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Combinatorial regulation of novel erythroid gene expression in zebrafish

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Abstract

Objective—The specification and differentiation of hematopoietic stem cells into red blood cells requires precise coordination by multiple transcription factors. Most genes important for erythroid maturation are regulated by the Gata family of DNA-binding proteins. Previously, we identified three novel genes *kelch-repeat containing protein (krcp), kiaa0650*, and *testhymin/glucocorticoid inducible transcript 1 (glcci1)* to be expressed in erythroid cells in a Gata-independent manner, and we sought to further understand how these transcripts are regulated during zebrafish hematopoiesis.

Methods—We employed a loss-of-function approach, using combinations of antisense morpholinos to hematopoietic transcription factors and assayed for changes in gene expression in zebrafish embryos.

Results—Upon examination of embryos deficient for Gata1, Gata2, Biklf and/or Scl, we found distinct gene combinations were required for expression of the novel genes. While *krcp* expression was dependent upon Gata1 and Biklf, *kiaa0650* expression was greatly reduced and *glcci1* was maintained in Gata1/Gata2/Biklf-deficient embryos. As with the *gata1* gene, *kiaa0650* and *krcp* required Scl for blood expression. Although reduced, *glcci1* was expressed in posterior blood precursors in the absence of Scl and Gata2.

Conclusions—This work identifies *glcci1* as having Scl-independent expression in the posterior hematopoietic mesoderm, suggesting that its posterior expression is activated by factors upstream or parallel to Scl and Gata2. Additionally, these studies establish that blood gene expression programs are regulated by transcription factors acting in combination during erythroid maturation.

Introduction

During mammalian embryogenesis, blood cells develop on the yolk sac (YS) and predominantly give rise to erythrocytes. The formation of these blood cells requires the transcription factor *stem cell leukemia gene (scl/tal1)* and the *lim-only 2 (lmo2)* gene[1],[2],[3],[4]. In the mouse, transcripts of these genes are first detected in the YS at embryonic day

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7.25 (E7.25)[5],[6], and are necessary for the specification of embryonic blood cells and proper angiogenesis[4],[7]. The zinc finger transcription factors *GATA1* and *GATA2*, also expressed in the YS, are involved in blood cell proliferation and maturation events[5],[6]. In *GATA1* mutant embryos, blood cells are formed, but are unable to differentiate, demonstrating an essential role for *GATA1* in terminal erythroid maturation[8],[9]. *GATA2* is necessary not only for maintaining erythroid cell number, but also for proper proliferation and survival of multipotential progenitor cells [9]. Additionally, there are erythroid lineage specific factors that have essential roles in red blood cell maturation. For instance, loss of the *Erythroid Krüppel-like factor (EKLF)*, a CACC box zinc finger transcription factor, in mice results in disrupted adult *β-globin* expression, severe anemia, and ultimately death by E16[10],[11].

Similar to mammals, zebrafish embryonic hematopoiesis is detected by the expression of *scl, lmo2* and *gata2* at the 2-somite stage in blood and endothelial cell precursors in the posterior mesoderm[12]. The first genes in zebrafish to mark erythroid precursors, *gata1* and the *blood island-enriched Krüppel-like factor (biklf/klf4)*, are expressed by 4 somites [13],[14],[15]. By 12 somites, these cells express embryonic *globins* and migrate towards the midline to form the intermediate cell mass (ICM), a group of blood cells surrounded by axial vein endothelium. By 24 hours post fertilization (hpf), erythroblasts residing in the ICM express genes important for terminal erythroid maturation, such as the heme synthetic enzyme *aminolevulinate synthase 2 (alas2)*, and eventually enter circulation[16]. Concomitant with erythropoiesis, myeloid cell development can be also detected by expression of the myeloid genes *l-plastin* and *myeloperoxidase (mpo)* in the ICM and an independent anterior location referred to as the rostral blood island (RBI)[17]. After 3 days post fertilization (dpf), adult hematopoiesis can be identified by *ikaros* and *rag1* staining in the thymic primordium [18],[19],[20]. Expression of blood markers such as *scl* and *c-myb*, as well as morphologically identifiable blood cells, are also observed in the developing kidney[21].

There are several zebrafish mutant lines that harbor defects at specific stages of hematopoiesis, and their study has improved our understanding of embryonic blood cell development. The *cloche* mutant, whose gene has not been identified, has virtually undetectable expression of *scl* and *gata1* and lacks blood and endothelial cells[22],[23],[24]. The *spadetail (spt)/tbx16* mutant, whose primary defect is in posterior paraxial mesoderm formation, lacks early *scl* and *gata1* expression in the posterior mesoderm at 4 somites[25],[26]. ICM blood cells are absent in *spt*, but unlike *clo*, these mutants begin expressing *scl, gata2* and *flk1* by 10 somites[27],[26]. The expression of these genes has been attributed to a later wave of angioblast development as *spt* mutants have endothelial cells that form severely disorganized vessels [26]. Consistent with the mouse *scl* knockout, Scl-deficient zebrafish have no *gata1* and *globin*-expressing blood cells as well as defects in the formation of intersegmental vessels [28],[29]. These findings highlight the dual functions of *scl*, early in blood cell specification and later in endothelial cell organization. The *moonshine (mon)* mutant, which has a disruption in the *transcriptional intermediary factor 1 γ(tif1γ)* gene, has defects specific to the erythroid blood lineage. In this mutant, *scl* and *gata1* expression are initiated normally, but by 18 somites most primitive erythroid cells are absent due to apoptosis[30]. Loss of zebrafish Gata1 also results in an absence of mature erythroid cells; however, most ICM precursors in Gata1-deficient embryos differentiate into myeloid cells that become granulocytes and macrophages [31],[32],[33]. Unlike Gata1, Gata2 deficiency does not cause a conversion to myelopoiesis, but results in a modest reduction in both myeloid and erythroid cell numbers, suggesting that like mammalian systems, Gata2 is required for multipotential cell survival and proliferation. Similar to mammalian EKLF, loss of Biklf also results in decreased numbers of red blood cells and a reduction in *globin* and *gata1* expression[34].

We have previously shown in zebrafish that expression of three genes, *testhymin* (hereafter referred to as *glucocorticoid inducible transcript 1 (glcci1)), kiaa0650*, and *kelch-repeat*

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containing protein (krcp), occurs independently of Gata1 and Gata2[32]. To understand the complex process underlying the regulation of these and known blood genes, a combinatorial gene knock-down approach was employed using morpholinos to abolish *gata1*, *gata2*, *biklf* and *scl* protein expression. *glcci1* was discovered to be a novel blood marker that is not expressed in ventral mesoderm or in endothelial cell precursors, but is specific to the hematopoietic system. Interestingly, *glcci1* is expressed independently of Scl/Gata2 in the posterior hematopoietic precursors. Our studies begin to uncover how programs of gene expression are regulated by multiple hematopoietic transcription factors that ultimately coordinate erythroid differentiation.

Materials and Methods

Zebrafish strains

Zebrafish were maintained and staged as described[35],[36]. Tü and TL strains were used for morpholino (MO) injection experiments and expression analysis. We incrossed heterozygous mutants of the following lines: *spt*^{b104}, *mon*^{tg234}, and *clo*^{m39} [22],[25],[30]. The *vlt*^{m651} line was genotyped as described[31].

Morpholino Injections

The *gata1*, *gata1* mismatch, *gata2* (also referred to as *gata2*E/I1), *gata2* mismatch[32], *scl*, *scl* mismatch[28], *biklf* [34] MOs were used. A four basepair mismatch MO to *biklf* (5'-TGGAAATGATAGGGTACTGAGAAGG-3') was designed. When the *biklf* mismatch MO was injected alone, *globin* expression was never affected, and when it was injected with the *gata1* MO *globin* expression was not abolished (n=22; 100%). A second *gata2* MO was designed against the splice donor site of exon 1 (*gata2e1D*: 5'-AATGAATCCTACCGTGCGCTTGTCC-3'), and it phenocopied the previously described *gata2* MO when 1 nL was injected at 0.4 μM. Loss of *globin* expression was used as a control for the *gata1/gata2* and the gata1/biklf MO injections and loss of *gata1* was used as a control for the *scl* MO injections.

Reverse Transcription PCR

RNA was isolated from approximately 30 uninjected or *gata2*E/I1 MO-injected embryos as described[28], and cDNA was prepared using the Superscript II RT (Invitrogen). PCR was performed using EF1α control primers [28] and primers designed to *gata2* (*gata2.E2* forward: 5'-CACACATCATCCCATCCCGACCTA-3' and *gata2.E5* reverse: 5'-GGAATGGTGCGGGTGGCTGAATGT-3'). The uninjected embryo pool yielded a normal size product of ~690 base pairs (bp) while the *gata2* E/I1 MO-injected embryo pool gave a ~540 kb product (Supplemental Figure 1A). After isolating, cloning, and sequencing the 540 bp product, it was determined that exon 3 (~150 bp) was absent in the *gata2* E/I1 MO-injected amplified cDNA. Further sequence analysis revealed that deletion of exon 3 causes a frame shift into a premature stop codon in exon 4 and truncates the Gata2 protein at amino acid 337 (Supplemental Figure 1B). Since exon 3 and 4 contain both zinc fingers and the DNA-binding domain, their loss most likely renders the protein non-functional.

Whole mount *in situ* hybridizations

Whole mount *in situ* hybridization was performed as described[37]. Digoxigenin-labeled antisense RNA was transcribed from linearized plasmids using a Roche SP6/T7 RNA labeling kit. The following antisense riboprobes were generated as described: *glcci1/testhymin, kiaa0650, krcp, carbonic anhydrase* [32], *gata1, gata2*[13], *biklf*[38], *scl*[24], *draculin*[39], *βe3 globin*[40], and *ikaros*[19]. Embryos were mounted in glycerol and photographed using a Nikon Eclipse compound microscope.

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Results

Expression analysis of *glcci1, kiaa0650*, and *kcrp*

From a zebrafish *in situ* hybridization screen for genes expressed in developing blood cells, three genes were discovered to not require Gata1 or Gata2 for their blood expression[32]. Further evaluation of *glcci1, kiaa0650*, and *krcp* revealed that these genes are expressed in two bilateral stripes of the posterior mesoderm at early stages of somitogenesis (Figure 1). A time-course analysis indicated that *kiaa0650* and *glcci1* are first expressed in blood precursors at the 3-somite stage, while *krcp* transcripts are not detected until the 5 somites. Expression of all three genes is maintained in the blood cells as they migrate medially to form the ICM at 24 hpf (Figure 1). *glcci1* and *kiaa0650* are expressed in the circulating primitive blood precursors until 36–48 hpf (Figure 1, arrowheads). *krcp* is also expressed at these stages, but its expression is decreased in the ICM after 20 hpf and found in fewer circulating blood cells than *glcci1* or *kiaa0650*. Diffuse expression of *glcci1, kiaa0650* and *krcp* was observed in regions of the brain at 24 hpf and 36 hpf (Figure 1). *krcp* was expressed in the somites and the tailbud from early somitogenesis to 24 hpf, and after 36 hpf, *krcp* transcripts were found in the fin buds and in the gut endoderm (Figure 1 and data not shown). Virtually no expression of these genes was detected in the blood, kidney or thymus between 48 hpf and 5 dpf although modest levels of *kiaa0650* were found in the ventral tail region between 3–5 dpf in a pattern similar to *scl* (data not shown)[24].

To test if these genes were present in blood and/or angioblasts, we analyzed their expression in *cloche (clo)* mutants, which specifically lack both hematopoietic and endothelial cell precursors. ICM expression of *glcci1, kiaa0650*, and *krcp* was absent in *clo* mutants, indicating these genes are expressed in blood or endothelium (data not shown). To determine that these genes were expressed in erythroid blood precursors and not endothelial cells, their expression was examined in *spt* and *mon* mutants. Upon inspection of *spt* embryos, a mutant that lacks blood but retains endothelial cells, we found that expression of all three genes was lost at 5 and 12 somites, indicating that their expression is confined to the hematopoietic compartment (Figure 1B and data not shown). ICM expression of *glcci1, kiaa0650*, and *krcp* was also virtually lost at 18 somites in the *mon* mutants compared to their wild type and heterozygous siblings (Figure 1C). At this time, *mon* mutant embryos have lost most erythroid precursors due to cell death[30]. Taken together, these results indicate that *glcci1, kiaa0650*, and *krcp* are expressed in blood precursors destined to become erythrocytes.

Loss of Biklf and GATA factors selectively affects erythroid gene expression

To determine which hematopoietic transcription factors are mediating blood cell expression of *glcci1*, *kiaa0650*, and *krcp* in the absence of GATA factors, loss-of-function experiments were performed using morpholino (MO) antisense oligonucleotides. *biklf* is important for *globin* and *gata1* expression in zebrafish[34], and is expressed independently of Gata1 and Gata2[32]. Therefore, we sought to determine whether *biklf* could regulate the blood expression of these genes. First, we examined the effect of loss of Biklf and Gata1 on the blood program, using well-characterized blood markers. Loss of Gata1 alone caused reduced numbers of ICM cells expressing the erythroid markers *globin*, *carbonic anhydrase*, and *gata1* compared to controls at 20 somites (Figure 2). Alternatively, expression of *ikaros*, a transcription factor found in myeloid and erythroid lineages, was unaltered compared to controls. This result is consistent with the ICM cells of Gata1-deficient embryos converting to the myeloid lineage [32].

Similar to loss of Gata1, loss of Biklf caused a reduction in the number of cells expressing *globin*, *carbonic anhydrase*, and *gata1* at 20 somites (Figure 2, Supplemental Figure 1). We next determined whether genes previously found to require Gata1 for expression were

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dependent on Biklf. Biklf MO-injected embryos were examined for the expression of *SH3 domain binding protein-5 (SH3BP5)*, *epsin/mitotic phosphoprotein* and *biliverdin reductase*, Gata1-dependent genes. While loss of Biklf reduced the number of blood cells expressing *SH3BP5*, *epsin*, and *biliverdin reductase* compared to controls at 24 hpf, it did not completely eliminate their ICM expression as in *gata1* MO-injected embryos[32] (data not shown). This result suggests that Biklf is not required for expression of these Gata1-dependent genes, and instead may regulate erythroid cell number. In contrast to Gata1 deficiency, loss of Biklf caused a decrease in *ikaros* staining at 24 hpf compared to controls and did not result in a myeloid cell expansion in the ICM (Figure 2;Supplemental Figure 1). These results indicate that Biklf is required for maintaining proper ICM blood cell number and for erythroid differentiation, but not important for determining myeloerythroid fates (Supplementary Figure 1). These results are consistent with a study demonstrating reduced *gata1* and *globin* expression in *biklf* MO-injected embryos[34].

To determine the effect of loss of Biklf and Gata1 and/or Gata2 on blood development, we examined gene expression in embryos deficient for specific combinations of these factors. Similar to Gata1/Gata2-deficient embryos, Biklf/Gata1-deficient embryos lost *globin*, *carbonic anhydrase* and *gata1* transcripts in the ICM at 20 hpf, indicating that both genes are necessary for expression (Figure 2). As loss of Gata2 causes a reduction in blood cells, a combined loss of Gata2 and Biklf also resulted in a reduction in the number of ICM blood cells expressing *globin*, *carbonic anhydrase*, and *gata1* (Figure 2). Since injection of all three MOs simultaneously was toxic, the *biklf* and *gata2* MOs were injected into *vlad tepes/gata1* heterozygous mutant incrosses and embryos were analyzed. Interestingly, the number of *ikaros*-expressing or *cmyb*-expressing ICM cells in Biklf/Gata1- and Biklf/Gata1/Gata2-deficient embryos resembles that of *gata1* morphants rather than the *biklf* MO-injected embryos (Figure 2, data not shown). This result is consistent with the Gata1-deficient ICM containing myeloid precursor cells, and suggests that *biklf* acts downstream of *gata1* in the erythroid lineage. Biklf/Gata1/Gata2-deficient embryos have less *cmyb*- or *ikaros*-staining cells in the ICM compared with Gata1- and Biklf/Gata1-deficient embryos, demonstrating that loss of Gata2 affects both myeloid and erythroid cell number.

As loss of Biklf, Gata1 and Gata2 affected erythroid gene expression at 20 somites, we investigated if their loss affected hematopoietic precursor genes at earlier stages. *draculin*, a gene expressed in erythroid and myeloid cell precursors, and *scl*, *lmo2*, and *gata2*, genes found in blood and endothelial cell precursors, were present in Biklf-, Biklf/Gata1-deficient embryos at 12 somites, and their expression was reduced in the Biklf/Gata2- and Biklf/Gata1/Gata2-deficient embryos (Figure 3B). This result is likely due to decreased blood cell number caused by loss of Gata2. Compared to control embryos at 12 somites, expression of *biklf* was reduced in the blood precursors of all MO-injected embryos especially the Biklf/Gata1/Gata2-deficient embryos (Figure 3B). The hatching gland expression of *biklf* was completely lost, demonstrating that it is required for hatching gland formation and providing an internal control for the morpholino injections. Expression of the previously identified erythroid genes *zinc finger-like gene 2 (znfl2)* and *nuclear receptor coactivator 4 (ncoa4)* were also examined in these morphants[41]. *ncoa4* expression was lost in Gata1-deficient embryos at 24 hpf, suggesting it requires Gata1 (Supplemental Figure 2). At 12 somites, *znfl4* expression was present in Biklf morphants, reduced in Gata1 morphants and absent in Gata1/Gata2-deficient embryos, suggesting it requires Gata1 and Gata2 but not Biklf for expression (Figure 3B and Supplemental Figure 2). Taken together, loss of Gata1/Gata2, Gata1/Biklf, or Gata1/Gata2/Biklf abolishes most erythroid gene expression as demonstrated by the loss of genes such as *globin* and *znfl2*, whereas expression of erythroid precursor genes such as *biklf*, erythroid and myeloid precursor genes such as *draculin*, *biklf*, *ikaros*, and *cmyb* or the early hematopoietic and endothelial markers, *scl*, *lmo2* and *gata2*, are maintained.

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Loss of key hematopoietic transcription factors has a differential effect on *glcci1*, *kiaa0650* and *krcp* expression

To determine how loss of combinations of Biklf and Gata1 and/or Gata2 affected expression of *glcci1*, *kiaa0650*, and *krcp*, *in situ* hybridizations were performed on 12 somite-stage MO-injected *vlt* mutant embryos. Hematopoietic expression of *krcp* was virtually absent in the Gata1/Biklf-deficient embryos (Figure 3A, arrowheads) and expression of *kiaa0650* was markedly reduced in Biklf/Gata1/Gata2-deficient embryos compared with the Gata2/Biklf-deficient or Biklf-deficient animals alone (Figure 3A). This suggests that Gata1 and Biklf are necessary for *krcp* expression and Gata1, Gata2, and Biklf are necessary for robust *kiaa0650* expression in the blood. In contrast, *glcci1* was expressed in the absence of Biklf, Gata1, and Gata2, demonstrating that Biklf and GATA factors are not required for blood expression (Figure 3A). This also demonstrates that the novel gene, *glcci1* is unique in that it is regulated in a manner similar to the early endothelial markers such as *scl*, *lmo2*, and *gata2*, yet it is expressed specifically in the erythroid blood lineage.

Based on this data, we hypothesized that genes such as *scl* that are involved in early blood cell specification may be important regulators of *glcci1* and *kiaa0650* expression. To test this, a *scl* MO was injected, and expression was examined. Scl-deficiency results in a loss of *gata1* and *biklf* expression[28]. We found that expression of *krcp* was lost in *scl* MO-injected embryos (Figure 4), consistent with *krcp* dependence on *gata1* and *biklf*. Expression of *kiaa0650* was virtually ablated in *scl* MO-injected embryos compared to control MO-injected embryos, indicating that it is also dependent on Scl for blood expression (Figure 4). *glcci1* expression was reduced but not lost in Scl-deficient embryos, indicating that Scl is not required for posterior blood precursor expression. Previous studies have shown that some hematopoietic genes such as *gata2* are expressed in the blood of *scl* MO-injected embryos[28]. To eliminate the possibility that Gata2 may be regulating posterior expression of *glcci1* in the absence of Scl, *scl* and *gata2* MOs were co-injected and expression of *glcci1* was examined in 12 somite-stage embryos. Although expression of *glcci1* was restricted to the posterior axis of the embryo compared with control MO-injected animals, its expression persisted, suggesting that factors upstream or parallel to Scl and Gata2 regulate its expression in this region (Figure 4). Interestingly, *draculin* expression, while significantly reduced in *scl* and *scl/gata2* morphants, remained in some posterior hematopoietic precursor cells (Figure 4). Taken together, these findings demonstrate how genes with similar expression patterns can be dependent on different factors for expression, and identify *glcci1* as having an Scl-independent expression domain in the more posterior blood cell precursors.

Discussion

While most erythroid genes are dependent on Gata1 or Gata2 for expression, our *in situ* hybridization screen discovered three novel genes that did not require Gata1 or Gata2 for their hematopoietic expression[32]. Similar to *gata1* and *biklf*, these genes were expressed in blood but not endothelial cell precursors, and specific transcription factors were necessary for their expression. Expression of *krcp* required Gata1 and Biklf, consistent with its expression initiating later than *gata1* and *biklf*. Expression of *kiaa0650*, was found to be dependent upon Gata1, Gata2, and Biklf together as well as Scl alone. Its regulation, therefore, resembles that of *biklf*, which also had significantly reduced expression in Biklf/Gata1/Gata2-deficient embryos and requires Scl for expression. Unlike these Scl-dependent genes, expression of *glcci1* is present but reduced in Scl and Scl/Gata2-deficient embryos. While there is the possibility that this results from hypomorphic Scl function rather than a complete loss-of-function situation, efficient knockdown of RNA levels was demonstrated at the concentration used[28]. The phenotype was also assessed in parallel with *scl* MO-injected sibling controls, in which a complete loss of *gata1* was always observed. Additionally, the persistent posterior

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expression of *glcci1* most resembles that of *draculin* expression in *scl/gata2* MO-injected embryos and may indicate similar regulatory mechanisms[28],[29]. Unlike *glcci1*, however, *draculin* expression is much more reduced in the *scl* morphants. In addition, it is expressed in ventral mesoderm during gastrulation and in myeloid cell precursors at later stages. Therefore, *glcci1* represents a unique gene that is specific to the blood lineage and not dependent on Scl and Gata2 for its early posterior hematopoietic expression.

Given the unique expression pattern of *glcci1*, analysis of its regulatory regions may give insight into which transcription factors are crucial for blood specification. Examination of 1 kb sequence of the 5'UTR of *glcci1* revealed the presence of Ets-, C/ebp-, Oct-1 and Gata-binding sites. The Ets domain transcription factors *fli1a*, *fli1b*, *ets1*, *mef*, and *ets1-related protein* are expressed during early embryogenesis. However, *fli1b* and *ets1-related protein* are only expressed in endothelial cell precursors, making them unlikely regulators of *glcci1* [42],[43],[44]. Alternatively, *fli1a*, *ets1*, and *mef* are expressed in domains coinciding with *lmo2* expression, and *ets1*, in particular, can activate the *lmo2* promoter[44]. *lmo2* is also expressed in the absence of Scl and Gata2 [28] (data not shown); however, in contrast to *glcci1*, *lmo2* is expressed in endothelial and blood cells. *CCAAT/enhancer binding proteins* (C/ebp), transcription factors, are unlikely regulators of *glcci1* expression as *c/ebpα*, *c/ebp β*, and *c/ebp1* are found in hematopoietic cells of the myeloid lineage[15],[45]. Interestingly, Oct-1 can cooperate with Smads to activate the zebrafish *gata2* promoter, and although these factors were shown to regulate *gata2* in ventral mesoderm, *glcci1* induction may depend upon a similar mechanism[46]. Ultimately, the examination of the regulation of genes such as *glcci1* that are only expressed in blood cell precursors and not myeloid or endothelial cells is crucial for gaining insight into how cell fate decisions are made during development.

Phylogenetic analysis of the amino acid sequence of the three novel genes revealed orthology to mammalian genes. While no known structural motifs exist in Glcci1, there is strong synteny and moderate identity with human (54%) and murine (62%) orthologs. Mouse *glcci1* was originally identified in a screen for genes regulated by glucocorticoids, but no mammalian expression pattern has been reported[47]. Zebrafish Kiaa0650 is syntenic with the mouse and human *SMC hinge domain containing protein*, and has 43% and 45% identity with them, respectively. Zebrafish Krcp shares modest identity with mouse (33%) and human (33%) F-box only protein 42 and was not found to be syntenic with any kelch domain-containing gene. Overall, the degree of conservation, among the Glcci1 and Kiaa0650 orthologs, suggests that the functional role of these genes may be preserved among vertebrates and future studies are aimed at determining the expression of their mammalian counterparts.

The specification and differentiation of HSCs into mature blood cells involves the activation of transcription factors that regulate expression of lineage-specific genes. The function of these genes has been well established, however, the mechanisms governing lineage-specific gene expression remain unclear. Additionally, several of these transcription factors have redundant or overlapping functions, and only by eliminating the function of multiple genes simultaneously can their roles in blood development be elucidated. Our study sought to dissect how the complex regulation of erythroid gene expression is orchestrated by a subset of known essential transcription factors. Zebrafish provided an ideal system as they permitted the knockdown of multiple genes at one time in an embryo. Using this approach, we identified *glcci1* to be expressed early and specifically in the erythroid blood lineage and have a Scl-independent expression domain in the most posterior hematopoietic precursors. We also established that despite their similar blood expression patterns, *krcp*, *kiaa0650*, and *glcci1* required distinct combinations of known transcription factors for their erythroid gene expression during development.

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Supplementary Material

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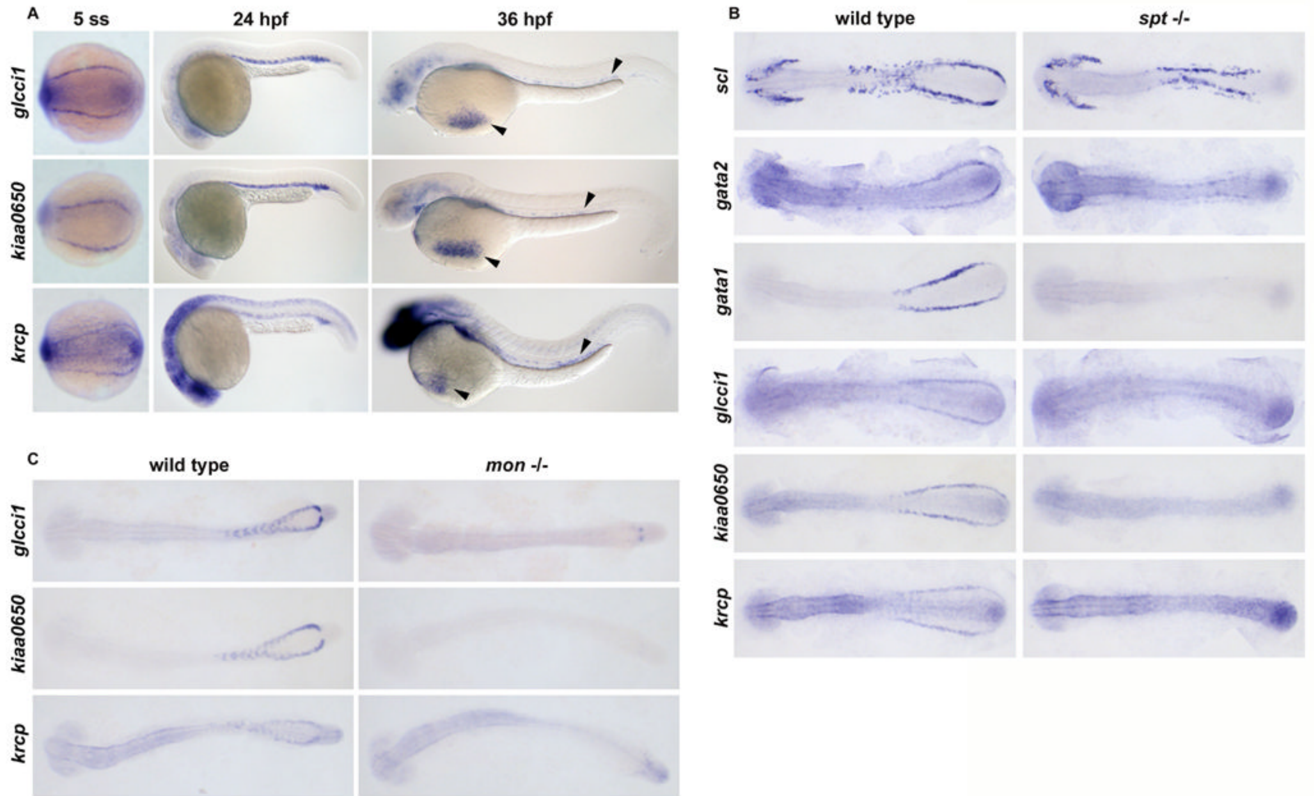

Figure 1. *glcci1*, *kiaa0650*, *krcp* are expressed in erythroid blood cell precursors

(A) Wild type embryos exhibiting expression of *glcci1*, *kiaa0650*, *krcp* in blood cells in the posterior mesoderm at 5 somites (dorsal view, left panels), in the ICM blood cells at 24 hpf (lateral view, middle panels), and in circulating blood cells in the vessels and over the yolk (arrowheads, lateral view, right panels). (B) Flat mount images of embryos are oriented with anterior on the left and posterior on the right. Expression of *gata1* (100%, n=9), *glcci1* (100%, n=7), *kiaa0650* (100%, n=3), and *krcp* (100%, n=6) was lost in spt^{b104} mutant embryos compared with wild-type and heterozygous siblings at 12 somites. Expression of *scl* (100%, n=5) and *gata2* (100%, n=7) was present but disorganized in putative angioblasts of spt^{b104} mutant embryos compared with wild-type and heterozygous siblings. (C) At 18 somites, normal expression of *glcci1* (5/27), *kiaa0650* (7/37), and *krcp* (6/27) in ICM blood cell precursors of wild-type and heterozygous siblings was virtually absent in mon^{tg234} mutant embryos. Numbers in parentheses represent the number of embryos lacking blood expression out of total number of embryos in an incross clutch.

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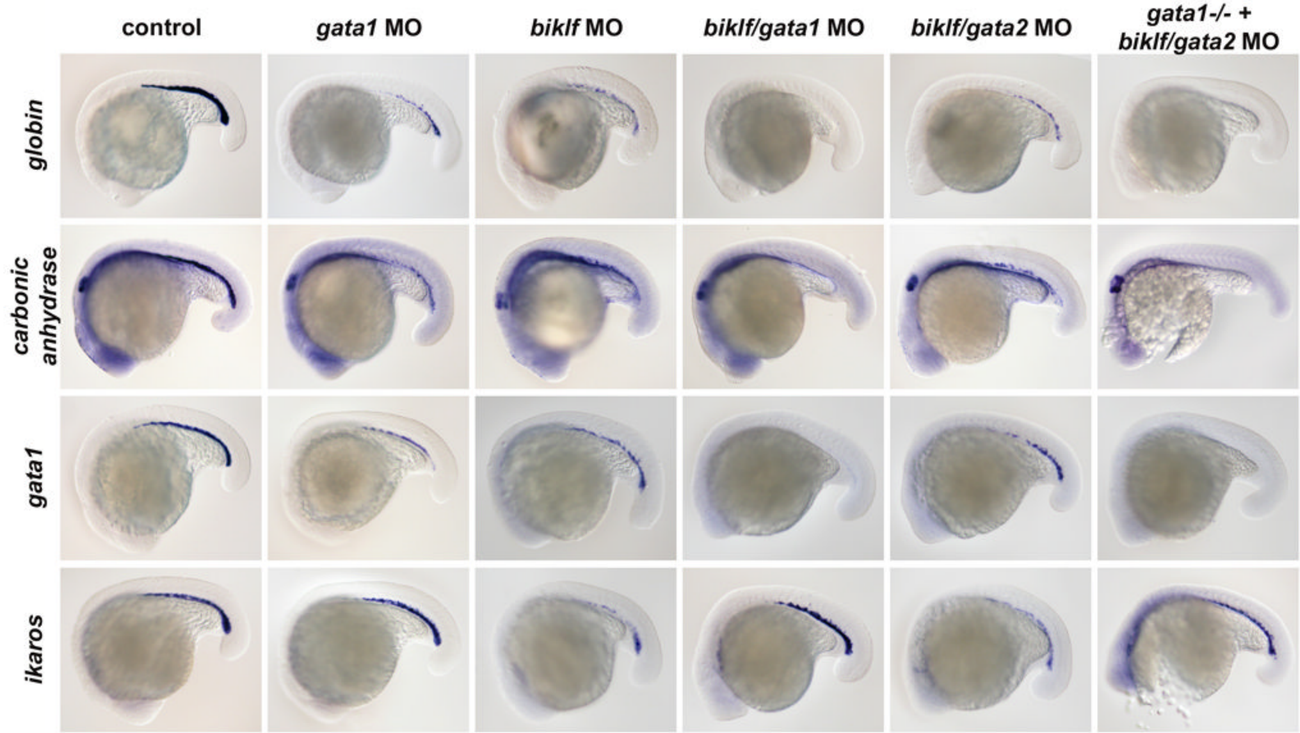

Figure 2. Loss of Biklf and GATA factors selectively effects erythroid gene expression

Wild type ICM blood expression of *globin*, *carbonic anhydrase*, *gata1*, and *ikaros* in control embryos at 20 hpf (left column). There is a decrease in the number of cells expressing *globin* (100%, n=67), *carbonic anhydrase* (100%, n=22), and *gata1* (97%, n=34) in *gata1* and *biklf* MO-injected embryos. While expression of *ikaros* is maintained in the myeloid ICM cells of *gata1* MO-injected embryos (100%, n=13), the number of *ikaros*-expressing ICM blood cells is reduced in *biklf* MO-injected embryos (100%, n=36). There is a loss of *globin* (100%, n=39), *carbonic anhydrase* (63%, n=16), and *gata1* (100%, n=29) expression in the ICM blood cells of *gata1/biklf* MO-injected and *gata2/biklf* MO-injected *gata-/-* embryos. Relatively normal *ikaros* expression is observed in ICM cells of *gata1/biklf* MO-injected and *gata2/biklf* MO-injected *gata-/-* embryos (100%, n=8). A decrease in the number of ICM cells expressing *globin* (100%, n=53), *carbonic anhydrase* (60%, n=30), *gata1* (100%, n=24), and *ikaros* (100%, n=45) is found in *gata2/biklf* MO-injected embryos.

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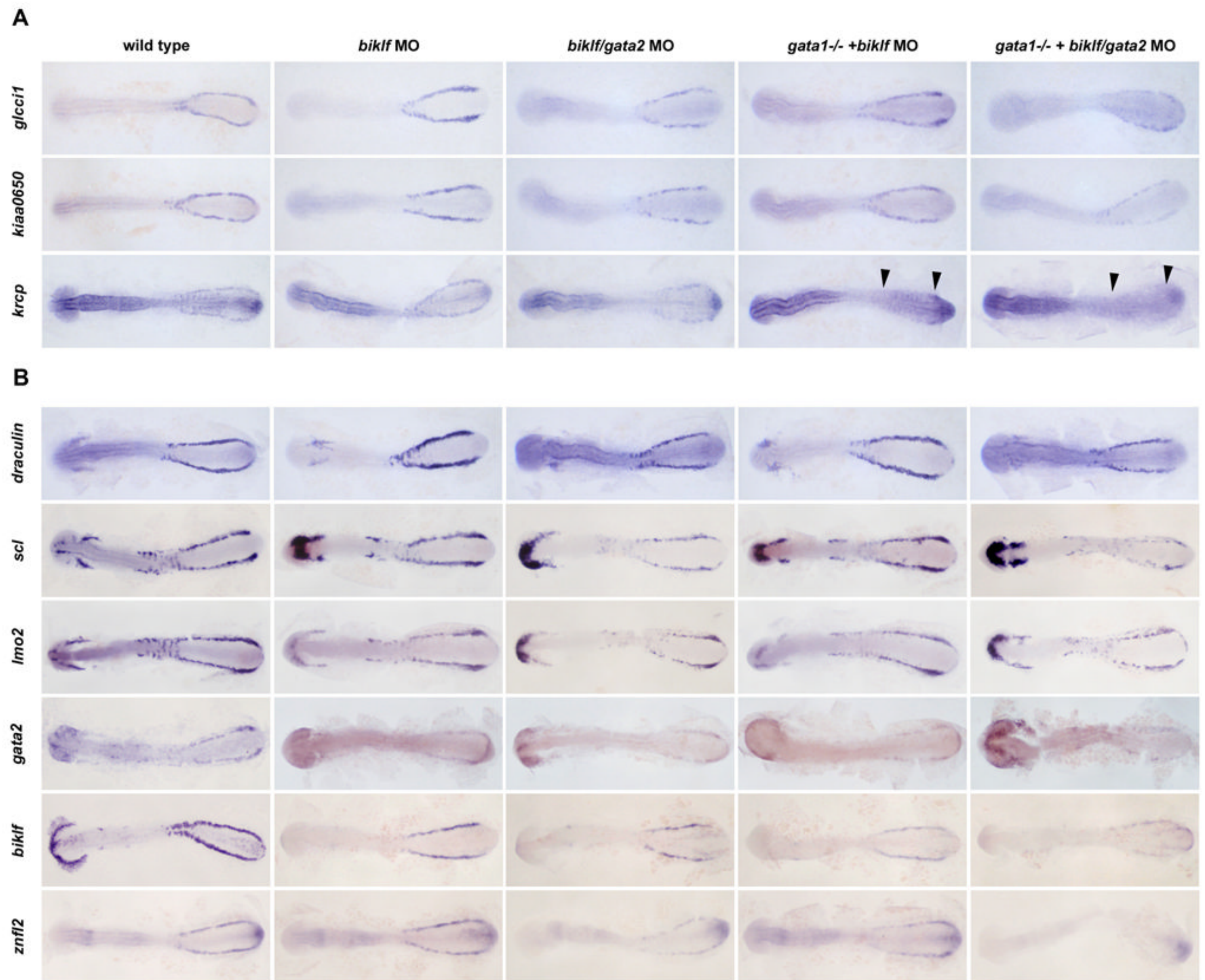

Figure 3. Loss of Gata1, Gata2 and Biklf has differing effects of *krcp*, *kiaa0650* and *glcci1* expression

(A) In control embryos at 12 somites, *glcci1*, *kiaa0650*, and *krcp*are expressed in ICM blood cell precursors. Expression of *glcci1* in the ICM precursors was not lost in *biklf* (100%, n=21), *gata1/biklf* (100%, n=43), *gata2/biklf* (100%, n=7) or *gata2/biklf* MO-injected *gata-/-* embryos (100%, n=5). Similarly, *kiaa0650* expression was not absent in *biklf* (100%, n=18), *gata1/biklf* (100%, n=63), or *gata2/biklf* (100%, n=9) injected embryos, but was greatly reduced in *gata2/biklf* MO-injected *gata-/-* embryos (70%, n=10). *krcp* was expressed in *biklf* (100%, n=10) and *gata2/biklf* (70%, n=20), but unlike *glcci1* not in *biklf* MO-injected *gata1-/-* embryos (100%, n=11) or *gata2/biklf* MO-injected *gata-/-* embryos (100%, n=2). (B) While reduced in *biklf/gata2* MO-injected and *biklf/gata2* MO-injected *gata-/-* embryos, *draculin* expression was present in all MO-injected embryos (100%, n=54). *scl*, *lmo2* and *gata2* are expressed in both the anterior and posterior blood and endothelial cell precursors in wild type embryos. Expression of *scl* (100%, n=25), *lmo2* (100%, n=27) and *gata2* (100%, n=28) was maintained in *biklf* MO-injected and *biklf* MO-injected *gata-/-* embryos. *scl* (100%, n=33), *lmo2* (100%, n=40) and *gata2* (97%, n=31) expression was reduced in *biklf/gata2* MO-injected and *biklf/gata2* MO-injected *gata-/-* embryos. Expression of *biklf* was reduced in the blood precursors and absent in the hatching gland of *biklf* MO-injected, *biklf/gata2* MO-injected, *biklf* MO-

injected *gata-/-*, and *biklf/gata2* MO-injected *gata-/-* embryos (100%, n=63). *znfl2* expression was maintained in *biklf* MO-injected embryos (100%, n=18), reduced in *biklf/gata2* MO-injected (100%, n=26) and *biklf* MO-injected *gata-/-* embryos (100%, n=4), and virtually absent in *biklf/gata2* MO-injected *gata-/-* embryos (100%, n=10).

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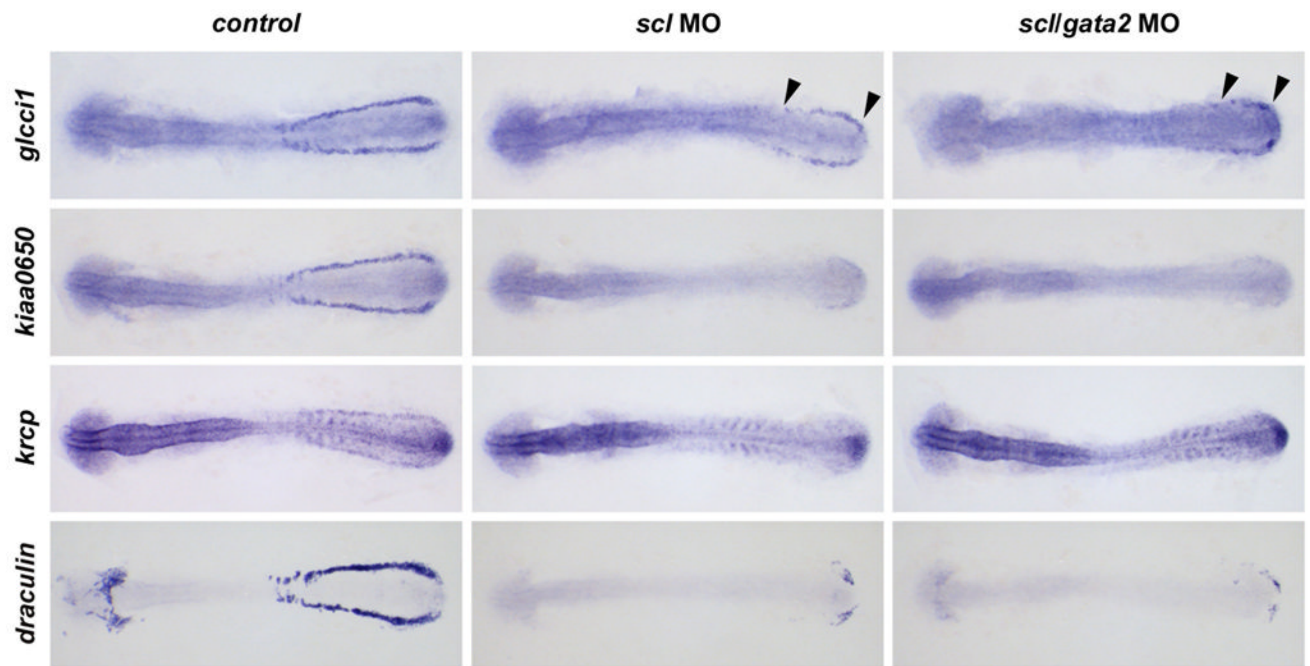

Figure 4. Posterior blood precursor expression of *glcci1* is not dependent on Scl and Gata2

Compared with control MO-injected embryos, expression of *kiaa0650* was virtually lost in the ICM precursors of both *scl* (39/39 gone) and *scl/gata2* (18/18 gone) MO-injected embryos at 12 somites. Similarly, *krcp* expression was absent in the posterior region of *scl* (100%, n=38) and *scl/gata2* (100%, n=18) MO-injected embryos. *glcci1* was reduced but not absent in *scl* (100%, n=41) and *scl/gata2* (89%, n=37) MO-injected embryos at 12 somites. Expression of *draculin* was significantly reduced in *scl* MO-injected embryos (100%, n=21) and *scl/gata2* MO-injected embryos (100%, n=16) at 12 somites compared with control MO-injected embryos.