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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 \textit{gfi1aa} and \textit{gfi1b} have distinct roles in regulating primitive and definitive hematopoietic progenitors, respectively. Loss of \textit{gfi1aa} silences markers of early primitive progenitors, \textit{scl} and \textit{gata1}. Conversely, loss of \textit{gfi1b} silences \textit{runx-1}, \textit{c-myb}, \textit{ikaros} and \textit{cd41}, indicating that \textit{gfi1b} is required for definitive hematopoiesis. We determine the epistatic relationships between the \textit{gfi} factors and key hematopoietic transcription factors, demonstrating that \textit{gfi1aa} and \textit{gfi1b} join \textit{lmo2}, \textit{scl}, \textit{runx-1} and \textit{c-myb} as critical regulators of teleost HSPC. Our studies establish a comparative paradigm for the regulation of hematopoietic lineages by \textit{gfi} transcription factors.

\textbf{Keywords}

primitive and definitive hematopoiesis; differentiation; lineage commitment

\section*{INTRODUCTION}

\textit{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, \textit{Growth factor independence-1b (Gfi1b)} was subsequently cloned based on high nucleotide homology to \textit{Gfi} (Grimes et al., 1996). Both \textit{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, \textit{Gfi1a} and \textit{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 \textit{Gfi1a} and \textit{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 \textit{Gfi1a} or \textit{Gfi1b} is dispensable for maintenance of mouse embryonic HSC, while loss of both \textit{Gfi} genes in mouse is incompatible with HSC maintenance (Khandanpour et al., 2010).

Although \textit{Gfi1a} and \textit{Gfi1b} have overlapping roles in HSC, in downstream lineages, \textit{Gfi1a} and \textit{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 \textit{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 \textit{GFI1A} suffer from severe congenital neutropenia (Person et al., 2003), underscoring a conserved function of the mammalian \textit{Gfi} genes. In addition to expression in hematopoietic tissues, \textit{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 \textit{Gfi1a}, loss of \textit{Gfi1b} has no effect on myelopoiesis (Saleque et al., 2002). Rather, loss of \textit{Gfi1b} specifically impairs erythrocyte and megakaryocyte development (Saleque et al., 2002). \textit{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>m651</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 GFPlo and GFPhi 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 ~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

Digoxygenin-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 prfdcl 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 FACSVantage 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 GFP<sup>lo</sup> 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 GFP<sup>lo</sup> 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 oncprotein Gfi family members. Three clones encode full-length polypeptides containing a conserved 20 amino acid SNAG transcriptional repressor domain and 6 C<sub>2</sub>H<sub>2</sub> 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 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 3A,h,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 gata-1 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 gfi genes would have a greater impact on hemoglobinization than knockdown of either gene alone. Knockdown of both 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 gfi morphants at 120 hpf. Remarkably, gfi1aa morphants recover from their initial anemia as observed by their abundance of hemoglobinized cells at 120 hpf (Figure 4A, h–i). Conversely, gfi1b morphants are severely anemic by 120 hpf (Figure 4A, j). To rigorously quantify the change in erythrocytes over time in gfi morphants, we knocked down gfi1aa and gfi1b in the Tg(globin-LCR:eGFP) line. Consistent with the hemoglobinization trend observed by o-dianisidine staining, gfi1aa morphants showed a significant reduction in erythrocytes at 48 hpf, subsequently recovering by 120 hpf, while gfi1b morphants showed a significant decrease in erythrocytes at later time points (Figure 4B). In toto, our erythrocyte data demonstrated that gfi1aa and gfi1b have discrete roles at different stages of erythropoiesis and suggested that the loss of each gfi gene could be differentially impacting primitive and definitive hematopoietic lineages.

**Zebrafish gfi1b is required for definitive hematopoiesis**

To evaluate the roles of gfi1aa and gfi1b in definitive hematopoiesis, we knocked down each gene and used WISH to evaluate the impact on definitive HSC using runx-1, c-myb and ikaros as markers (Davidson and Zon, 2004). Loss of gfi1b, but not gfi1aa, reduces the expression of runx-1, c-myb and 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 Tg(cd41:eGFP) gfi morphants and examined the GFPlo expressing definitive HSC population (Ma et al., 2011). Consistent with our WISH results, loss of gfi1b reduces the population of definitive HSC, while loss of gfi1aa has no effect (Figure 5B). We also used the Tg(cd41:eGFP) line to evaluate the thrombocyte lineage in 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 gfi1b morphants, loss of gfi1b reduces the population of downstream thrombocytes (Figure 5B). Loss of 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 cd41 in gfi morphants using itga2b. itga2b encodes the platelet glycoprotein GPIIb and is a member of the cd41 protein complex in zebrafish thrombocytes (Davidson and Zon, 2004). Consistent with our thrombocyte FACS data, gfi1b morphants showed a significant reduction in expression of itga2b relative to controls (Figure 5B). gfi1aa morphants, in contrast, showed no significant change in itga2b expression (Figure 5B).

To assess the role of gfi1aa and gfi1b in lymphocyte development, we performed WISH of rag-1 in gfi morphants. Consistent with their lack of ikaros expression, gfi1b morphants showed a reduction in rag-1 expressing thymic lymphocytes, while the thymic expression of rag-1 was preserved in 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 gfi morphants. Alcian blue staining showed dysplastic development of the jaw cartilage in gfi1b morphants, while gfi1aa morphants showed normal jaw cartilage development (Figure 5C, d–f).

**Genetic epistasis of gfi1aa and gfi1b in primitive and definitive hematopoiesis**

To understand the epistatic relationship of the gfi genes in the context of other hematopoietic transcription factors, we evaluated the ICM expression of gfi1aa and 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$^{21384}$ (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 *fil* 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, t test, *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 mfnl 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) Neither lmo2 nor gata2 is expressed in the ICM of clo mutants, confirming the presence of hematopoietic progenitors. (e–h) 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. (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).