Drl.3 governs primitive hematopoiesis in zebrafish

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The molecular program controlling hematopoietic differentiation is not fully understood. Here, we describe a family of zebrafish genes that includes a novel hematopoietic regulator, draculin-like 3 (drl.3). We found that drl.3 is expressed in mesoderm-derived hematopoietic cells and is retained during erythroid maturation. Moreover, drl.3 expression correlated with erythroid development in gata1a- and spi1b-depleted embryos. Loss-of-function analysis indicated that drl.3 plays an essential role in primitive erythropoiesis and, to a lesser extent, myelopoiesis that is independent of effects on vasculature, emergence of primitive and definitive progenitor cells and cell viability. While drl.3 depletion reduced gata1a expression and inhibited erythroid development, enforced expression of gata1a was not sufficient to rescue erythropoiesis, indicating that the regulation of hematopoiesis by drl.3 extends beyond control of gata1a expression. Knockdown of drl.3 increased the proportion of less differentiated, primitive hematopoietic cells without affecting proliferation, establishing drl.3 as an important regulator of primitive hematopoietic cell differentiation.

Vertebrate hematopoiesis occurs in successive waves that originate from distinct anatomical regions in the developing embryo. In zebrafish, primitive granulocytes arise predominantly from the anterior lateral mesoderm (ALM) whereas primitive erythrocytes arise exclusively from the posterior lateral mesoderm (PLM), which later becomes the intermediate cell mass (ICM). Subsequently, a transient wave of definitive cells with myeloid and erythroid potential (EMPs), form in the tail posterior blood island (PBI) between 24–40 hours post-fertilization (hpf). Overlapping with this wave, by 28 hpf the hemogenic endothelium in the aorta-gonad-mesonephros (AGM) region gives rise to definitive hematopoietic stem cells (HSC). Cell tracing experiments demonstrated that primitive granulocytes are present through 3 days post-fertilization (dpf) and cell structure analysis showed that primitive erythrocytes can be detected at 4 dpf, indicating the persistence of primitive cells despite the emergence of definitive waves.

While the spatiotemporal production of hematopoietic cells is well described, the molecular circuitry controlling this process continues to unfold. In vertebrate embryos, scl and lmo2 are expressed at the earliest stages of hematopoietic specification and are essential for the generation of primitive hematopoietic lineages. Thus, scl or lmo2 depleted zebrafish embryos display decreased expression of erythroid gata1a and myeloid spi1b in mesoderm-derived cells, and certain markers of more mature cells. In contrast, runx1 and cmyb are key regulators of definitive HSC development. There are a number of transcriptional regulators that direct hematopoietic lineage specification/differentiation, cell proliferation and/or survival. Within this framework, gata1a is essential for primitive erythropoiesis while spi1b is necessary for primitive myelopoiesis, although cross-antagonism between these regulators also contribute to cell fate outcomes. To better understand the regulation of hematopoietic differentiation, we used zebrafish to examine still elusive components of this regulatory network.

Here, we report the discovery of three new genes, drl.1, drl.2, and drl.3, that exhibit very high homology to the draculin (drl) gene and are predicted to encode zinc-finger proteins of, so far, unknown function. Drl was previously identified in a whole mount RNA in situ hybridization (WISH) screen of zebrafish CDNA libraries. We examine the role of the drl gene family in zebrafish embryonic and hematopoietic development and focus on one family member, drl.3, that is specifically required for primitive hematopoietic differentiation.

Results
Identification of the draculin-like gene family. Basic Local Alignment Search (BLAST) of the drl gene to the zebrafish genome identified three draculin-like genes. Drl, drl.1, drl.2, and drl.3 are clustered consecutively on
chromosome 5. All four genes contain three exons with very high homology in coding and non-coding exon sequences, with\( \text{drl.3} \) having the longest coding sequence of the group (Supplementary Fig. S1a).\( \text{Drl.1}, \text{Drl.2} \), and\( \text{Drl.3} \) contain 13 consecutive Cys2-His2 (C2H2) zinc-finger domains, while\( \text{Drl.3} \) contains 20 C2H2 domains (Supplementary Fig. S1b). Multiple adjacent C2H2 motifs are known to confer DNA binding activity, which suggests a role for these factors as transcriptional modulators\(^{33,34}\). Consistent with this idea, a\( \text{Drl.3} \)-specific antibody detected the protein in nuclear and cytoplasmic lysates from zebrafish embryos (Supplementary Fig. S1c).

To examine the conservation of the\( \text{drl} \) family between species, we performed BLASTP analysis of\( \text{Drl.3} \) protein to non-redundant protein sequences in various metazoans. The phylogenetic relationship between homologous proteins shows segregation into species-specific clusters, but not protein-specific clusters (Supplementary Fig. S2a). The zebrafish\( \text{drl} \) genomic region is similar to a region on chicken chromosome 19, but is not syntenic to the human or mouse genomes (Supplementary Fig. S2b). The genes flanking the\( \text{drl} \) cluster are located on human 17 and murine 11 chromosomes, however 6 of the 21 genes examined have not been identified in chicken (Supplementary Fig. S2c). While clear\( \text{drl} \) family homologs were not identified, the human genes encoding proteins homologous to\( \text{Drl.3} \) are clustered to several distinct chromosomal regions (Supplementary Fig. S2d), supporting the idea that the zebrafish\( \text{drl} \) cluster has a corresponding cluster in the human genome. As the functions of the human genes are largely unknown and many of the corresponding mouse homologs have not been determined (Supplementary Fig. S2e), understanding\( \text{drl} \) family activity will likely guide the identification of their functional equivalents in higher vertebrates.

**Expression analysis of\( \text{drl} \) family members.** To determine the expression profile of\( \text{drl} \) family members, we performed reverse transcription-PCR (RT-PCR) from a series of developmentally staged embryos (Fig. 1a). The primers were gene-specific, with two exceptions: first, primers for\( \text{drl.2} \) also amplified\( \text{drl} \) DNA (Supplementary Fig. S1d) and, second,\( \text{drl} \) primers amplified an additional upper band from total RNA, which was sequenced and determined to be\( \text{drl.1} \) (Fig. 1a). Our results from embryo samples revealed that\( \text{drl} \) family genes are not maternally expressed. Expression was detected by 30% epiboly through 120 hours post-fertilization (hpf). Semi-quantitative RT-PCR analysis of\( \text{drl} \) family members in 14 somite stage embryos showed varied levels of expression, with\( \text{drl.2} \) and\( \text{drl.3} \) having the highest and lowest levels of expression, respectively (Fig. 1b). While high homology between\( \text{drl} \),\( \text{drl.1} \) and\( \text{drl.2} \) prohibits the real-time quantification of these genes, this method could be used to analyze\( \text{drl.3} \) expression. Real-time PCR determined that\( \text{drl.3} \) expression was highest at 30% epiboly and progressively decreased through 48 hpf (Fig. 1c). This may reflect a progressive restriction in cell type-specific expression during development or a decrease in cellular expression levels during embryogenesis.

To address this question, we used whole mount RNA in situ hybridization (WISH).\( \text{Drl} \) expression is widespread at 30% epiboly, is expressed in developing mesoderm tissue during gastrulation (50%–90% epiboly) and becomes restricted to hematopoietic cells during somitogenesis (data not shown)\(^{35,36}\). WISH indicated that the expression patterns of\( \text{drl.1} \) (data not shown),\( \text{drl.2} \), and\( \text{drl.3} \) (Fig. 1d and e, respectively) are identical to\( \text{drl} \). Markers of early hematopoietic cells are expressed in the ALM and PLM/ICM of 10 and 18 somite stage embryos\(^{37}\); at these stages\( \text{drl} \) family members are expressed exclusively in these hematopoietic regions. At 24 hpf,\( \text{drl} \) family transcripts were detected in developing erythroid cells and very weakly in myeloid cells. Expression at 48 hpf was detected in erythroid cells and cells in the aorta- gonad-mesonephros (AGM) region.

Using\( \text{drl.3} \) as an example, we examined whether this gene is expressed in the hemogenic endothelium in the ventral aorta (VDA), the AGM region that gives rise to definitive\( \text{runx1} \)-expressing HSC. Transverse sections determined that\( \text{drl.3} \) was expressed in cells within the VDA in 28 hpf embryos, similar to\( \text{runx1} \)-expressing cells (Supplementary Fig. S3a). Double WISH analysis of 36 hpf embryos showed that a portion of the\( \text{runx1} \) cells in the VDA coexpress\( \text{drl.3} \) (Supplementary Fig. S3b–c), indicating that\( \text{drl.3} \) is expressed in definitive stem or/and progenitor cells.

Next, we performed RT-PCR analysis of purified hematopoietic cells (Fig. 1f). Flow cytometric purification of the hematopoietic populations was based on the transgenic expression of fluorescent labels controlled by the promoters marking the vascular/hemangio- blasts (\( \text{flh} \)), myeloid progenitor cells (\( \text{spi1} \)), erythroid progenitor cells (\( \text{gata1} \)), mature myeloid cells (\( \text{mpx} \)), definitive HSC and throm- bocytes (\( \text{itga2b} \)), and lymphoid cells (\( \text{rag2} \) and\( \text{ltk} \)).\( \text{Drl} \) gene family transcripts were detected in all of these cell types, with several exceptions. To determine if a lack of a band was due to RT-PCR failure, genes that were not detected in a sample were retested for expression in those cell types. Initial evaluation did not detect\( \text{drl.3} \) in 2 dpf (Fig. 1f). However, two additional independent experiments clearly detected\( \text{drl.3} \) in these samples (Supplementary Fig. S1e and data not shown), indicating that\( \text{drl.3} \) is expressed in these cells. In contrast, triplicate independent analyses failed to detect\( \text{drl.1} \) expression in 5 dpf (Fig. 1f). Supplemental Fig. S1e and data not shown), suggesting that\( \text{drl.1} \) is not expressed in this cell type at this age. Quantitative real-time PCR analysis showed that\( \text{drl.3} \) has significantly higher expression in\( \text{gata1:RFP}^{+} \) cells than cells sorted from\( \text{flh:GFP}^{+}, \text{spi1:GFP}^{+} \) and\( \text{mpx:GFP}^{+} \) 24 hpf embryos (Fig. 1g), indicating that\( \text{drl.3} \) expression is retained during primitive erythropoiesis. Further studies are needed to quantify the cell-type-specific levels of\( \text{drl.1} \) and\( \text{drl.2} \).

Since\( \text{drl.3} \) is expressed in\( \text{runx1} \) cells during embryogenesis, we examined whether\( \text{drl} \) family members are expressed in adult hematopoietic cells. RT-PCR analysis of\( \text{gata1:RFP}^{+} \) transgenic cells from adult zebrafish detected expression of each\( \text{drl} \) family member (Supplementary Fig. S3d), although the\( \text{drl.2} \) band may reflect\( \text{drl.2} \) and/or\( \text{drl} \) expression. Next, we purified mature myeloid\( \text{mpx:GFP}^{+} \) from adult kidney marrow to compare to\( \text{gata1:RFP}^{+} \) cells (Supplementary Fig. S3g–h). Murine\( \text{Scl} \) and\( \text{Gata1} \) expression increase as cells differentiate into erythroid progenitors and decrease during myeloid specification\(^{38,39}\), whereas expression of mammalian\( \text{Sptl} \) increases during myeloid differentiation\(^{38,39}\). The\( \text{gata1:RFP}^{+} \) cells were separated into large and small size groups based on the prediction that the larger cells would be less differentiated with lower levels of\( \text{scl} \) and\( \text{gata1a} \) expression. Indeed,\( \text{scl} \) and\( \text{gata1a} \) expression was detected in both\( \text{gata1:RFP}^{+} \) cell size pools, but was significantly lower in the larger\( \text{gata1:RFP}^{+} \) cells compared to the smaller cells (Supplementary Fig. S3g,\( P = 0.0003 \) and\( P = 0.0097 \), respectively). This expression pattern suggests that the small cell fraction is more differentiated than the larger cell fraction, although both likely contain a mix of cells. Similar levels of\( \text{spi1b} \) and\( \text{drl.3} \) expression were detected in both\( \text{gata1:RFP}^{+} \) cell size pools, but was significantly lower in the larger\( \text{gata1:RFP}^{+} \) cells compared to the smaller cells (Supplementary Fig. S3h,\( LC = 0.0115, SC = 0.0221 \), similar to the expression pattern of\( \text{drl.3} \) during embryonic hematopoiesis.

**The draculin gene family regulates embryonic patterning.** To gain insights into the roles of\( \text{drl} \) family members in embryogenesis, we performed gene knockdown experiments using antisense morpholinos. The target sites are diagrammed in Fig. 2a. The translation blocking MO2 was used in a previous study, but its effects were not carefully tested\(^{16,17}\). The morpholino sequences predict that (1) MO2 should inhibit the protein translation of all 4 family members while (2) MO3 should inhibit splicing of\( \text{drl.1} \) and\( \text{drl.3} \).
Figure 1 | Drl gene family members are expressed throughout embryonic development and in multiple hematopoietic lineages. (a) RT-PCR analysis of the drl family members and β-actin from pooled embryos at the indicated ages. (b) Semi-quantitative (q) RT-PCR of drl genes and β-actin from pooled 14 somite stage embryos (left panel). Arrows indicate the bands that were used for quantitation, which is shown in the right panel. Compared to drl, *P = 0.0150, **P = 0.0052, *P = 0.0142, **P = 0.0029 and ***P < 0.001 (Student’s t-test). (c) Quantitative real-time PCR analysis of drl.3 in whole embryos at the indicated ages. The relative expression of drl.3 was normalized to the expression of gapdh. (d–e) WISH of drl.2 (d) and drl.3 (e) during embryonic development. Ages of embryos are indicated. Embryos at 2-cell and 30% epiboly stages are shown in a lateral view, animal pole at the top. 50% epiboly embryos are shown in an animal pole view, dorsal to the right. Embryos at 75% and 90% epiboly are shown as lateral views, dorsal to the right. Staged embryos at 10 somites, 18 somites, 24 and 48 hpf are shown as lateral views, anterior to the left. White arrows indicate ALM; asterisks indicate ICM; black arrows indicate PLM; arrowheads indicate AGM cells. Insets show magnified AGM region of corresponding embryo. (f) RT-PCR analysis of the drl family members and β-actin from purified populations of hematopoietic cells. The age of the embryos and the transgenic lines from which cells were purified are indicated. (g) Quantitative real-time PCR analysis of drl.3 in 24 hpf sorted hematopoietic cells. The relative expression of drl.3 was normalized to the expression of gapdh. ***P < 0.0001 (Student’s t-test). (b–c, g) Bars show mean ± S.D. (a–b, f) Full-length gel images are provided in Supplemental Figure 8.
and drl.2 without affecting drl.3; thus, MO3 is likely to have overlapping or similar effects as MO2. In contrast, (3) L3MO should disrupt splicing of drl.3 specifically without altering the processing of other family member transcripts. Consistent with these expectations, RT-PCR analysis of RNA from 24 hpf morpholino and mismatch control-injected individual embryos (morphants) determined that splice site-directed MO3 decreased drl, drl.1 and drl.2 transcripts by 67% (P = 0.0382), 100% (P < 0.0001), and 97% (P = 0.0088), respectively, but did not affect drl.3 transcripts (P = 0.4588) (Fig. 2b). Conversely, the splice-site morpholino specifically targeting drl.3 (L3MO) significantly decreased this transcript (80%, P = 0.0064) without affecting other family members (Fig. 2b). Detection of endogenous Drl.3 protein showed that MO2 and L3MO decreased Drl.3 protein in 24 and 48 hpf morphant lysates (Fig. 2c–d).

Simultaneous knockdown of multiple drl family genes with either MO2 or MO3 resulted in the same developmental defects, including decreased ventrally-derived tail tissues and a shortened body axis at 24 hpf.
18 hpf (Fig. 2e). Knockdown of bloody fingers (blf) has been shown to cause similar morphological defects40. The Blf zinc finger protein is similar to Drl and Drl.3 (Supplementary Fig. 4a), but RT-PCR analysis showed that the drl morpholinos do not alter blf expression levels (Supplementary Fig. 4b–c). WISH analysis of MO2- and MO3-injected embryos at 9–10 somites revealed convergence/extension defects as indicated by the increased lateral extension of krox20 marking rhombomeres 3 and 5, myoD labeled somites, and decreased anterior-posterior expanse of scl expression (Fig. 2f). MO2 and MO3 morphants appear dorsalized at 72 and 48 hpf, with some variation in the severity of the phenotype (Supplementary Fig. S5a–b and data not shown). A higher morpholino dose correlated with a higher proportion of embryos displaying severe or moderate loss of tissue and body axis truncation at 24 hpf, whereas lower doses resulted in fewer affected embryos (Supplementary Fig. S5c). This suggests that the morpholino effects are specific and establishes a role for this family in the development of ventral tissue during zebrafish embryogenesis. Depletion of drl.3 alone did not cause morphological defects (Fig. 2e–f).

Drl.3 is required for erythropoiesis. Since drl.3 is strongly expressed in the (gata1α) erythroid lineage, we examined whether drl.3 is essential for erythropoiesis. We found that 40–47% of 24 hpf drl.3 morphants (3 WISH experiments, 218 total embryos) had decreased numbers of gata1α cells (Fig. 3a). Manual counting of gata1α or o-dianisidine stained hemoglobin+ cells determined that morphants with decreased erythropoiesis had on average about half the number of cells as outwardly normal morphants (Supplementary Fig. S5d–e). In the studies below, affected embryos have obviously decreased erythroid cell numbers that are estimated to be ≤60% of normal. Consistent with gata1α deficiency, mature erythroid marker hbae1 was decreased in 23–51% of 24 hpf drl.3 morphants (186 total embryos, triplicate experiments) compared to controls (Fig. 3a). In contrast, at 24 hpf, expression of the fli1:EGFP transgene was normal in drl.3 morphants (Fig. 3a), indicating that drl.3 is not required for vasculogenesis.

At 35 hpf, drl.3 morphants with decreased erythrocytes in circulation had normal fli1:EGFP expression despite showing slight edema in the posterior blood island (PBI) (Fig. 3b). The PBI edema (8–15% in triplicates; 167 total embryos) was only present in embryos with a severe decrease in numbers of circulating cells, as assessed by bright field microscopy (data not shown). Similarly, gata1α transgenic drl.3 morphants showed that 8–10% of the embryos had decreased erythroid cells in circulation (data not shown). Consistent with an ablation of mature erythroid cells, 16–25% of drl.3 morphants (triplicate; 213 total embryos) showed decreased numbers of o-dianisidine+ cells (Fig. 3b). RT-PCR analysis revealed that drl.3 morphants with normal or decreased gata1α:RFP circulating cells had decreased numbers of gata1α cells (Fig. 3a). Manual counting of gata1α or o-dianisidine stained hemoglobin+ cells determined that morphants with decreased erythropoiesis had on average about half the number of cells as outwardly normal morphants (Supplementary Fig. S5d–e). In the studies below, affected embryos have obviously decreased erythroid cell numbers that are estimated to be ≤60% of normal. Consistent with gata1α deficiency, mature erythroid marker hbae1 was decreased in 23–51% of 24 hpf drl.3 morphants (186 total embryos, triplicate experiments) compared to controls (Fig. 3a). In contrast, at 24 hpf, expression of the fli1:EGFP transgene was normal in drl.3 morphants (Fig. 3a), indicating that drl.3 is not required for vasculogenesis.
comparable levels of drl.3 knockdown (Fig. 3c–d), suggesting that drl.3 knockdown gives rise to an erythroid phenotype that is not fully penetrant. By 48 hpf, expression of gata1a was indistinguishable between drl.3 morphants and controls at 48 hpf (Fig. 3e) while drl.3 morphants continued to display decreased erythroid slc4a1a" (Fig. 3e; 51/63 embryos) and o-dianisidine" cells (Supplementary Fig. S5e; 22/65 embryos). The numbers of o-dianisidine" cells remained low in 4 dpf drl.3 morphants (Fig. 3f; 16/132 embryos), suggesting that primitive erythropoiesis does not recover.

Monteiro et al. established that the genetic interactions of gata1a are different in primitive versus definitive hematopoietic cells, a concept that is likely to apply to other factors such as drl.3. It remains unclear why slc4a1a WISH at 48 hpf showed a higher percentage of embryos that were affected by drl.3 knockdown compared to o-dianisidine staining. Nonetheless, our findings show that drl.3 is important for the generation of erythrocytes during primitive erythropoiesis. Drl family morphants also display decreased o-dianisidine stained cells at 2 dpf (Supplementary Fig. S5f–g). The percent of embryos with severe and moderate decreases in erythrocytes was dependent on the morpholino dose, and the severity of the erythroid phenotype correlated with the severity of the morphological defects (Supplementary Fig. S5h–i). This suggests that decreased erythropoiesis in MO2 and MO3 morphants are likely secondary to the developmental abnormalities, although the possibility remains that the drl genes contribute to erythropoiesis.

**Drl.3 deficiency impairs primitive myelopoiesis.** Since drl.3 deficiency inhibits erythroid lineage development, we postulated that drl.3 functions at a very early stage of hematopoiesis. Scl precedes gata1a expression in the PLM and scl is essential for the derivation of all embryonic hematopoietic lineages. Figure 1f shows that scl expression is normal in 9–10 somite stage drl.3 morphants. Drl.3 morphants continued to show a normal pattern of scl expression at 24 and 48 hpf (Fig. 4a). At 48 hpf, definitive hematopoietic stem/progenitor cell marker runx1 was easily detected in AGM cells even when erythroid slc4a1a expression was decreased (Fig. 4a). These results indicate that drl.3 is not essential for the emergence of primitive hematopoietic progenitors or definitive stem cells.

As drl.3 expression was detected in myeloid cells, we questioned whether drl.3 depletion would impact myelopoiesis. At 24 hpf, drl.3 deficiency resulted in decreased numbers of spi1b-expressing primitive myeloid cells in 40–43% of the morphants (triplicate; 175 total embryos) (Fig. 4b–c) and l-plastin-expressing monocytes/macrophages in 45–49% of the morphants (Fig. 4d). While L3MO/L3MO:GFP morphants showed decreased numbers of cells expressing the four drl genes in the ICM and anterior hematopoietic regions compared to the control-injected embryos (Supplementary Fig. S6a), ruling out increased drl family member expression as a compensatory mechanism in drl.3 morphants. To determine that inhibition of hematopoiesis is specifically due to knockdown of drl.3, we rescued the L3MO-induced defects by injecting drl.3 mRNA (Fig. 5a–c). Enforced expression of drl.3 significantly reduced the proportion of drl.3 morphants with increased gata1a" (L3MO = 36%; L3MO + drl.3 mRNA = 9%; P < 0.0001), slc4a1a" (L3MO = 49%; L3MO + drl.3 mRNA = 13%; P < 0.0001), and l-plastin" (L3MO = 46%; L3MO + drl.3 mRNA = 27%; P = 0.0445) cells, confirming that the defects in erythropoiesis and myelopoiesis are caused by drl.3 deficiency. Although there is extremely high homology between drl family members and slightly decreased expression of other family members in drl.3 morphants (Fig. 2b and Supplementary Fig. S6a), enforced expression of drl did not compensate for the loss of drl.3 (Supplementary Fig. S6b). Thus, drl.3 and drl have non-redundant, or at least partially non-overlapping, functions.

**Knockdown of gata1a and spi1b affect drl.3 expression.** Since spi1 and gata1 play key roles in the development of myeloid and erythroid lineages, respectively, we were curious about the genetic relationship of drl.3 with these other factors. To address this question, we morpholino-silenced gata1a and spi1b and assessed drl gene family expression (Fig. 6a). At 24 hpf, gata1a morphants showed a loss of slc4a1a" cells, increased numbers of anterior l-plastin" cells and a robust spi1b" population in the ICM while spi1b morphants showed decreased numbers of l-plastin-expressing cells (Fig. 6a). These data are consistent with published studies showing that Gata1a and Spi1b control primitive hematopoietic cell fate and confirm the activity of these morpholinos. Turning to the drl family, 24 hpf gata1a morphants displayed decreased drl family-expressing cells in the ICM and anterior hematopoietic region compared to control embryos (Fig. 6a–b). As drl family expression closely tracks with the derivation of erythroid cells, this result is likely due to the altered ICM cell composition in gata1a morphants. However, it remains to be determined whether Gata1a regulates transcription of the drl family genes, which could also underscore these findings. The numbers of cells expressing drl family members was increased in the anterior hematopoietic region of spi1b morphants (Fig. 6a–b), consistent with the acquisition of erythroid features by spi1b-deficient anterior hematopoietic cells.

At 48 hpf, gata1a morphants show an absence of slc4a1a-expressing cells and increased numbers of l-plastin-expressing cells (Fig. 6c and published studies). Gata1a morphants displayed decreased Myeloid cells accumulate over time at sites of acute injury in zebrafish embryos. To evaluate the myeloid inflammatory response, tail transections were performed in 48 hpf spil1-GFP and non-transgenic embryos, and live GFP" or WISH lycC" cells were quantified in equal sized areas surrounding the injury 6 hours later. Spil1-GFP drl.3 morphants were visually selected based on low numbers of cells in circulation; control-injected embryos were randomly selected and had normal circulation. Drl.3 morphants with decreased circulation or slc4a1a expression had significantly fewer injury-localized GFP" (Fig. 4f–g, P = 0.007) and lycC-expressing (Fig. 4h–I, P = 6.8E−5) cells, respectively, compared to control embryos. In contrast, gata1a morphants, which lack erythrocytes and have increased lycC" cells at 2 dpf, had a slight, but not significant increase in lycC" cells near the acute injury site. These data suggest that an absence of primitive erythrocytes does not necessarily block the myeloid cell response to injury, and that drl.3 morphant myeloid cell function is compromised despite the recovery of normal numbers of l-plastin-, mpx- and lycC-expressing cells.
Figure 4 | Knockdown of *drl.3* transiently decreases myeloid cells without altering the emergence of primitive progenitor and definitive stem cells. (a) WISH of *scl* at 24 and 48 hpf, and *runx1* (dark blue)/*slc4a1a* (red) at 48 hpf in L3MM- and L3MO-injected embryos, as labeled. Embryos shown as lateral views. (b) WISH of *spii1*, *l-plastin* and *mpx* at 24 hpf and *l-plastin* and *mpx* 48 hpf. Dorsal, anterior (24 hpf only, right panels) and lateral views are shown. (c) Quantitation of the number of the WISH *spii1*, *l-plastin* and *mpx* cells in the anterior of the embryo at 24 hpf and total body *l-plastin* and *mpx* cells at 48 hpf in L3MM (blue) and L3MO-injected (red) embryos (N = 8 for each column except for *mpx* at 24 hpf where N = 15, bars show mean ± S.E.). **P < 0.0044, ***P < 0.0001 and *P = 0.0156 (Student’s t-test). (d) Quantitative real-time PCR analysis of *drl.3* in whole embryo RNA samples from 24 and 48 hpf *drl.3* morphants (red) and controls (L3MM, blue, set to 1, arbitrary units). **P = 0.0042 and ***P < 0.0001 (Student’s t-test). (e) Quantitative real-time PCR analysis of *scl*, *spii1*, *gata1a*, *gata1b* and *drl.3* in whole embryo RNA samples from pools of 30 hpf *drl.3* morphants (red) compared to control-injected embryos (blue, set to 1, arbitrary units). *P = 0.0114, **P = 0.0083 and ***P = 0.0005 (Student’s t-test). (d–e) Bars show mean ± S.D., from three independent experiments. Expression was normalized to *gapdh*. (f) Tail region of *spii1*:GFP embryos at 54 hpf, 6 hours after tail transection. Selected L3MO embryos had decreased circulating cells; controls were randomly selected, and had normal circulation. Red boxes = tail cut region. (g) Number of *spii1*:GFP+ cells in tail cut region in control or *drl.3* morphants. *P = 0.007 (Student’s t-test). (h) WISH of *lysozymeC*:lysC (blue) and *slc4a1a* (red) at 54 hpf, 6 hours after tails were cut. (i) Number of lysC+ cells in an equal sized region surrounding the tail in the indicated embryos at 54 hpf, 6 hours after tail cuts were performed. *P = 6.77E-5, Student’s t-test.
drl family-expressing cells in hematopoietic pools on the yolk, which are likely erythrocytes, compared to controls (Fig. 6c). Spi1b morphants had decreased numbers of l-plastin-expressing cells, as expected32; however, at this time point, drl family expression appeared normal (Fig. 6c). These data suggest that gata1a promotes the derivation or survival of drl family-expressing cells or transcription of these genes, while spi1b knockdown transiently impacts the expression pattern of drl family members during primitive hematoipoiesis. Additional studies are necessary to establish the transcriptional versus non-transcriptional control Gata1a exerts on drl family expression.

Enforced expression of gata1a mRNA did not alter the proportion of drl.3 morphants with defective erythropoiesis (Fig. 6d, \( P = 0.6282 \)). Similarly, we determined that 98% of both gata1a morphants and gata1a morphants co-injected with drl.3 mRNA had decreased o-dianisidine\(^{-}\) erythrocytes (Fig. 6e, \( P = 1.000 \)). These results demonstrate that enforced expression of drl.3 or gata1a is not sufficient to rescue the defects caused by depletions of the other gene, suggesting that either they do not have a linear relationship to one another and/or represent only a portion of the differentiation program that they control.

**Drl.3 is important for hematopoietic cell differentiation.** Acridine orange staining and immunodetection of cleaved Caspase-3 revealed that drl.3 deficiency does not alter cell survival at 24 hpf (Supplementary Fig. S7a–b), suggesting that other cellular mechanisms underlie the loss of maturing hematopoietic populations. Using phosphorylated histone H3 (pH3) as a mitotic indicator, we found no difference in the percent of mitotic cells in the ICM of control and drl.3 morphants (Supplementary Fig. S7c). Gata1a and pu.1b mark cells destined to become erythroid and myeloid, respectively, partly due to the expression of these genes as well as the distinct regional localization of the cells. The gata1:RFP and spi1:GFP transgenes mark primitive hematopoietic progenitors even when endogenous gene expression is depleted32. Fluorescence-activated cell sorting (FACS) analysis showed no difference in the percent of gata1:RFP\(^{+}\) cells from 24 hpf drl.3 morphants (2.89%) and control embryos (2.17%) (Fig. 7a). Furthermore, we did not detect significant differences in the percent of spi1:GFP\(^{+}\) cells at 24 hpf (control 6.91%, L3MO 8.94%; \( P = 0.3083 \)) (Supplementary Fig. S8a–b) or fli1a:GFP\(^{+}\) cells at 30 hpf (control 14.70%, L3MO 16.50%; \( P = 0.8473 \)) (Supplementary Fig. S8c). Together, these findings indicate that survival and proliferation are normal in drl.3-deficient hematopoietic cells.

Erythroid cell size decreases during maturation. Using forward light scatter as an indicator of cell size, we found an increased proportion of gata1:RFP\(^{+}\) large cells (16.98%) in 24 hpf drl.3 morphants compared to the control population (9.72%) (Fig. 7a–b). At 48 hpf,
the *drl.3* morphant cell population continued to be skewed towards larger cells (Fig. 7b–c). May-Grundwald-Giemsa (MGG) staining of purified 24 hpf *gata1:RFP* cells distinguished cells with large nuclei, diffuse chromatin and a cell size that is larger, which is consistent with a less differentiated state compared to the smaller, more granular cells (Fig. 7d). From 24 hpf embryos, we found 34% (148 cells counted) of the *drl.3*-deficient cells appeared less differentiated compared to 14% (105 cells counted) of cells from control-injected embryos (*P* < 0.0001) (Fig. 7d–e). Similarly, *drl.3*-deficient 48 hpf *gata1:RFP* cells showed an increased proportion of less differentiated

**Figure 6** | **Loss of *gata1a* and *spi1b* affect *drl* gene family expression. (a) WISH of *drl* family members in *gata1a* and *spi1b* morphants (MO) compared to uninjected embryos at 24 hpf. From top to bottom: *drl*, *drl.1*, *drl.2*, *drl.3*, l-plastin (dark blue)/slc4a1a (red) and *spi1b*. Lateral views, head to the left (left panels); anterior, dorsal views (right panels). The number of the embryos with the representative phenotype out of the total number of embryos is indicated. Arrows in the panels showing dorsal views indicate an increase or decrease in the numbers of WISH+ cells. (b) Quantitation of the number of *drl* gene family-expressing cells in the anterior hematopoietic region of control (UI), *gata1a* morphants and *spi1b* morphants at 24 hpf. *N* = 10 for each column. Bar shows mean ± S.D. *P* = 0.0013, **P** = 0.0002, *P* = 0.0003 and ***P* ≤ 0.0001 (Student’s t-test). (c) WISH of *drl* family members in the indicated embryos at 48 hpf. Embryos shown as lateral views. Horizontal arrows indicate the region where cells in circulation can be visualized. Downward facing arrows indicate decreased WISH+ cell numbers. (d) Percent of L3MM-injected, L3MO-injected, and L3MO/*gata1a* mRNA co-injected embryos that have normal (blue, wt) or low numbers of erythroid cells (red, *) based on α-dianisidine staining at 48 hpf. (e) Quantitative analysis of uninjected, *gata1a* MO-injected, and *gata1a* MO/*drl.3* mRNA co-injected embryos that have normal (blue, wt) or low numbers of erythroid cells (red, *) based on α-dianisidine staining at 48 hpf. (d–e) Numbers of embryos are indicated in the columns. Statistical significance was analyzed using Fisher’s exact test.
**Figure 7** | *Drl.3* is necessary for hematopoietic cell differentiation. (a) FACS plots of 24 hpf cells from wild-type uninjected (UI) and Tg(*gata1:DsRED*) embryos injected with L3MM or L3MO as indicated. (b) Quantitation of the percent of large cells RFP<sup>+</sup> populations from FACS analysis of L3MM- (blue) and L3MO-injected (red) Tg(*gata1:DsRED*) embryos, as indicated. *P* = 0.0107; **P** = 0.0021 (Chi-Squared test). (c) FACS plots of 48 hpf cells from wild-type uninjected (UI) and Tg(*gata1:DsRED*) embryos injected with L3MM and L3MO as indicated. (a, c) The percent of RFP<sup>+</sup> cells out of total cells and the percent of large (FSC high) and smaller sized cell (FSC low) populations are indicated. (d) May–Grunwald–Giemsa (MGG) staining of purified Tg(*gata1:DsRED*) cells from the indicated 24 hpf morphants. *p* = less differentiated progenitor cell; p = progenitor cell, more differentiated. (e) Cell type distribution in purified Tg(*gata1:DsRED*) cells at 24 hpf based on MGG staining. L3MM versus L3MO (*p* and p cells), *P* = 0.0004 (Fisher’s exact test). (f–g) MGG staining of purified 48 hpf Tg(*gata1:DsRED*) cells (f) and quantitation of MGG cell type distribution (g). p = progenitor cell; e = erythroid cells (<8 μm); m = myeloid; l = lymphoid. L3MM versus L3MO (e and p cells), *P* < 0.0001 (Fisher’s exact test). The yellow-blue color balance of merged L3MM and L3MO images was slightly adjusted in Photoshop. (e, g) The cell count for each cell type is indicated in the appropriate column. (h) FACS plots of cells from 24 hpf Tg(*spi1:EGFP*) L3MM- (top) and L3MO- (bottom) injected embryos. An arrow indicates the less mature population (*P* = 0.0069, Chi-squared test). Supplementary Figure S6a–b shows gating for GFP<sup>+</sup> cells. (i) Real-time PCR of *scl, spi1b, gata1a, gata1b,* and *drl.3* in purified large and small size populations of *gata1:RFP*<sup>+</sup> cells from 24 hpf L3MM- and L3MO-injected embryos. The relative expression in small L3MM cells was set to 1, arbitrary units. Expression was normalized to *gapdh*. Bars show mean ± S.D., triplicate experiments. **P** = 0.0083, ***P** = 0.0004, and ****P** = 0.0009 (Student’s t-test).
highly homologous to these data suggest that dsrl.3 deficiency severely compromised erythropoiesis while myelopoiesis was only modestly affected. Drl.3 is highly expressed in erythroid lineage cells compared to myeloid lineage cells, which may explain the sensitivity of erythropoiesis to drl.3 knockdown. The mechanism underlying the partial penetrance of the erythroid defect remains unclear. Possible explanations for the transient decrease in myelopoiesis are that (1) primitive myelopoiesis is delayed but recovers, perhaps by activating intrinsic compensatory mechanisms and/or that mesoderm-derived cells are not absolutely dependent on dsrl.3 for myeloid differentiation, (2) primitive myelopoiesis is rescued by definitive hematopoiesis and/or (3) cell non-autonomous signals promote myeloid cell homeostasis. It remains to be determined whether, given time, the myeloid inflammatory response to injury would recover in dsrl.3 morphants.

Embryonic knockdown of zebrafish gata1a and spib alters erythroid and myeloid lineage specification32,41,45, respectively, whereas depletion of dsrl.3 disrupts both the myeloid and erythroid lineages. Enforced expression of gata1a and dsrl.3 could not rescue the erythroid defects due to knockdown of the other gene. These data suggest that dsrl.3 and gata1a govern distinct aspects of erythropoiesis.

Drl.3 knockdown increases the proportion of less differentiated primitive erythroid-lineage cells, and possibly primitive myeloid-lineage cells, and causes a corresponding decrease of mature myeloid and erythroid cells during primitive hematopoiesis without affecting mesoderm specification, the emergence of mesoderm-derived progenitors or HSC, cell proliferation and viability. The patterns of scl and runx1 expression suggest that the normal controls over of the regulation of these genes are engaged despite the block in cell differentiation. Nevertheless, our data suggest that dsrl.3 is important for the homeostatic balance between undifferentiated cells and mature, functional cells during hematopoietic development.

**Discussion**

We have identified new zebrafish genes, dsrl.1, dsrl.2, and dsrl.3, that are highly homologous to dsrl. Knockdown of dsrl.1 and dsrl.2 together result in convergence/extension defects, however silencing of dsrl.3 uncovered a specific requirement for this gene in hematopoiesis. The relatively low expression levels of dsrl.3 in embryos compared to other family members raise the possibility that dose may be important for their roles. Enforced expression of dsrl is not sufficient to rescue dsrl morphants, supporting the idea that dsrl.3 activity is not entirely redundant with other family members and highlighting the functional divergence of dsrl.3. These studies do not rule out the possibility that dsrl, dsrl.1, and dsrl.2 may contribute to hematopoiesis. Likewise, dsrl.3 may contribute to embryonic patterning, although ablation of dsrl.3 alone is not sufficient to induce defects in non-hematopoietic tissues. Moreover, expression in definitive stem/progenitors and adult cells raises the possibility that dsrl.3, and perhaps other family members, participate in the production of hematopoietic cells throughout the lifespan of zebrafish.

The dsrl gene family encodes proteins with multiple consecutive C2H2 zinc-finger domains, an architecture that has been shown to direct sequence-specific DNA binding activity46,57. Given the unique requirement for dsrl.3 in hematopoiesis compared to other family members, we postulate that the 7 additional zinc-finger domains that are only present in Drl.3 and/or the unique amino- and carboxyl-terminal regions may underlie its hematopoietic-specific function. Alternately, the different levels of dsrl family expression may correlate to their different developmental roles. Further studies are necessary to better understand the molecular activity of Drl.3. Moreover, given the conservation of the hematopoietic regulatory program and the presence of C2H2 gene clusters in the human genome, it is likely that a human equivalent of dsrl.3 exists, although additional studies are needed to identify this gene/s.

**Methods**

**Zebrafish maintenance.** Wild-type AB stocks of Danio rerio and the transgenic lines Tg(gata1a:dsRED), Tg(fli1:EGFP), Tg(spib:EGFP), Tg(mpx:EGFP), Tg(spi1a:EGFP), and Tg(lck:EGFP) were raised and maintained under standard laboratory conditions. The FCCC IACUC approved all zebrafish procedures.

**Cloning of dsrl family genes.** The zebrafish dsrl genes were PCR-amplified from a zebrafish cdNA library with gene-specific primers (Supplementary Table S1). Full-length coding sequences were cloned into TOPO-TA (Invitrogen) then subcloned into the EcoRI site of the pCS2+ vector and confirmed by DNA sequencing. The accession numbers of dsrl.1, dsrl.2, and dsrl.3 are NM_130977, JX844126, JX844127, respectively (Supplementary Table S1). Drl.2 cdNA sequence is in Supplementary Fig. S1. The Ensembl designators are listed in Supplementary Table S1.

**Microinjections and whole mount RNA in situ hybridization (WISH).** Gene Tools, LLC (http://www.gene-tools.com/) designed the morpholinos. Sequences are provided in Table S1. Published gata1a and spib morpholinos were used as previously described42. Morpholinos (1–4 ng per embryo) were injected into the yolk of 1-cell-stage embryos, except L3MO, which was heated at 50°C for 3–5 minutes and then injected into the cell of 1-cell-stage embryos. To prepare mRNAs, dsrl.3, and gata1a clones in pCS2+ were linearized by digestion with NotI (high fidelity, NEB) and in vitro transcribed with the mMessage mMachine SP6 Kit (Ambion). mRNAs were purified using NucAway Spin Columns (Ambion) and injected into the cell of 1-cell-stage embryos. All 4 dsrl anti-sense riboprobes were prepared by using BstBI (NEB) and T3 RNA polymerase (Ambion), while sense riboprobes were prepared by using Apal (NEB) and SP6 RNA polymerase (Ambion). RNA probes were generated with digoxigenin or fluorescein RNA labeling mix (Roche) and purified by NucAway Spin Columns according to the manufacturer protocols. Digoxigenin- and fluorescein-labeled anti-sense riboprobes for zebrafish scl, krox20, myod3, gata1a, hba1e, sclata1a, spib, lplastin, and runx1 were synthesized according to published literature44.

Embryo fixation and WISH were performed as previously described43,44. Vector Labs BCIP/NBT substrate kit and Roche Fast Red Tablets were used to detect alkaline phosphatase conjugated secondary antibodies (Roche). WISH embryos were embedded in Optimum cutting temperature (Tissue-Tek OCT, Sakura Finetek) freezing medium for 8-micron thick transverse cryosections.
O-dianisidine staining and cell quantitation: Hemoglobin was detected by incubating live embryos in o-dianisidine (Sigma) + H2O2 as previously described. To determine the threshold for whether whole mount embryos are scored as having normal or decreased numbers of erythroid cells, we used control and Drl3 morphants analyzed by gata1a WISH at 24 hpf and o-dianisidine staining at 48 hpf. We then scored the embryos as having apparently normal or low numbers of erythroid cells by microscopy and from individual embryos, we counted the numbers of gata1a+ cells within a matching 3 somite region of the trunk and the o-dianisidine+ cells on comparable regions of the yolk surface. The average number of cells in embryos observed to have decreased erythropoiesis was about half that of apparently normal embryos, although there was some variation (Supplementary Fig S2–F). Based on these data, we can estimate that embryos visually scored as having decreased erythropoiesis have ~60% the normal numbers of cells.

FACS analysis and cytospin. For each embryonic sample, 100–200 dechorionated transgenic embryos were pooled, washed with ice-cold FACS buffer (1 mM HEPES, 5% FBS, 0.1% EDTA, 1% Triton X-100, 5% FBS, 1% deoxycholate, 1% Nonidet P-40, 1% SDS with 0.2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail and 10% FBS) and fixed with 500 mM sodium chloride (NaCl) and 4% PFA. Whole embryos were cut into pieces and filtered through a 1 μm mesh. Whole kidneys from 15 wild-type AB strain adult fish (31 months old), 6 gata1a:dsRed adults (15–21 months old) and 6 mpeg:EGFP adults (13 months old) were mechanically dissociated in ice-cold FACS buffer and filtered through an 85 μm mesh. Propidium iodide (PI) was added to preparations from GFP transgenics. Up to 100,000 fluorescently labeled cells were collected for each sample using Becton-Dickinson FACS Vantage SE or Aria II cell sorters. Sorted cells were stained with May-Grünewald-Giemsa stain as previously described.

RT-PCR and quantitative real-time PCR. Reverse Transcription-PCR (RT-PCR) analysis of drl family gene expression was performed on RNA extracted with TRIzol (Invitrogen) from individual or pools of 10 embryos. RNA was extracted from sorted hematopoietic populations using a phenol/chloroform/isoamyl alcohol protocol and DNase treatment (Ambion). The primers for these studies are listed in Table S1. For quantitative real-time PCR, cDNA was prepared from whole embryo or purified cell RNA extracts with SuperScript II First Strand Synthesis System (Invitrogen) and oligo-dT primers (Ambion). Gel images were acquired from a UVP DigiDoc-It Imaging system with a Canon Powershot G9 camera and Canon Utilities RemoteCapture DC 3 software. Quantitative real-time PCR was performed using the Taqman Master Mix (Applied Biosystems) according to the manufacturer protocols. Oligonucleotide sequences were designed and provided by Applied Biosystems. Gene expression changes were normalized to gpdh.

Drl3 antibody production and immunodetection. A rabbit polyclonal antibody was generated in the FACC animal facility against Drl3 carboxy-terminal peptide sequence CEGEHDQTSLLKG (Alpha Diagnostic International). Antibody was purified from 6 bleeds of rabbit using the Sulfolink Immunobilization Kit for Peptides (Pierce) according to manufacturer’s instructions, except 0.5% Acetic Acid with 150 mM sodium chloride (NaCl) was used as elution buffer. Anti-Drl3 was diluted 1:25 in 5% milk in PBST (Fisher Scientific) and incubated overnight at 4°C with western blots. Monoclonal Tubulin antibody (Sigma) was used at a 1:5,000 dilution. Peroxidase conjugated secondary antibodies (Jackson Immunoresearch Labs Inc) and ECL Substrate (Pierce) were used for detection.

HEK-293T cells were cultured using standard procedures (www.ATCC.org) and transfected with pcDNA3.1+ or a construct encoding a FLAG-Drl3 fusion protein using LipoFectamine2000 (Invitrogen). Cells were treated with 1% Triton X-100, 150 mM NaCl, 20 mM HEPES, 5 mM EDTA, 5 mM sodium fluoride, 0.2 mM sodium orthovanadate and Complete protease inhibitor cocktail (1 tablet/10 ml buffer, Roche). Whole embryo lysates were prepared by pooling randomly selected embryos per sample, removing the yolk using mechanical dissociation and lysing in 20 mM HEPES, 150 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, 1% SDS with Complete protease inhibitor cocktail (Roche). Cytoplasmic lysates were prepared from dissociated embryos lysed in 10 mM HEPES, 10 mM potassium chloride, 1.5 mM magnesium chloride (MgCl2), 1 mM diithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail and 10% Nonidet P-40. Following centrifugation (cytosol), cells were (supernatant) was collected. The nuclear fraction (pellet) was lysed using 20 mM HEPES, 400 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, 5% glycerol, and protease inhibitor cocktail (Roche). Standard procedures were used for 10% polyacrylamide electrophoresis and transfer of proteins to nitrocellulose.

Whole mount immunofluorescent detection was performed as previously described. Anti-cleaved-Caspase 3 (BD Biosciences) and anti-phospho-Histone H3 (Santa Cruz) were diluted 1:100 and 1:200, respectively. Alexa-488-conjugated secondary antibodies (Life Technologies) were diluted 1:200 dilution. Samples were mounted for fluorescence microscopy in Vectashield mounting medium with DAPI (Vector Labs).

Imaging. Bright field images were captured on a Nikon SMZ 1500 microscope with a Spot Insight camera using Spot Basic software. Fluorescent and cytospin images were captured on a Nikon SMZ 1500 microscope with a Nikon Digital Sight camera or a Nikon Eclipse E80 microscope with a CRI Nuance Multispectral camera and a Nikon Digital Sight camera. Cells were obtained using NPs Element AR software or CRI Nuance 2.1.0 software. Composite images of different focal planes were compiled in Adobe Photoshop without altering the characteristics of the images. Confocal images were acquired on a Nikon Eclipse TE-2000E/C1 laser scanning confocal microscope using EZ-C1 3.80 software (Nikon) and analyzed with Fiji software (distributed by ImageJ, National Institutes of Health).
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