davidson and Zon, 2004). Definitive HSC expressing *runx-1* and *c-myb* can be detected at 36 hpf in the AGM (Davidson and Zon, 2004). Whole-mount and section analyses indicated that both *gfi1aa* and *gfi1b* are expressed in the AGM along with *runx-1* and *c-myb* at 36 hpf (Figure 2B, a–f).

**Zebrafish gfi1aa is required for primitive hematopoiesis**

To evaluate the roles of *gfi1aa* and *gfi1b* in hematopoiesis, we designed two different MO to knockdown each *gfi* gene. The MO target either: (1) the 5’UTR and putative ATG methionine initiator site of their respective *gfi* transcript (translation-blocker), or (2) intron/exon splice junction sequences unique to each *gfi* gene (splice-blocker). Injection of both the translation-blocker and splice-blocker MO resulted in identical, distinct phenotypes for each *gfi* gene. To verify the specificity of each ATG MO, we performed an in vitro translation in the absence or presence of increasing concentrations of matched and mismatched MO to evaluate the inhibition of protein synthesis. As shown in Supplementary Figure 1A, an
increasing concentration of *gfi1aa* ATG MO specifically inhibits the *in vitro* translation of *gfi1aa*, whereas it has no effect on the translation of *gfi1b* mRNA. Similarly, the *gfi1b* ATG MO has no effect on the translation of *gfi1aa* mRNA, but effectively inhibits translation of its cognate *gfi1b* mRNA. qRT-PCR analysis of embryos injected with the ATG MO showed no significant change in the level of *gfi* transcripts, indicating a lack auto- or cross-regulatory feedback mechanisms (Supplementary Figure 1B). To verify the efficacy of each splice-blocker MO, qRT-PCR analysis showed that each *gfi* MO efficiently and selectively targets its respective pre-mRNA (Supplementary Figure 1C). As with the ATG MO, qRT-PCR showed no auto- or cross-regulatory feedback with the splice-blocker MO (Supplementary Figure 1C).

Embryos injected with either *gfi1aa* or *gfi1b* MO (hereafter referred to as “morphants”) show normal expression of hematopoietic progenitors, as evident by lmo2 and *gata2* expression at the 20 ss (Figure 3A, a–f). However, consistent with prior analyses (Wei et al., 2008), *gfi1aa* morphants show a reduction in *scl* and *gata1* expression, the respective markers of primitive hematopoietic progenitors (Figure 3Ah,k). These results are consistent with observations that loss of either *scl* or *gata1* does not impact the initial specification of hematopoietic progenitors, but does impair subsequent differentiation (Dooley et al., 2005; Amigo et al., 2011). In contrast to *gfi1aa* morphants, *gfi1b* morphants show no change in either *scl* or *gata1* expression, indicating that *gfi1b* is not essential for the initial commitment or differentiation of primitive hematopoietic progenitors (Figure 3A,i,l). To validate and quantify our *gata1* results, we knocked down *gfi1aa* and *gfi1b* in the Tg(*gata1:eGFP*) reporter line and subjected the morphants to FACS analysis at the 20 ss. We found a significant decrease in the population of *gata1*+ cells in *gfi1aa* morphants (Figure 3B, a). Consistent with our WISH results, no change in the population of *gata1*+ cells was observed after knockdown of *gfi1b* (Figure 3B, a).

Primitive hematopoiesis generates *gata1* regulated erythroid progenitors and *pu.1* regulated myeloid progenitors (Lieschke et al., 2002). The cross antagonism of *gata1* and *pu.1* in primitive hematopoiesis is well-characterized (Rhodes et al., 2005). Since our analysis of *gfi1aa* morphants showed a significant reduction in the pool of primitive *gata1*+ erythroid progenitors, we sought to determine whether the population of *pu.1* progenitors was affected. Consistent with the antagonistic relationship between *gata1* and *pu.1*, WISH showed an increase in the expression of *pu.1* expressing cells in *gfi1aa* morphants, while *gfi1b* morphants showed no change in *pu.1* expression (Figure 3A, m–o). Also consistent with the increase in *pu.1*+ cells, the *mpo*+ myeloid population downstream of *pu.1* is expanded in *gfi1aa* morphants at 48 hpf (Figure 3A, p–r). To rigorously quantify the change in the population of primitive myeloid progenitors, we performed FACS in the Tg(*pu.1:eGFP*) line (Hsu et al., 2004). FACS analysis of *gfi1aa* morphants at the 20 ss revealed a significant increase in the population of *pu.1*+ myeloid cells (Figure 3B, b). The increase in *pu.1*+ cell number is consistent with previous observations showing that overexpression of *gfi1aa* expands the pool of erythroid progenitors at the expense of myeloid progenitors (Wei et al., 2008). In contrast to the knockdown of *gfi1aa*, the knockdown of *gfi1b* had no effect on the population of *pu.1*+ myeloid progenitors (Figure 3B, b), thereby reinforcing our conclusion that *gfi1b* has no functional role in primitive hematopoiesis.

**gfi1aa and gfi1b have distinct roles in erythropoiesis**

To further evaluate the roles of *gfi1aa* and *gfi1b* in erythropoiesis, we stained *gfi* morphants with o-dianisidine and assessed their degree of hemoglobinization. At 48 hpf, *gfi1aa* morphants have reduced hemoglobinization relative to control embryos (Figure 4A, a–b). In contrast, *gfi1b* morphants showed normal levels of hemoglobinization (Figure 4A, c). At 72 hpf, both *gfi1aa* and *gfi1b* morphants show a partial reduction in hemoglobinized erythrocytes (Figure 4A, d–f). This incomplete reduction motivated us to investigate
whether knockdown of both \textit{gfi} genes would have a greater impact on hemoglobinization than knockdown of either gene alone. Knockdown of both \textit{gfi} genes using the same total dosage of MO showed an absence of hemoglobinized cells at 72 hpf (Figure 4A, g). Extending our time course, we evaluated hemoglobinization in \textit{gfi} morphants at 120 hpf. Remarkably, \textit{gfi1aa} morphants recover from their initial anemia as observed by their abundance of hemoglobinized cells at 120 hpf (Figure 4A, h–i). Conversely, \textit{gfi1b} morphants are severely anemic by 120 hpf (Figure 4A, j). To rigorously quantify the change in erythrocytes over time in \textit{gfi} morphants, we knocked down \textit{gfi1aa} and \textit{gfi1b} in the \textit{Tg(globin-LCR:eGFP)} line. Consistent with the hemoglobinization trend observed by \textit{o-dianisidine} staining, \textit{gfi1aa} morphants showed a significant reduction in erythrocytes at 48 hpf, subsequently recovering by 120 hpf, while \textit{gfi1b} morphants showed a significant decrease in erythrocytes at later time points (Figure 4B). \textit{In toto}, our erythrocyte data demonstrated that \textit{gfi1aa} and \textit{gfi1b} have discrete roles at different stages of erythropoiesis and suggested that the loss of each \textit{gfi} gene could be differentially impacting primitive and definitive hematopoietic lineages.

**Zebrafish \textit{gfi1b} is required for definitive hematopoiesis**

To evaluate the roles of \textit{gfi1aa} and \textit{gfi1b} in definitive hematopoiesis, we knocked down each gene and used WISH to evaluate the impact on definitive HSC using \textit{runx-1}, \textit{c-myb} and \textit{ikaros} as markers (Davidson and Zon, 2004). Loss of \textit{gfi1b}, but not \textit{gfi1aa}, reduces the expression of \textit{runx-1}, \textit{c-myb} and \textit{ikaros} in the AGM at 36 hpf, indicating a reduction of definitive HSC (Figure 5A, a–f). To quantify the reduction in definitive HSC, we performed FACS of \textit{Tg(cd41:eGFP)} \textit{gfi} morphants and examined the GFP$^{lo}$ expressing definitive HSC population (Ma et al., 2011). Consistent with our WISH results, loss of \textit{gfi1b} reduces the population of definitive HSC, while loss of \textit{gfi1aa} has no effect (Figure 5B). We also used the \textit{Tg(cd41:eGFP)} line to evaluate the thrombocyte lineage in \textit{gfi} morphants using GFP$^{hi}$ as a marker. Zebrafish thrombocytes are the hemostatic cellular equivalent of mammalian platelets (Lin et al., 2005). Consistent with the loss of definitive HSC in \textit{gfi1b} morphants, loss of \textit{gfi1b} reduces the population of downstream thrombocytes (Figure 5B). Loss of \textit{gfi1aa} caused an unexpected increase in the thrombocyte population, possibly due to reactive thrombocytosis often observed in anemias (Dan, 2005) (Figure 5B). To confirm the defect in thrombopoiesis, we analyzed the expression of \textit{cd41} in \textit{gfi} morphants using \textit{itga2b}. \textit{itga2b} encodes the platelet glycoprotein GPIIb and is a member of the \textit{cd41} protein complex in zebrafish thrombocytes (Davidson and Zon, 2004). Consistent with our thrombocyte FACS data, \textit{gfi1b} morphants showed a significant reduction in expression of \textit{itga2b} relative to controls (Figure 5B). \textit{gfi1aa} morphants, in contrast, showed no significant change in \textit{itga2b} expression (Figure 5B).

To assess the role of \textit{gfi1aa} and \textit{gfi1b} in lymphocyte development, we performed WISH of \textit{rag-1} in \textit{gfi} morphants. Consistent with their lack of \textit{ikaros} expression, \textit{gfi1b} morphants showed a reduction in \textit{rag-1} expressing thymic lymphocytes, while the thymic expression of \textit{rag-1} was preserved in \textit{gfi1aa} morphants (Figure 5C, a–c). Since other hematopoietic factors have been shown to participate in ectoderm and endoderm development (Amigo et al., 2009), we further analyzed whether the jaw cartilage supporting the thymic anlage was properly formed in \textit{gfi} morphants. Alcian blue staining showed dysplastic development of the jaw cartilage in \textit{gfi1b} morphants, while \textit{gfi1aa} morphants showed normal jaw cartilage development (Figure 5C, d–f).

**Genetic epistasis of \textit{gfi1aa} and \textit{gfi1b} in primitive and definitive hematopoiesis**

To understand the epistatic relationship of the \textit{gfi} genes in the context of other hematopoietic transcription factors, we evaluated the ICM expression of \textit{gfi1aa} and \textit{gfi1b} in mutant zebrafish with genetic blocks at sequential stages of hematopoietic differentiation.
Since both gfi1aa and gfi1b are strongly expressed in the ICM at the 20 ss in wild type zebrafish (Figure 6A, a–b, arrowhead), we chose this stage to evaluate gfi expression in mutant embryos. The earliest blood mutant, cloche (clo), is defective in a gene at the hemangioblast level that specifies the formation of hematopoietic and vascular progenitors (Stainier et al., 1995). Neither gfi1aa nor gfi1b are expressed in the ICM in clo embryos (Figure 6A, c–d), indicating that the clo gene product functions upstream of gfi1aa and gfi1b and confirming the hematopoietic specificity of our WISH results. We next evaluated gfi expression in the scl mutant, scl^{2138} (Bussmann et al., 2007). Previous epistatic analyses have confirmed that scl acts downstream of clo in primitive hematopoietic progenitors (Gering et al., 1998). Expression of both gfi1aa and gfi1b is absent in scl^{2138} mutants (Figure 6A, e–f), suggesting that the gfi genes function either downstream or in parallel to scl. Taken together with our prior loss-of-function analyses, gfi1aa and scl likely function in parallel, as loss of either gene silences expression of the other gene.

Moving further downstream in the epistatic hierarchy, we evaluated gfi expression in the gata1 mutant, vlad tepes (vlt) (Lyons et al., 2002). gata1 functions downstream of scl to drive the specification of erythroid progenitors (Davidson and Zon, 2004). The expression of gfi1aa is preserved in vlt mutants, indicating that gfi1aa is epistatically upstream of gata1 (Figure 6A, g). In contrast, gfi1b expression is absent in vlt mutants, indicating that gfi1b functions downstream of gata1 in primitive hematopoiesis (Figure 6A, h). To determine whether gata1 also regulates gfi1b in definitive hematopoiesis, we isolated RNA from control and gata1 morphants at 30, 36, and 96 hpf and performed qRT-PCR using probes for gfi1aa, gfi1b, and cd41 (itga2b). gfi1b expression is reduced in gata1 morphants at 30 and 36 hpf, reinforcing our conclusion that gfi1b functions downstream or in parallel to gata1 in the primitive program (Figure S2). In contrast to the primitive wave, both gfi1b and cd41 show normal levels of expression in gata1 morphants by 4 dpf, indicating that their expression is not dependent on gata1 in the definitive program (Figure S2) (Davidson and Zon, 2004).

We next evaluated gfi1aa and gfi1b expression in the frascati (frs) mutant (Shaw et al., 2006), which has a block in erythroid maturation at a later stage than vlt. frs mutants are defective for mitoferrin1 (slc25a37), the primary importer of mitochondrial iron in developing erythroblasts (Shaw et al., 2006). Both gfi1aa and gfi1b are expressed in frs mutants, indicating that the block in terminal erythroid differentiation in frs mutants is epistatically downstream of the expression of the gfi genes.

To strengthen our epistasis model, we analyzed the expression of lmo2 and gata2 in primitive hematopoietic progenitors in the ICM of mutants with defects in early hematopoiesis. Compared to wild type embryos, which have robust expression of lmo2 and gata2 in the ICM at the 20 ss (Figure 6B, a–b), clo mutants lack lmo2 and gata2 expression in the ICM due to an absence of hematopoietic progenitors (Stainier et al., 1995) (Figure 6B, c–d). Conversely, both lmo2 and gata2 are expressed in scl and vlt mutants (Figure 6B, e–h), thereby excluding the possibility that loss of gfi expression in scl and vlt mutants is due to a global loss of hematopoietic progenitors. The presence of lmo2 and gata2 in the scl mutant is consistent with previous observations in embryos depleted of scl by MO-mediated knockdown (Dooley et al., 2005; Patterson et al., 2005).

Extending our epistasis studies to definitive hematopoiesis, we examined the expression of gfi1aa and gfi1b in the AGM in runx-1 mutants, which lack definitive HSC (Sood et al., 2010). We included ikaros, another marker of definitive HSC progenitors (Davidson and Zon, 2004) as a reference control. The AGM expression of ikaros is silenced in runx-1 mutants as compared to wild type embryos (Figure 6C, a–b, brackets), thus confirming the defect in definitive HSC in runx-1 mutants. Consistent with our functional data, gfi1aa has
no role in definitive hematopoiesis, as loss of runx-1 does not impact gfi1aa expression in the AGM (Figure 6C, c–d, brackets). Conversely, expression of gfi1b is silenced in runx-1 mutants (Figure 6C, e–f), demonstrating that gfi1b is epistatically in parallel or downstream of runx-1. We further validated our genetic analysis using ASO probes specific for either wild type or mutant runx-1 alleles to genotype representative embryos. Taken together with our prior loss-of-function analyses, we conclude that gfi1b and runx-1 likely function in parallel, as loss of either gene silences expression of the other gene.

DISCUSSION

In mammalian models, Gfi factors have discrete roles as critical regulators of either myeloid and lymphoid lineages (Gfi1a) or erythroid and megakaryocyte lineages (Gfi1b). Previous studies in zebrafish have proposed that gfi1aa functions as the teleost ortholog of Gfi1a (Wei et al., 2008); however, the phenotype of gfi1aa morphants, particularly the severe anemia observed during early embryogenesis, is inconsistent with the mammalian Gfi1a paradigm. Through extending the functional studies of zebrafish gfi1aa in definitive hematopoiesis and functionally characterizing a novel gfi-family transcription factor, gfi1b, we suggest that zebrafish gfi factors are functionally distinct from their mammalian orthologs. In contrast to the mammalian Gfi paradigm, we demonstrate that the putative zebrafish ortholog of Gfi1a, gfi1aa, is specifically required in early embryogenesis as a regulator of primitive hematopoietic progenitors, while gfi1b is required at a later stage to regulate definitive HSC (Figure 7).

Studies of Gfi1a knockouts in mouse have established that Gfi1a is required to maintain HSC quiescence and preserve HSC self-renewal capacity (Hock et al., 2004; Zeng et al., 2004). Despite a critical role in HSC maintenance, loss of Gfi1a in mouse results in discrete, rather than global defects in specific downstream lineages (Karsunky et al., 2002; Hock et al., 2003). Two independent studies of Gfi1a knockout mice have demonstrated that Gfi1a deficiency impairs differentiation of granulocytes, neutrophils and lymphocytes while preserving erythrocytes and megakaryocytes (Karsunky et al., 2002; Hock et al., 2003). In contrast to the mouse model, we show that loss of gfi1aa in zebrafish results in severe anemia in early embryogenesis secondary to a reduction in scl and gata1 expression. By quantifying the change in erythrocytes over time and examining definitive HSC markers, we demonstrate that loss of gfi1aa specifically impacts primitive, but not definitive hematopoietic progenitors.

Interestingly, the defect in primitive hematopoiesis observed in gfi1aa morphants is strikingly similar to the phenotype of an unmapped zebrafish mutant named bloodless (bls) (Liao et al., 2002). The bls mutant, which has a specific defect in ICM hematopoiesis, starts to recover erythrocytes and other lineages after 5 dpf (Liao et al., 2002). Like gfi1aa morphants, bls embryos show reduced expression of scl and gata1 in primitive wave hematopoiesis, but subsequently recover during definitive hematopoiesis (Liao et al., 2002). Since the bls gene product has yet to be identified, gfi1aa might be a reasonable candidate gene for the bls mutant.

In contrast to the well-established role of Gfi1a as a regulator of HSC, evidence that Gfi1b plays a role in mammalian HSC regulation has only recently emerged. Like Gfi1a, Gfi1b also regulates HSC dormancy and quiescence (Khandanpour et al., 2010). Notably, mouse knockouts of Gfi1b result in an increase in HSC populations while specifically reducing the populations of downstream platelets and erythrocytes (Khandanpour et al., 2010). Myelopoiesis and lymphopoiesis is normal in Gfi1b knockout mice (Saleque et al., 2002; Khandanpour et al., 2010). In contrast to the mouse model of Gfi1b function, loss of gfi1b impacts definitive HSC populations and, consequently, the ability to generate downstream...
definitive hematopoietic lineages. While *gfi1aa* morphants are severely anemic during early embryogenesis, we showed that the erythrocyte population in *gfi1b* morphants is intact at this stage. However, as the production of erythrocytes transitions from the primitive to the definitive HSC program, *gfi1b* morphants show a steady reduction in erythrocytes over time. A similar phenotype is observed beginning at 3 dpf in the *grechetto* zebrafish mutant, which undergoes normal primitive wave hematopoiesis but lacks definitive HSC due to a defect in *cpsf1* (Bolli et al., 2011).

After establishing that *gfi1aa* and *gfi1b* represent a departure from the mammalian Gfi paradigm, we analyzed the epistatic relationship between *gfi1aa* and *gfi1b* and known teleost hematopoietic transcription factors. Using our analyses of *gfi* morphants and genetic mutants, we generated a model depicting the epistatic hierarchy of the *gfi* factors in primitive and definitive hematopoiesis. In primitive hematopoiesis, the *clo* gene product and *fil1* act at the top of the genetic cascade to drive the development of the hemangioblast from mesoderm precursors (Liu et al., 2008) (Figure 7A). The hemangioblast subsequently gives rise to endothelial and hematopoietic progenitors expressing *gata2*, *lmo2* and *scl* (Davidson and Zon, 2004). Our analysis of *scl* genetic mutants along with extensive studies of *lmo2* and *scl* morphants (Dooley et al., 2005; Patterson et al., 2005; 2007) demonstrated that *scl* and *lmo2* function in parallel, downstream of *gata2*. Through our analyses of *gfi1aa* morphants, we concluded that *gfi1aa* functions downstream of *gata2*, in parallel with *scl*. Recent murine studies have shown that *Scl* can regulate *Gfi1a* expression in early hematopoietic cells (Wilson et al., 2010); however, given the functional differences between *gfi1aa* and mammalian *Gfi1a*, it is unclear whether the specific regulatory mechanisms of higher vertebrates are directly applicable to the teleost *gfi* model. In more differentiated primitive populations, the reduction of erythroid progenitors and concomitant expansion of myeloid progenitors observed in *gfi1aa* morphants is a consequence of both reduced *gata1* expression and disinhibition of *pu.1* repression by loss of *gfi1aa*. Thus, the function of *Gfi1a* as a repressor of myeloid differentiation (Spooner et al., 2009) appears to be conserved in murine and teleost models.

While *gfi1aa* likely acts early in primitive hematopoiesis, our epistasis studies of genetic morphants indicate that *gfi1b* functions at a later stage, downstream or in parallel to *gata1* during the primitive wave. Extending our epistasis studies in definitive hematopoiesis, we further concluded that *gfi1b* acts in parallel with *runx-1*, *c-myb* and *ikaros* to regulate definitive HSC and subsequent downstream definitive lineages (Figure 7B). In contrast to the primitive program, the expression of *gfi1b* is not dependent on *gata1* in the definitive program.

The partitioning of complex mammalian Gfi transcription factors into teleost genes with different roles in zebrafish provides a unique opportunity to dissect discrete roles in hematopoiesis and embryogenesis. Through our cloning and functional analysis of a novel *gfi*-family transcription factor: *gfi1b*, and functional characterization of *gfi1aa* in definitive hematopoiesis, we showed that zebrafish have evolved a new paradigm for the regulation of primitive and definitive hematopoietic lineages. While mammalian *gfi* factors regulate the differentiation of hematopoietic lineages at the committed progenitor stage, teleost *gfi* factors regulate hematopoiesis at the HSPC level. This is not too dissimilar to *FOG-1*, which functions as a multi-versatile *GATA-1* cofactor in all hematopoietic and endodermal tissues in teleosts; in contrast, this multifaceted function is divided between *FOG-1* and *FOG-2* in higher vertebrates (Amigo et al., 2009).

We demonstrated that during early embryogenesis, zebrafish *gfi1aa* specifically regulates the primitive wave of hematopoiesis, while *gfi1b* regulates the subsequent, definitive wave.
By harnessing the genetic power of the zebrafish model, we placed the gfi factors within the hierarchy of genes whose native function is required for hematopoiesis.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Evolutionary conservation between members of zebrafish and mammalian growth factor independence family of zinc finger transcription factors

(A) Deduced amino acid alignment of zebrafish (Dr), human (Hs) and mouse (Mm) growth factor independence transcription factors. The conserved SNAG domain and zinc finger motifs are marked. Zinc finger domains are underlined and numbered. Conserved amino acid residues are highlighted in black. (B) The phylogenetic relationship of homologous zebrafish, mouse, and human Gfi factors was constructed based on the J. Hein method. The MegAlign application in DNASTAR software was used for multiple alignment and construction of the phylogenetic dendrogram. Zebrafish gfi1ab (NP_958495.1) and gfi1aa (NP_001018612.1) cluster most closely with human GFI1A (NP_005254.2) and mouse Gfi1a (NP_034408.1), while zebrafish gfi1b (HQ599192) clusters most closely with human GFI1B (NP_004179.3) and mouse Gfi1b (NP_032140.1). The corresponding GenBank accession numbers are given in parenthesis.
Figure 2. *gfi1aa* and *gfi1b* expression pattern during primitive and definitive hematopoiesis

The expression pattern of *gfi1aa* and *gfi1b* was detected by WISH of developing zebrafish embryos. (A) For the primitive wave of hematopoiesis, *FOG-1* was included as a reference control. Dark-blue staining indicates pre-zygotic maternal expression of *gfi1aa* and *gfi1b* transcripts, along with *FOG-1*, at the 2-cell and 1000-cell stages (a–f). At the 5 and 10 ss, zygotic *gfi1aa* and *FOG-1* expression is localized to the LPM (black arrowheads) (g–h, j–k), while *gfi1b* is expressed in the ectoderm (i,l). As the LPM converges to the ICM at the 15 ss, *gfi1aa* and *FOG-1* expression remains localized to the LPM (j–k, m,n), while *gfi1b* transitions from ectoderm to LPM expression (l,o). (The vertical arrow indicates the anterior-posterior axis and the horizontal arrow indicates the rostral-caudal axis.) *gfi1aa* and *gfi1b* are strongly expressed in the ICM at the 20 ss along with *FOG-1* (s–u). At 24 hpf, *gfi1b* and *FOG-1* remain strongly expressed in the ICM, while expression of *gfi1aa* shows a relative decrease in ICM expression (v–x, insert). (B) AGM expression of *gfi1aa* and *gfi1b* during definitive hematopoiesis was analyzed by whole-mount and sectioned *in situ* using *runx-1* and *c-myb* as reference controls (a–f). The white arrowhead indicates AGM expression of *runx-1* and *c-myb* in the ventral wall of the dorsal aorta (d).
Figure 3. gfi1aa regulates the primitive wave of zebrafish hematopoiesis

(A) gfi morphants and matching controls were subjected to WISH and analyzed for ICM expression (arrowhead) of primitive hematopoietic markers at the 20 ss. (a–f) ICM expression of the early hematopoietic markers, lmo2 and gata2, is preserved in gfi morphants and controls. (g–l) gfi1aa morphants show a reduction in ICM expression of scl and gata1, markers of primitive hematopoietic progenitors as compared to controls. Loss of gfi1b does not affect scl or gata1 expression in primitive hematopoiesis (c,f,i,l). (m–o) Loss of gfi1aa shows an expansion in the population of pu.1 myeloid progenitors, while no change is observed in gfi1b morphants. (p–r) The mpo myeloid population is expanded in gfi1aa morphants. (B) Transgenic reporter fish for primitive wave hematopoietic progenitors were subjected to FACS analysis after morpholino-mediated knockdown. (a) FACS of Tg(gata1:eGFP) embryos at the 20 ss reveals a significant decrease in the population of erythroid progenitors in gfi1aa morphants relative to controls. Erythroid progenitors are unaffected in gfi1b morphants (mean ± SE, t test, *p < 0.05, n = 3). (b) FACS of Tg(pu.1:eGFP) embryos at the 20 ss reveals a significant increase in the population of myeloid progenitors in gfi1aa morphants relative to controls. Myeloid progenitors are unaffected in gfi1b morphants (mean ± SE, t test, *p < 0.05, n = 5).
Figure 4. gfi1aa and gfi1b have distinct roles at different stages in erythropoiesis

(A) Morphant and control embryos were stained with o-dianisidine to detect hemoglobinized cells. At 48 hpf, knockdown of gfi1aa results in severe anemia, as indicated by the absence of hemoglobinized cells in the cardiac region (arrow) (a–b). Knockdown of gfi1b does not impact hemoglobinization at this stage (c). At 72 hpf, knockdown of either gfi1aa or gfi1b partially reduces hemoglobinized erythrocytes relative to control embryos, while knockdown of both genes results in a more profound reduction of hemoglobinized erythrocytes (d–g). At 120 hpf, gfi1aa morphants have recovered from their initial anemia, as indicated by the presence of hemoglobinized cells, whereas gfi1b morphants are severely anemic (h–j). (B) FACS of Tg(globin-LCR:eGFP) embryos injected with MO targeting either gfi1aa (green) or gfi1b (blue) quantifies the relative change in erythrocytes over time, showing that loss of either gfi1aa or gfi1b differentially impacts erythropoiesis at different stages of development (mean ± SE, *p < 0.05, n = 3).
Figure 5. Loss of gfi1b silences definitive HSC

(A) Loss of gfi1b silences runx-1, c-myb and ikaros expressing HSC in the AGM at 36 hpf relative to matched controls (a–f). Loss of gfi1b also reduces expression of GFP⁺ cd41 cells in Tg(cd41:eGFP) embryos at 96 hpf (d–f). (B) FACS of Tg(cd41:eGFP) embryos at 96 hpf injected with MO targeting either gfi1aa or gfi1b reveals a marked reduction in the GFPlo and GFPhi populations in gfi1b morphants (mean ± SE, t test, *p < 0.05, n = 3). qRT-PCR of gfi morphants shows significantly reduced expression of itga2b (cd41) in gfi1b morphants at 96 hpf (mean ± SE, t test, *p < 0.05, n = 3). (C) Loss of gfi1b silences rag-1 expressing thymic lymphocytes, while loss of gfi1aa has no effect on lymphopoiesis in the thymic anlage (a–c). (d–f) Embryos were stained with Alcian Blue to delineate the morphologic architecture of the jaw cartilages. gfi1aa morphants have normal jaw cartilage development compared with wild type controls (d,e), while gfi1b morphants show dysplastic development of the jaw cartilage (f).
Figure 6. *gfi1aa* and *gfi1b* function at different hierarchical levels in hematopoietic epistasis
(A) The ICM expression of *gfi1aa* and *gfi1b* was evaluated in mutants with genetic blocks at sequential stages in hematopoiesis at the 20 ss. (a–b) Both *gfi1aa* and *gfi1b* are expressed in the ICM (arrowheads) in wild type embryos. (c–d) Mutants with genetic defects at the hemangioblast level (*clo*) and hematopoietic progenitors (*scl*) specifically lack ICM expression of either *gfi1aa* or *gfi1b*. Note that the neural expression of the *gfi* genes is preserved in these genetic mutants, confirming the specificity of the assay. (g–h) ICM expression of *gfi1aa* is preserved in the *gata1* mutant, *vlt*, whereas *gfi1b* expression is deficient. (i–j) In contrast, both *gfi1aa* and *gfi1b* are expressed in the ICM of the *mfn1* mutant, *frs*. (B) To confirm the presence of hematopoietic progenitors in various genetic mutants, the expression of *lmo2* and *gata2* was evaluated in mutant embryos at the 20 ss. (a–b) Both *lmo2* and *gata2* are expressed in the ICM of wild type control embryos. (c–d) Expression of *lmo2* and *gata2* is preserved in *scl* and *gata1* (*vlt*) mutants, confirming that hematopoietic progenitors are present. (C) The AGM expression of *ikaros*, *gfi1aa* and *gfi1b* was evaluated in *runx-1* mutants at 36 hpf and embryos were genotyped for *runx-1* wild type and mutant alleles. *Ikaros* was included as a reference control. (a–b) AGM expression (brackets) of *ikaros* is silenced in *runx-1* mutants relative to wild type embryos (wt), confirming the defect in definitive hematopoiesis. (c–d) AGM expression of *gfi1aa* is preserved in *runx-1* mutants. (e–f) Loss of *runx-1* silences expression of *gfi1b* in the AGM.
Figure 7. Model of gfi1aa and gfi1b epistasis in primitive and definitive hematopoiesis

(A) The clo gene product and Fli1 function at the top of the hierarchal cascade to drive formation of the hemangioblast from mesoderm precursors. The hemangioblast subsequently gives rise to hematopoietic progenitors expressing gata2, lmo2 and scl. Expression of gata2 and lmo2 is independent of gfi1aa expression, while gfi1aa is required for scl expression, and vice-versa. Primitive hematopoietic progenitors expressing lmo2, scl and gfi1aa generate erythroid and myeloid progenitors, regulated by gata1 and pu.1, respectively. gata1 and pu.1 antagonize each other to specify erythroid vs. myeloid progenitor commitment. Downstream in the primitive wave, gata1 is required for gfi1b expression. (B) In definitive hematopoiesis, runx-1, c-myb, ikaros and gfi1b act in parallel to specify definitive HSC and subsequently drive formation of downstream definitive lineages marked by globin (erythrocytes), cd41 (thrombocytes) and rag-1 (lymphocytes).