Regulation of iron transport is required for terminal erythroid differentiation even under iron-replete conditions that are sufficient for hemoglobinization

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Abstract

Erythroid cells are the main driver of iron utilization in vertebrates, as their main role is to synthesize hemoglobin to oxygenate the body's tissues. As such, iron is a key nutrient for the development and function of erythroid cells. When iron deficient, erythroid cells are both lacking in hemoglobin and exhibit differentiation defects. Currently, the efficacy of iron supplementation is monitored by measuring indices of erythroid hemoglobinization. However, its effect on erythroid differentiation is less clear. In this study, we used zebrafish with genetic iron metabolism defects to determine if iron supplementation could rescue erythropoietic defects in organisms that are iron deficient at the cellular and systemic level. To carry out this study, we developed a technique to sort Tg(*globin lcr:EGFP*) erythrocytes from single zebrafish embryos onto slides for imaging. We found that iron supplementation in *mfrn1* mutant zebrafish, which carry a defect in mitochondrial iron trafficking, restored hemoglobinization but not erythroid cell number or terminal differentiation. Iron supplementation in *fpn1* mutant zebrafish, which have defects in export of iron from yolk syncytial cells and intestinal epithelium, functioning a model of dietary iron deficiency, restored erythroid cell number but not terminal differentiation deficiencies. Our data suggests that in addition to adequate iron levels, correct regulation of iron trafficking is required for optimal erythroid iron utilization and terminal erythropoiesis.

Introduction

Iron deficiency anemia (IDA) poses a formidable public health problem, afflicting 27% of the world's population and accounting for 8.8% of the world's total disability. Iron deficiency especially common problem among pregnant women, as pregnancy requires large quantities of iron to support the placenta, fetal growth and increased maternal blood volume (1-3). Iron deficiency is commonly treated by oral or IV supplementation. When assessing the efficacy of iron supplementation on iron deficient patients, the focus is on hematocrit, hemoglobinization status and iron levels, as erythroid cells utilize 90% of the body's iron for hemoglobin synthesis. The focus on hemoglobinization masks another important role of iron in erythroid cell biology,

namely its role in erythroid differentiation. Previous studies in model organisms have consistently shown erythroid differentiation defects in mutants of iron metabolism genes (4-10). The commonalities have included: decreases in erythroid cell number, enucleation defects, defects in nuclear condensation and impaired erythroid cell proliferation. A common assumption is that these differentiation defects are driven by iron deficient hemoglobinization defects, leading to anemia and embryonic lethality. However, this hypothesis has not been formally tested and neglects the possibility that iron metabolism may play alternative roles in erythroid differentiation. Addressing this hypothesis, and determining how iron facilitates erythroid development, is critical for optimizing therapeutics for iron deficiency anemia. Conceptually, this is an important question as little is understood mechanistically as to how transition metals are handled in different cellular contexts and employed as effectors of developmental signaling, despite the well-established role of transition metals in cellular differentiation (11-16).

In mammalian models, rescue of anemia in embryos by iron supplementation would require *in utero* iron supplementation or blood transfusions, which are both invasive and technically challenging. We have therefore decided to use the zebrafish as an *in vivo* model to dissect the role of iron metabolism in erythroid differentiation. Zebrafish embryos develop externally, allowing supplementation of exogenous iron to chemically complement iron metabolism defects. We have previously identified hinokitiol as an iron chelator that can bind to iron and transport it down a concentration gradient through cellular membranes (17). The iron-hinokitiol complex has previously been shown to complement hemoglobinization defects *in vivo* and enter the mitochondria (17), showing that iron-hinokitiol be used in heme synthesis.

In this work, we used iron-hinokitiol to supplement zebrafish with genetic iron transport defects with sufficient iron to rescue hemoglobinizatio. We then examined whether normal erythropoiesis was restored in iron-supplemented embryos. A key challenge of studying erythropoiesis at the cellular level in the zebrafish model is the small number of cells in embryos. We have therefore designed a method with which to sort erythroid cells from single zebrafish embryos onto microscope slides for imaging. Beyond our studies in iron biology, these studies increase the resolution at which we examine *in vivo* effects using the zebrafish model. We carried out studies with the *frs* mutant zebrafish line, which carry a defect in mitochondrial iron transport protein, Mfrn1, and the *weh* fish line, with a Fpn1 defect, causing defective dietary iron transport from the intestine or yolk syncytia. Our results demonstrate that defects in iron transport proteins cause defects in terminal erythropoiesis, even when sufficient iron is present for erythroid cells to make hemoglobin. Our work suggests a model where in iron transport proteins participate in multiprotein complexes that enable inter-organellar transport and trafficking of iron in its various chemical forms to facilitate terminal erythropoietic developmental pathways.

Results

The goal of this study was to investigate the role of iron in erythroid differentiaton. To circumvent the issue of genetic iron deficiency causing embryonic lethality, we used the zebrafish system to interrogate the effects of genetic iron deficiency on erythropoiesis. To do this, we optimized isolation of erythroid cells from zebrafish embryos to facilitate analysis of

their development. A number of studies have obtained embryonic blood by tail amputation (10) or from adult zebrafish, as there are larger numbers of cells in the kidney marrow, the site of adult hematopoiesis (8). However, to maximize the use of the cells in zebrafish embryos, we wanted to concurrently obtain quantifiable information about the numbers of differentiated erythroid cells and collect cells for imaging in the same experiment and from single embryos. We took advantage of the Tg(*lcr:eGFP*) zebrafish line to sort zebrafish erythroid cells during development (18). The zebrafish globin locus control region (LCR) controls globin gene expression during erythroid development, and erythroid cells in the Tg(*lcr:eGFP*) zebrafish line are marked by GFP expression (18). We did a first pass experiment by FACS sorting GFP^+ cells from 2-5 dpf zebrafish embryos onto microscope slides, followed by Wright-Giemsa staining of the cells to monitor their morphology. Average benzidine staining intensity in GFP^+ cells was not appreciably altered over time, suggesting that the kinetics for maximal hemoglobin production in embryonic zebrafish erythroid cells was much faster than that in mice (19) (Figure 1A, B). Unlike other vertebrate erythroid cells, the size of zebrafish nuclei, as measured by the areas delineated by DAPI staining, did not change significantly during differentiation (Figures 1A, B). This suggests that condensation of zebrafish erythroid nuclei occurs early in differentiation, unlike in vertebrate erythroid cells (20-22). We also analyzed erythroid cellular morphology in Giemsa stained erythroid cells. Consistent with the literature, as zebrafish erythroid cells mature, they elongate and become more elliptical (23-25) (Figure 1C). These data show that our FACS sorting method of isolating erythroid cells robustly recapitulates what was observed from previous methods of zebrafish erythroid cell isolation.

We then asked if we could carry out this technique on single zebrafish embryos. Zebrafish are an excellent model for studying development *in vivo*. Among their many advantages, the optical transparency of zebrafish allows the use of transgenic reporter lines to track the fate and localization of specific cell types. FACS analysis is often used to quantitate the abundance of specific cell types in zebrafish. However, because of the small size of zebrafish embryos and small number of cells arising from each cell type, FACS analysis is normally performed on pools of embryos. This precludes comparisons between groups that are phenotypically similar (for example, between wild-type and heterozygote embryos) as these animals are typically not pre-genotyped and could be grouped together during analysis. We therefore designed a workflow where we collect cells from a single zebrafish embryo for genotyping, analyze erythropoiesis in the same embryo using FACS, and finally, carry out imaging analyses on the sorted erythroid cells.

To begin, we crossed zebrafish hematopoietic mutants into the *Tg (globin lcr:eGFP)* transgenic zebrafish line and homogenized 3 dpf single embryos in PBS by dounce homoginization. Single cell suspensions for cell sorting were obtained by filtration of homogenized cells. The single cells were washed, and fixed with glutaraldehyde prior to sorting. GFP^+ erythroid cells were quantitated by FACS analysis, and sorted onto a microscope slide at a density of 100 cells per spot. Cell aggregates that were trapped in the cell filter were washed, spun down and lysed to obtain genomic DNA. Lysates were then subjected to PCR with primers flanking the gene mutation. The PCR band was extracted and sequenced (Figure S1).

Using this approach, we were able to sort and phenotype erythroid cells from single embryos resulting from incrosses between *frs/+; Tg (globin lcr:eGFP)* zebrafish, which carry a mutation in the *Mfrn1* mitochondrial iron transporter (8). Consistent with previous results, we found a significant decrease in the number of GFP^+ erythroid cells in *frs/frs* embryos relative to wild-type siblings (Figure 2A). Benzidine staining of sorted *frs/frs* erythroid cells showed that

they had a hemoglobinization defect, consistent with previous reports in zebrafish and mouse models (7,8). DAPI staining of nuclear DNA indicated a DNA condensation defect in the *frs* embryos. A nuclear size defect in *frs* embryonic blood was further indicated by Giemsa staining (Figure 2B). We isolated DNA from unsorted cells to genotype individual embryos. The region flanking the mutation was amplified by PCR, and genotypes were obtained by Sanger sequencing. Of note, we were able to isolate sufficient quantities of gDNA from unsorted cells obtained from single embryos that produced good quality sequencing data.

To determine if iron supplementation rescues the erythropoiesis in zebrafish with a defect in mitochondrial iron transport caused by *Mfrn1* deficiency *in vivo*, iron supplementation was carried out by treating 24 hpf zebrafish embryos iron-hinokitiol which enables the transport of iron through a lipid membrane down a concentration gradient (17). At 72 hpf, embryos were homogenized and processed as described in Figure S1. Iron supplementation caused increased erythroid hemoglobinization in *frs/frs* erythroid cells (Figure 3A,D), as was previously described in the literature (17). Strikingly however, iron supplementation did not rescue the defect in erythroid cell number in *frs/frs* embryos, suggesting that the presence of *Mfrn1* was required for erythroid cell proliferation, survival or differentiation, even under iron-sufficient conditions (Figure 3B). Iron supplementation also does not rescue erythroid nuclear size defects (Figure 3C, D).

Is the requirement for iron transporters in erythroid differentiation limited to intracellular iron transport, or are extracellular iron transporters also required for optimal levels of erythroid differentiation? To address this question, we investigated erythroid differentiation in *weh/weh*; *Tg (globin lcr:eGFP)* zebrafish (9,26), which carry a mutation in *Fpn1*, an iron exporter that functions in intestinal epithelial or yolk syncytial cells. Because these fish have defective export of iron from iron stores, they serves as a genetic model for dietary iron deficiency. *weh/weh* zebrafish did not have as severe a defect in erythroid cell number as *frs/frs* erythroid cells (Figure 4A, B); however, their erythroid cells had a hemoglobinization defect and they had enlarged nuclei (Figure 4B). Similar to the *frs/frs* zebrafish, we were also able to obtain high quality data and genotyping from single embryos (Figure 4). To determine if iron supplementation could rescue the erythropoietic defect in *weh/weh* zebrafish, we supplemented WT and *weh/weh* zebrafish with exogenous iron, as described above. Consistent with previously published results (Burke), iron-hinokitiol was able to compensate for the genetic defect and rescue erythroid hemoglobinization (Figure 5A). Notably, in this case iron supplementation was able to lead to a significant increase in the number of *lcr:eGFP* erythroid cells in *weh/weh* zebrafish (Figure 5B). The nuclei of *weh/weh* erythroid cells remained enlarged even after iron supplementation (Figure 5C, D). These data suggest a heterotypic role for iron supplementation in the rescue of erythropoietic defects in genetic iron deficiency that is dependent on the type of iron transport defect present.

We have developed a method that allows us to analyze erythroid cell development *in toto* using FACS and imaging analysis. The use of zebrafish embryos to analyze the effects of iron deficiency on early erythroid development and differentiation provides a noninvasive *in vivo* method by which the ability of iron supplementation to correct the effects of iron deficiency can be reliably monitored and tested. Our ability to phenotype single zebrafish embryos at high resolution, and genotypes of each embryo in tandem, increases the standard of rigor in these experiments. This method can be extended to studying early development in other cell types such as liver or hematopoietic cell types for which transgenic fluorescent zebrafish lines exist. Collectively, these data demonstrate that iron supplementation, while effective in restoring

hemoglobinization, does not fully rescue the erythropoietic defects resulting from deficiencies in cellular iron transporters. Further, the difference in erythroid cell number in the *frs/frs* versus *weh/weh* mutants in response to iron indicates a qualitative difference in the erythroid response to iron status. This difference depends on whether the defect is one of intrinsic cellular iron processing (*frs/frs*), or whether organisms have a defect in systemic iron absorption but in which cell intrinsic iron processing is normal (*weh/weh*).

Discussion

Our studies confirm previous work demonstrating that genetic defects in iron metabolism causes defects in erythropoiesis. However, in addition, we leveraged the external development of zebrafish embryos to determine the extent to which *in vivo* iron supplementation can rescue erythropoietic defects in genetic models of iron deficiency. Using the Tg(*lcr:eGFP*) transgenic zebrafish line, we sorted erythroid cells from control or iron treated wild-type or mutant zebrafish embryos. We developed a method which enabled us to accurately quantify and sort erythroid cells from single zebrafish embryos for imaging, as well as to analyze the genotypes of the same embryos in tandem. This significantly increases the experimental rigor of these experiments, significantly adding to our arsenal of tools to study development *in vivo*. Imaging of zebrafish erythroid cells revealed that while iron supplementation rescued hemoglobinization in mutants with iron transport defects, iron treatment did not fully restore normal erythropoiesis in mutant zebrafish.

Our results revealed several differences between zebrafish and higher vertebrates in their terminal erythropoietic program. In mice and humans, hematopoietic cells commit to the erythroid lineage. After erythropoietic commitment, erythroblasts undergo 4 cell divisions, during which they become progressively differentiated. During this differentiation process, hemoglobin production, heme synthesis and iron uptake/utilization is transcriptionally upregulated. Concomitantly, morphological changes, such as nuclear condensation, occur during the terminal cell divisions of terminal erythropoeisis (19-22). In contrast, our studies show that zebrafish erythroid cells contain high levels of hemoglobin even at the earliest stages of erythropoiesis. Their nuclear size also remains fairly constant during terminal erythroid differentiation. The morphological changes associated with terminal erythropoiesis seem to consist of cells gradually becoming more elongated and increase of cytoplasmic area (Figure 1). Our studies and others demonstrate that zebrafish with mutations causing erythropoietic defects cause enlarged erythroid nuclei, indicating that nuclear condensation is still part of the erythropoietic program in zebrafish. Our data suggest that in contrast to vertebrates (where nuclear condensation progressively occurs at the final steps of terminal differentiation), nuclear condensation occurs much earlier in the zebrafish erythropoietic differentiation process, possibly even at the initiation of globin LCR activation (18,20-22).

Iron metabolism regulates erythropoiesis at multiple levels. Aside from the expected requirement for iron metabolism for hemoglobinization, our findings show that iron metabolism is required for regulation of erythroid cell number, nuclear condensation and later morphological changes associated with terminal erythropoiesis (Figures 2-4). The requirement for iron for erythroid cell number indicates that iron is required even at the earliest stages of erythropoiesis. This is consistent with previous studies showing that iron deficiency in mice favors the differentiation of megakaryocytic-erythroid progenitors (MEPs) towards the megakaryocytic lineage, while decreasing hematocrit. In iron deficient mice, MEPs contain decreased labile iron and have lower levels of cellular proliferation (27). The stark decrease in erythroid cell numbers

that we observed in our iron metabolism mutants is consistent with these studies. Further, our studies add to this by carrying out chemical complementation of iron deficiency. While we were able to restore erythroid hemoglobinization, iron supplementation could not rescue erythroid cell number and differentiation in *frs/frs* mutants, demonstrating that in addition to systemic iron, *intracellular* iron metabolism mediated by correct transport pathways plays a major role in erythroid differentiation and cell number.

How may intracellular iron transport regulate cellular differentiation and cell numbers? Little is known about interorganellar iron trafficking through iron transport proteins. Recent studies have shown that formation of multiprotein complexes is essential for the proper regulation of heme synthesis (28,29) and iron-sulfur cluster formation (30-33). An intriguing paper by Martinez-Guzman *et al*. demonstrated that labile heme traffics from the mitochondrial inner membrane, where it is synthesized, to the nucleus more rapidly than to the more proximate cellular compartments such as the mitochondrial matrix or cytosol (34). These data raise the possibility of direct inter-organelle contacts that transport heme (or iron) between reactions in different subcellular compartments via transporters. The requirement for iron transporters in erythropoiesis, even under iron-replete conditions that support hemoglobinization, supports this hypothesis. So then what do these observations tell us about the mechanism of ferroportin, which supplies iron indirectly to erythroid cells by cellular export into serum from intestinal epithelia? One possibility is that ferroportin transfers iron to carrier proteins or chelators, which are then efficiently transferred to transferrin and taken up by erythroid cells via a transferrin mediated pathway.

Collectively, these data indicate that intact iron transport machinery is required for iron to facilitate terminal erythropoiesis. Iron, in its various chemical forms, is a signal to the nucleus during terminal erythropoiesis. Deficiencies in mitochondrial iron metabolism cause generalized defects in erythropoiesis, including altered nuclear morphology and cell number (which may be caused by defective cell proliferation, cell death or defects in cell fate decisions) which all involve nuclear processes. These studies indicate that detailed studies on iron metabolism and handling, and the investigation of how iron metabolism facilitates erythropoiesis, continue to be essential for design of therapeutics for iron deficiency anemia.

Together, our study has illuminated a number of differences between zebrafish and mammalian erythropoiesis. However, the zebrafish model continues to be a valuable model for understanding erythroid cell development because of our ability to examine development, and the effects of clinically relevant treatments of hematopoietic diseases, *in vivo.* In summary, we have developed a technique with which to rigorously interrogate erythroid development *in vivo*, which will be applicable to the studies of other cell types. Our studies have revealed that genetic iron deficiency due to defects in iron transport causes erythropoietic defects that cannot be effectively treated by exogenous iron supplementation, despite the ability of iron supplementation to ameliorate hemoglobinization defects. Conceptually, this reveals a role for iron transporters in developmental signaling. Clinically, our studies are significant as they reveal the need for detailed studies of iron transport in order to develop effective therapeutics for iron deficiency anemias.

Methods and materials

Complete methods are described in the Supporting Information.

Acknowledgements

Author contributions

M.C., A.D., Y.Y.Y. conceptualization; M.C., A.D., Y.Y.Y., data curation; M.C., A.D., R.W.. Y.Y.Y., formal analysis and validation. M.C., A.D., R.W., A.S., Y.Y.Y., investigation. M.C., A.N.S., Y.Y.Y. writing-review and editing. A.N.S.,Y.Y.Y. funding acquisition; M.C., Y.Y.Y. writing-original draft; Y.Y.Y. project administration.

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Figures

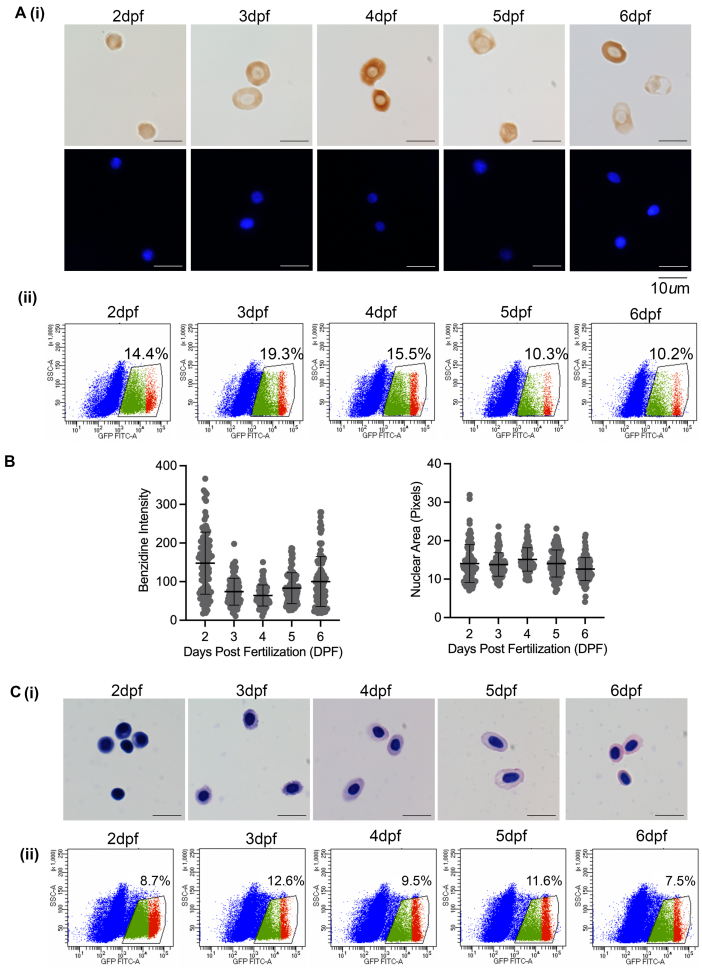

Figure 1. Sorting of erythroid cells from a bulk population of Tg(*lcr:eGFP*) zebrafish embryos. (A)(i) Benzidine and DAPI staining of erythroid cells from 2-5 dpf Tg(*lcr:eGFP*) zebrafish embryos. (ii) FACS plots denoting sorted population. Boxed regions denote cell population that was sorted onto microscope slides. Numbers indicate percentages of GFP^+ cells in homogenized zebrafish embryos. (B) Quantitation of benzidine staining intensity and nuclear area (DAPI stained area) of sorted cells. (C) (i) Wright-Giemsa staining of sorted erythroid cells. (ii) FACS plots denoting sorted population.

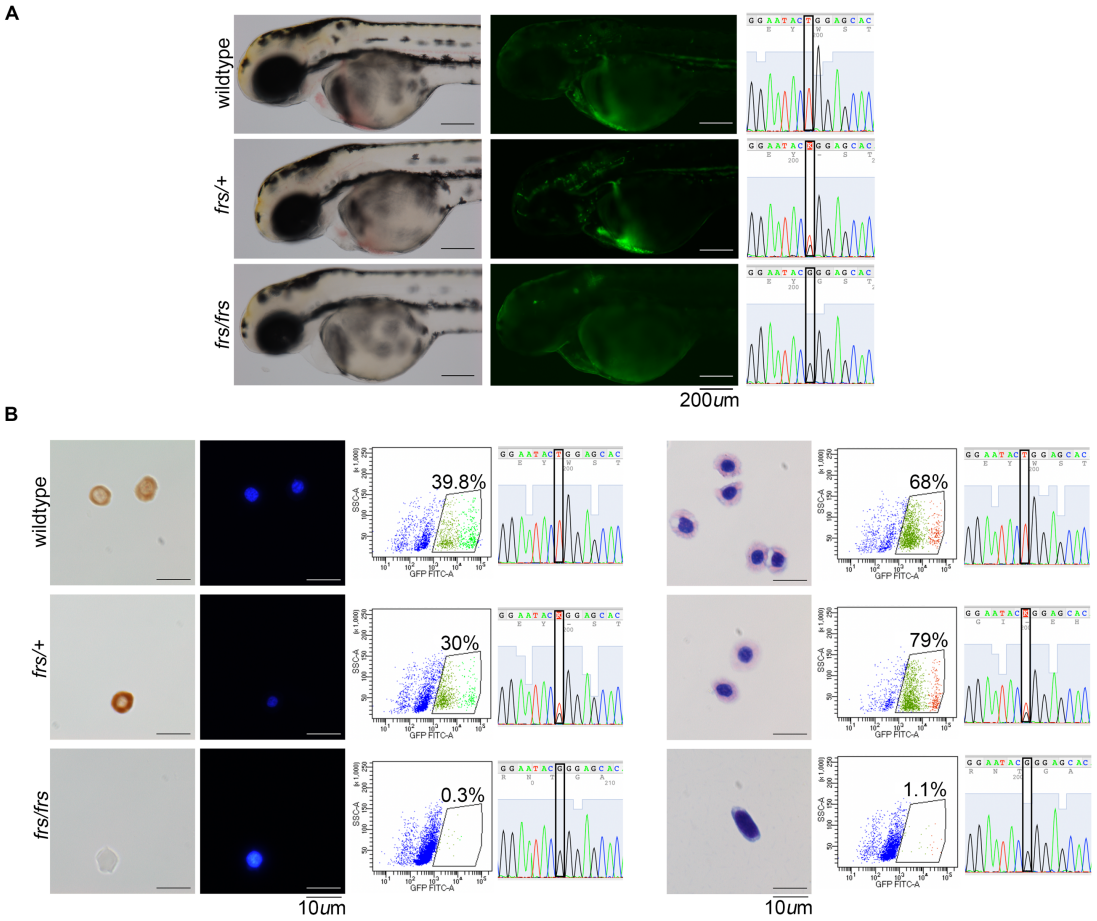

Figure 2. Sorting of erythroid cells and genotyping of individual WT, *frs/+* and *frs/frs* zebrafish embryos. (A) Imaging and genotyping of 3 dpf wild-type Tg(*lcr:eGFP*), *frs/+;* Tg(*lcr:eGFP*), and *frs/frs;* Tg(*lcr:eGFP*) zebrafish embryos. As previously shown, *frs/frs* zebrafish embryos have starkly decreased numbers of erythroid cells, as denoted from the numbers of GFP^+ cells. (B) Imaging of erythroid cells sorted from single zebrafish embryos. (Left) Benzidine and DAPI staining of erythroid cells. (Right) Wright-Giemsa staining of erythroid cells. Sanger sequencing indicates that good quality sequencing data can be obtained from unsorted cells obtained from single embryos; FACS plots indicate that within individual clutches, *frs/frs* zebrafish have sharply decreased numbers of GFP^+ erythroid cells compared to wild-type and heterozygous fish.

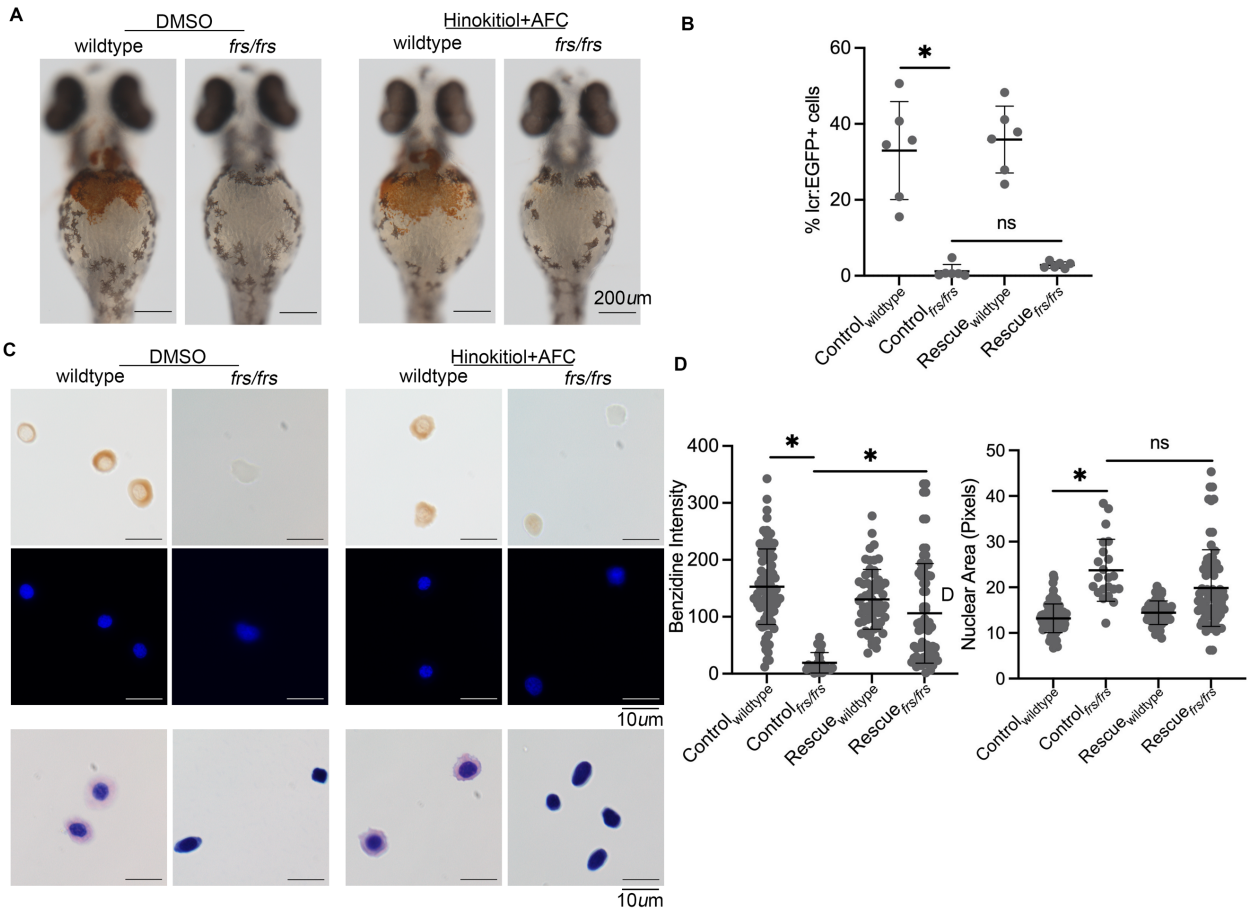

Figure 3. Iron supplementation does not rescue the erythroid cell number or differentiation defect in *frs/frs* zebrafish embryos. (A) Whole mount imaging of DMSO (vehicle) or iron-hinokitiol (hinokitiol + ammonium ferric citrate (AFC)) treated 3 dpf wild-type or *frs/frs* zebrafish embryos. Iron-hinokitiol treatment of *frs/frs* zebrafish embryos partially restores the overall hemoglobinization of the embryo. (B) FACS analysis shows that treatment of *frs/frs* zebrafish embryos with iron does not increase the number of erythroid cells. (C) Sorting of control or iron-hinokitol treated erythroid cells from wild-type and *frs/frs* embryos onto slides for imaging, followed by benzidine/DAPI (top) or Wright-Giemsa staining. (D) Quantitation of benzidine intensity or DAPI staining of imaged erythroid cells shows that iron-hinokitiol restores hemoglobinization in erythroid cells that are formed in *frs/frs* zebrafish (left) but does not reverse the nuclear size defect in *frs/frs* erythroid cells.

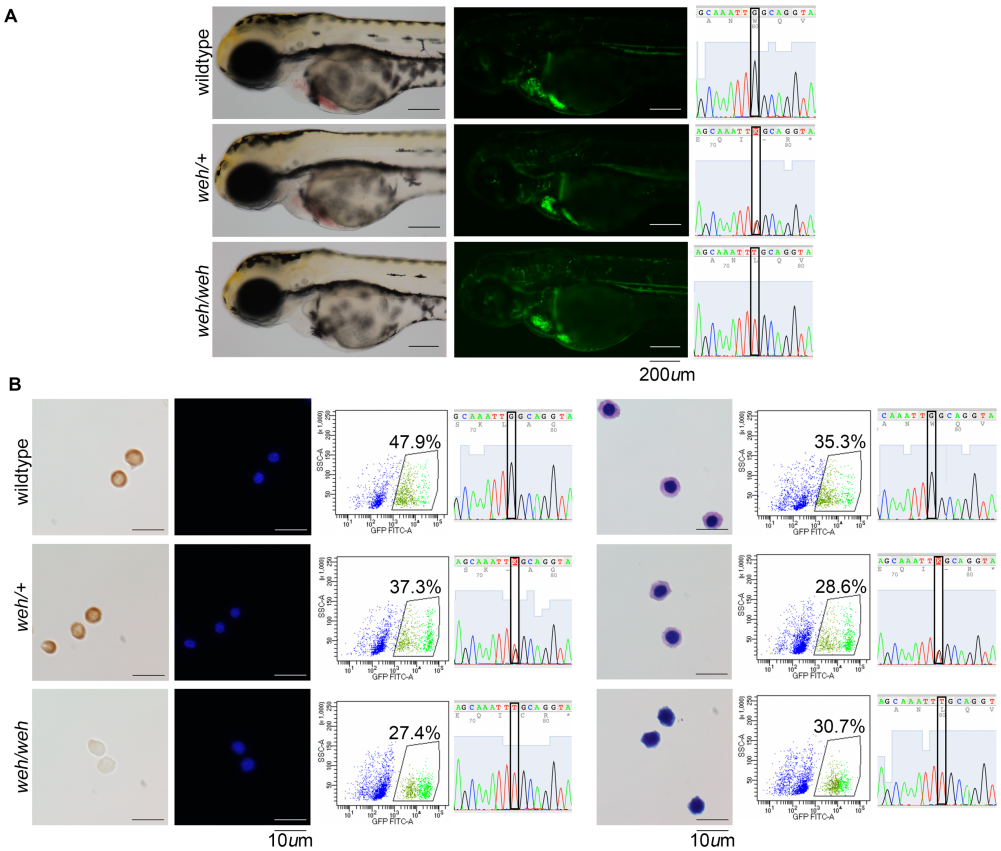

Figure 4. Sorting of erythroid cells and genotyping of individual WT, *weh/+* and *weh/weh* zebrafish embryos. (A) Imaging and genotyping of 3 dpf wild-type Tg(*lcr:eGFP*), *weh/+;* Tg(*lcr:eGFP*), and *weh/weh;* Tg(*lcr:eGFP*) zebrafish embryos. *weh/weh* zebrafish embryos do not have a stark decrease in the number of of erythroid cells, as denoted from the numbers of GFP^+ cells. (B) Imaging of erythroid cells sorted from single zebrafish embryos. (Left) Benzidine and DAPI staining of erythroid cells. (Right) Wright-Giemsa staining of erythroid cells. Sanger sequencing indicates that good quality sequencing data can be obtained from unsorted cells obtained from single embryos; FACS plots indicate that within individual clutches, *weh/weh* zebrafish have slightly decreased numbers of GFP^+ erythroid cells compared to wild-type and heterozygous fish.

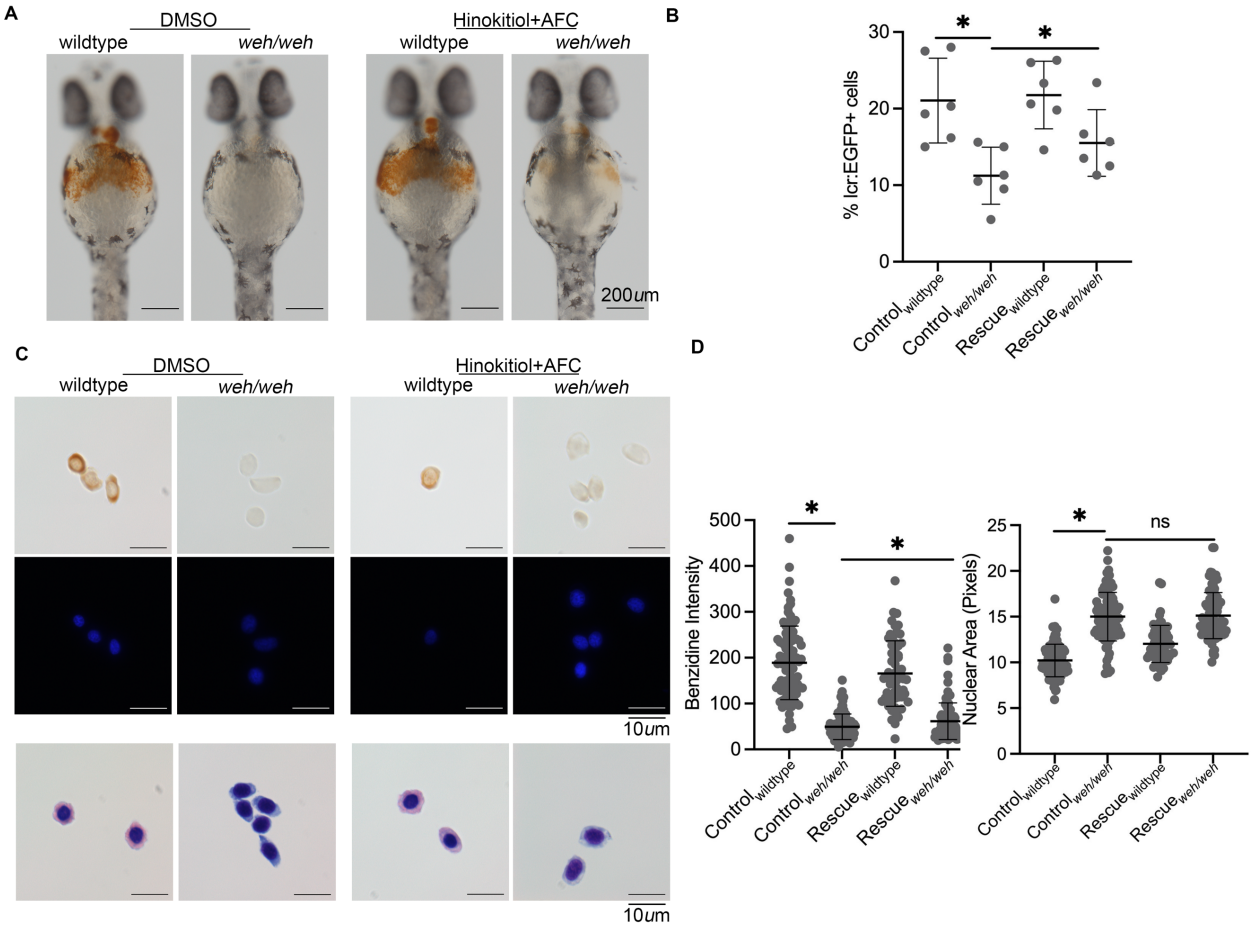

Figure 5. Iron supplementation does not rescue the erythroid cell number or differentiation defect in *weh/weh* zebrafish embryos. (A) Whole mount imaging of DMSO (vehicle) or iron-hinokitiol (hinokitiol + AFC) treated 3 dpf wild-type or *weh/weh* zebrafish embryos. Iron-hinokitiol treatment of *weh/weh* zebrafish embryos partially restores the overall hemoglobinization of the embryo. (B) FACS analysis shows that treatment of *weh/weh* zebrafish embryos with iron increases the number of erythroid cells. (C) Sorting of control or iron-hinokitol treated erythroid cells from wild-type and *weh/weh* embryos onto slides for imaging, followed by benzidine/DAPI (top) or Wright-Giemsa staining. (D) Quantitation of benzidine intensity or DAPI staining of imaged erythroid cells shows that iron-hinokitiol increases hemoglobinization in erythroid cells that are formed in *weh/weh* zebrafish (left) but does not reverse the nuclear size defect *weh/weh* erythroid cells.

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