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Ze brafish as a model system to delineate the role of heme and iron metabolism during erythropoiesis

Jianbing Zhang, **Iqbal Hamza***

Department of Animal & Avian Sciences and Department of Cell Biology & Molecular Genetics, University of Maryland, College Park, MD 20742, USA

Abstract

Coordination of iron acquisition and heme synthesis is required for effective erythropoiesis. The small teleost zebrafish (*Danio rerio*) is an ideal vertebrate animal model to replicate various aspects of human physiology and provides an efficient and cost-effective way to model human pathophysiology. Importantly, zebrafish erythropoiesis largely resembles mammalian erythropoiesis. Gene discovery by large-scale forward mutagenesis screening has identified key components in heme and iron metabolism. Reverse genetic screens using morpholino-knockdown and CRISPR/Cas9 have further accelerated gene functional studies, taking advantages of genetic tractability of zebrafish embryos. Ultimately, the *ex utero* development of zebrafish embryos combined with their transparency and developmental plasticity could provide a deep understanding of the role of iron and heme metabolism during early vertebrate embryonic development.

Keywords

zebrafish; porphyria; heme transport; iron metabolism; erythropoiesis

1. Introduction

The teleost fish, *Danio rerio*, also known as zebrafish, has gained greater attention as a model organism for studying vertebrate development owing to its advantage in developmental biology. Adult zebrafish are relatively small and one breeding pair can prod uce 100–200 progenies per spawning each week, which allows easy maintenance of animal strains with small space and ensures production of numerous embryos for experiments (genetic screening work and building transgenic lines). The external fertilization and rapid development *ex utero*, combined with the property of transparency, allow direct visualization

*Corresponding author: University of Maryland, College Park, 2413 ANSC, Bldg 142, College Park, Maryland 20742, hamza@umd.edu.

I.H. is the President and Founder of Rakta Therapeutics Inc. (College Park, MD), a company involved in the development of heme transporter-related diagnostics. He declares no other competing financial interests. Correspondence and request for materials should be addressed to I.H. (hamza@umd.edu).

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and manipulation of developmental processes in early embryos, which are not possible in mice. The techniques for transgenesis and gene manipulation are well-developed, rendering zebrafish as a genetically-tractable vertebrate animal model [1].

2. Zebrafish erythropoiesis resembles those of higher vertebrates

Zebrafish has a similar hematopoietic program compared to higher vertebrates, spanning from developmental waves of hematopoiesis to conservation of producing comparable blood cell components. Two successive waves of hematopoiesis, primitive and definitive, are also found in the zebrafish [2, 3]. Additionally, crucial regulators have been isolated as orthologs of many essential mammalian hematopoietic regulators and functional conservation of these factors in zebrafish is validated by morpholino-mediated knockdown or identified mutants [4]. These conserved properties of zebrafish hematopoiesis make it a good model for hematopoietic study and further applicable to mammals.

Primitive erythropoiesis in zebrafish occurs in the intermediate cell mass (ICM) of developing embryos, which is functionally equivalent to extra-embryonic yolk sac blood island region in mammals [5]. Primitive erythroid lineage arises from ICM which is previously developed from posterior lateral mesoderm (PLM) region. Primitive erythropoiesis in zebrafish begins at 4-somite stage with the onset expression of erythroid-specific transcription factor GATA1, a zinc-finger transcription factor for early primitive erythroid progenitors differentiation [6]. The circulating primitive erythroblasts further proliferate and differentiate into mature erythrocytes through a series of morphological alterations, with elongated nucleus around 4 dpf (days post fertilization), and functionally survive to 10 dpf in peripheral blood stream [7]. Mature erythrocytes display unique morphological characteristics, distinguishable from adult counterparts by less cytoplasm and remaining nucleus.

Consistent with hematopoietic programs in mammals, definitive hematopoiesis in zebrafish marks its distinctive wave by generating all blood cell lineages throughout the whole lifespan. The initiation site of zebrafish definitive hematopoiesis has been identified by expression patterns of mammalian definitive hematopoietic regulator orthologues, C-MYB and RUNX1. These two transcription factors dictate the formation of definitive HSPCs (hematopoietic stem/progenitor cells) [8]. In zebrafish, initial expression of *runx1* and *c-myb* is located in the VDA (ventral wall of dorsal aorta) from 26–48 hpf (hours post fertilization), suggesting that definitive HSPCs are generated in the VDA. The initiation of definitive hematopoiesis in VDA happens as early as 36 hpf [9]. At around 4–5 dpf, hematopoietic cells are identifiable in pronephros/kidney and thereafter by 13 dpf. The fish kidney functions as a counterpart of the mammalian bone marrow to sustain definitive hematopoiesis throughout its lifespan [10]. Posterior blood island (PBI), also called caudal hematopoietic tissue (CHT) and located between the caudal artery and the caudal vein at the end of the tail, is recognized as a fetal hematopoietic organ in zebrafish and an intermediate hematopoietic site covering the period between VDA and kidney as a counterpart to fetal liver in mammals [11, 12]. Definitive erythrocytes are postulated to populate the circulation at around 5 dpf as RBCs emerge at this stage in *bloodless* mutants with defects in producing primitive erythrocytes [13]. Transfusion experiments reveal that primitive erythrocytes are

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the major circulating erythroid components for the first 5 days and thereafter are gradually replaced by presumptive definitive erythrocytes [7]. Like mammals, these definitive erythrocytes switch to express adult form of globins and could be discriminated morphologically from primitive counterparts [14, 15].

The kidney marrow (head kidney) is an adult hematopoietic organ in zebrafish which is functionally equivalent to BM in mammals. Based on forward scatter (cell size) and side scatter (granularity), hematopoietic cells in the kidney marrow could be fractioned into four populations: immature precursors of all lineages, lymphoid cells, mature erythroid cells and myelo-monocytic cells (neutrophils, monocytes, macrophages, and eosinophils) [16]. Lethal irradiation followed by transplantation rescue in zebrafish reveals presence of definitive HSCs in the kidney marrow maintaining lifelong hematopoiesis program [17].

Spleen is postulated to be another hematopoietic tissue in the adult zebrafish, although clear evidence is lacking to define its hematopoietic activity. Compared to the kidney marrow, zebrafish spleen is not well-characterized. It was proposed that zebrafish spleen may function as a reservoir for RBCs where erythrocytes are stored and destroyed [18]. Splenic macrophages can be found in the red pulp and contain phagosomes with erythrocytes and other cellular debris, indicating the possibility of active EP in the zebrafish spleen [19, 20].

3. Iron acquisition and heme biosynthesis during erythropoiesis

The need for iron for heme synthesis during erythropoiesis mandates an efficient pathway to uptake extracellular iron (Fig.1). Erythroid cells rely on a high affinity system composed of transferrin (TF) and transferrin receptor (TFR). One molecule of tra nsferrin can bind to two ferric iron atoms with an association coefficient of 10^{-20} M at physiological pH [21]. Transferrin receptor 1 (TFR1, also known as CD71) tightly binds to TF, permitting developing erythroid cells to uptake iron efficiently from circulation. The complex of iron-bound TF and TFR1 is internalized by receptor-mediated endocytosis and the ferric iron is then released from TF as the endosomes acidify. The released ferric iron is reduced to ferrous by STEAP3 (six-transmembrane epithelial antigen of prostate 3 reductase) [22]. The ferrous iron is transported out of the endosome by DMT1 (divalent metal transporter 1, also known as NRAMP2 and SLC11A2) [23]. The apo-TF/TFR1 complex is then recycled back to the cell surface, where apo-TF dissociates from the TFR1 and re-enters the circulation. The holo-TF/TFR1 and apo-TF/TFR1 cycle ensures optimal iron uptake from the circulation for hemoglobin production.

Since free iron is cytotoxic due to fenton chemistry, iron is either stored or utilized upon transport into the cytosol [24]. Iron is stored in the cytosol by binding to ferritin with the aid of Poly r(C)-binding protein (PCBP) [25]. PCBP1 and PCBP2 exhibit high affinity for ferritin *in vitro* and PCBP1 and its paralog PCBP2 co-immuoprecipitates with ferritin in HEK293 cells. Co-expression of PCBP1 and PCBP2 together with human ferritins in yeast causes iron deficiency response by increasing iron deposition into ferritin. While PCBP1 mediates delivery and integration of iron into ferritin, nuclear receptor coactivator 4 (NCOA4) promotes release of iron from ferritin by directing the ferritin complex to autophagosomes for degradation [26]. Binding of PCBP1 to ferritin precedes NCOA4-

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ferritin interaction and coincides with globin synthesis during erythroid maturation. NCOA4-deficient cells exhibit massive accumulation of iron in ferritin with impaired hemoglobinization and enhanced erythroid cell death by ferritinophagy, since ferritin is an essential source of iron for heme production during terminal erythroid differentiation.

Upon release from ferritin, iron is transported into the mitochondria mediated by SLC25A37 (mitoferrin1, MFRN1), a protein belonging to the family of mitochondrial solute carrier proteins. MFRN1 is expressed in the inner mitochondrial membrane and transports iron across mitochondrial membranes [27]. Mouse erythroblasts derived from *Mfrn1*-deficient embryonic stem cells show a complete block of iron incorporation into heme. Defect in Mfrn1 results in profound hypochromic anemia and erythroid maturation arrest owing to insufficient mitochondrial iron uptake in zebrafish. Deletion of two yeast Mfrn homologs, Mrs3 and Mrs4 impairs incorporation of iron into PPIX and formation of Fe-S cluster assembly, collectively resulting in poor growth under low iron conditions. Deletion of *Mfrn1* in mice is embryonic lethal and mice with targeted deletion of *Mfrn1* in adult hematopoietic tissues show severe anemia owing to deficits in erythroblast formation [28].

Large amount of heme is synthesized during differentiation and maturat ion of RBCs and therefore must be coupled with iron acquisition. The efficiency of converting iron to heme in maturing erythroid cells is extremely high, resulting in heme-iron concentrations to be over 40,000-fold greater than non-heme iron in mature RBCs [29, 30]. One mode of tackling this large requirement for heme would be upregulating heme synthesis in the mitochondria and then mobilizing heme out of the mitochondria for insertion into cytoplasmic globin to couple heme synthesis with increasing globin production. In contrast, it is also essential to downregulate heme synthesis and decrease intracellular iron content when globin production is low in the early stage of erythropoietic development, since both free heme and iron are toxic to erythroid cells by inducing oxidative stresses. Failure to regulate heme synthesis during erythropoiesis will cause either iron-deficient or iron-overload anemia [31].

Although there are eight enzymatic steps for heme biosynthesis, the rate-limiting step is the synthesis of ALA from glycine and succinyl-coenzyme A, catalyzed by ALAS [32] (Fig.1). Two forms of ALAS exist, ALAS1 and ALAS2. ALAS1 is ubiquitously expressed in all tissues, while ALAS2 (or ALAS-E), is exclusively expressed in developing erythroid cells [33, 34]. ALAS2 is activated by the transcription factor GATA1, a master regulator for erythropoiesis. Chip-Seq analysis using G1E-ER4 erythroid progenitor cell line derived from *Gata1* mutant mice identified two GATA-1 *cis* elements in the first and eighth introns of *Alas2* [35–37]. Disruption of these two GATA1-binding elements abrogates expression of ALAS2 and subsequent inhibition of heme synthesis, which can be rescued by supplementing cells with high concentrations of ALA, the product of ALAS [36, 37]. Another regulation of ALAS2 occurs at the post-transcriptional level, modulated by iron responsive elements (IREs) in the 5’UTR. IREs interact with iron regulatory proteins (IRPs), linking the regulation of heme biosynthesis in erythroid cells to iron availability [38]. Under iron-deficient conditions, IRPs bind to IREs and inhibit *Alas2* mRNA translation. Conversely, when intracellular iron levels increase, IRPs are either degraded or converted to an aconitase by an iron-sulfur [4Fe-4S] and *Alas2* mRNA translation resumes to promote heme synthesis. The regulation of ALAS2 expression is coordinated with the cellular iron

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levels to tightly control cellular heme content. Another rate-limiting enzyme in the heme biosynthetic pathway is FECH. Transcription of *Fech* spikes during terminal erythroid differentiation and is controlled by the transcription factors SP1, NFE2 and GATA1[39]. Furthermore, the enzymatic activity of FECH is dependent on the presence of [4Fe–4S] cluster, again linking iron levels to heme synthesis [40]. Distinct erythroid-specific elements have been identified in the promoter region of *Fech*, together with erythroid-specific alternative splicing in the 3′ noncoding region of *Fech* mRNA in the mouse genome [41, 42], underscoring the unique regulatory mechanisms for heme synthesis in RBC maturation. Beside regulation of heme synthesis in erythroid cells, globin synthesis is controlled by the heme/BACH1 axis, in which heme binds to BACH1, a transcription suppressor, to relieve the depression for globin gene expression [43]. These regulations collectively coordinate cellular iron levels, heme synthesis, and globin protein expression, in order to maintain heme and iron homeostasis in erythroid cells.

4. Heme Transport and erythropoiesis

The hydrophobicity and cytotoxicity of free heme suggests the existence of heme trafficking pathways [44] (Fig.2). The final step of heme biosynthetic pathway occurs in the inner mitochondria. Thus heme must be exported out of the mitochondria for incorporation into hemoproteins located in various subcellular compartments [45]. Studies have shown that ABCB10 may facilitate transport of heme out of the mitochondria in erythroid cells [46]. ABCB10 is a mitochondrial ABC transporter located in the inner mitochondrial membrane. ABCB10 interacts with MFRN1 and FECH and stabilizes the complex [47]. Expression of ABCB10 is highly induced during erythroid differentiation and ABCB10 overexpression strengthens hemoglobin synthesis in erythroid cells. It has been shown that ABCB10-null mice display defective erythropoiesis and lack of hemoglobinized RBCs, indicating that ABCB10 is essential for erythropoiesis *in vivo* [46]. However, conclusive evidence for direct heme transport by ABCB10 is still lacking.

The Feline leukemia virus subgroup C receptor-related protein 1 (FLVCR1) was identified as a heme exporter [48]. FLVCR1 belongs to the family of MFS (major facilitator superfamily) proteins which transport small solutes across membranes facilitated by a counter ion gradient. Overexpression of FLVCR1 significant reduces cellular heme content, suggesting that FLVCR1 is involved in heme export [48]. FLVCR1-null mice are embryonic lethal with deficiencies in definitive erythropoiesis and suffer from craniofacial and limb deformities resembling those of patients with Diamond-Blackfan anemia (DBA). *Flvcr1*$^{-/-}$ mice develop a severe macrocytic anemia with proerythroblast maturation arrest, suggesting that erythroid precursors may be exporting excess heme to avoid heme toxicity [49]. Two isoforms of FLVCR has been identified, FLVCR1a and FLVCR1b. While FLVCR1a encodes a plasma membrane-localized heme transporter, FLVCR1b was identified to be a mitochondrial isoform e ncoded from an alternate transcription start-site, resulting in a shortened N-terminus containing a mitochondrial-targeting signal [50]. Thus, FLVCR1a and FLVCR1b contributes to intercellular and intracellular heme transport respectively. FLVCR1a is expressed in different hematopoietic cells and shows weak expression in the fetal liver, pancreas and kidney [51]. FLVCR1a may export heme during erythrophagocytosis (EP), a process in which macrophage phagocytose senescent RBCs, as

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FLVCR1a has been showed to interact with the extracellular heme-binding protein hemopexin and mediate heme export that is at least 100-fold more efficient than in the absence of hemopexin [52]. FLVCR1a expression is increased during erythropoiesis and is at its greatest during intermediate stages of RBC maturation when HMOX1 expression is low, implying that FLVCR1a helps to maintain stoichiometric amounts of heme and globin by exporting excess heme and preventing heme toxicity to RBCs [53]. Depletion of FLVCR1b in HeLa cells results in accumulation of mitochondrial heme, indicating that FLVCR1b may play a role in heme export from the mitochondria [50]. It is not known whether FLVCR1b resides on the inner or outer mitochondrial membrane. Moreover, if mitochondrial heme export by FLVCR1b is indispensable and indeed attenuated in *Flvcr1*$^{-/-}$ mice, it cannot explain why embryos from *Flvcr1*$^{-/-}$ null mice can survive until E14.5. Yeast does not appear to have an obvious FLVCR homolog, yet is able to export heme from the mitochondria indicating that alternate mechanisms must exist for mitochondrial heme export.

ABCG2, also known as breast cancer resistance protein (BCRP) has been identified as a heme exporter in mammals [54]. *ABCG2* is expressed in hematopoietic stem cells (HSCs) and erythroid progenitors. Compared to high FLVCR1 expression during erythropoietic differentiation, the expression levels of ABCG2 are particularly high at the early stages of hematopoiesis [55]. ABCG2 binds to heme directly through an extracellular loop 3 with a porphyrin-binding domain [56]. Ectopically expressed ABCG2 exports ZnMP, a heme analog, in K562 cells. However, direct evidence that ABCG2 exports heme is still lacking [56]. Whether FLVCR1 and ABCG2 can function synergistically to export heme during erythropoiesis is not clear. ABCG2-null human patients are defined as Jr(a–) blood group with a unique side population of HSCs, but no apparent deficiencies in erythropoiesis [57, 58].

MRP-5 was identified as a heme exporter in *Caenorhabditis elegans.* This roundworm is a unique model for uncovering heme trafficking pathways because it cannot synthesize heme but acquires environmental heme for sustenance. Thus, worms need to acquire dietary heme via the intestine and distribute heme from the intestine to other tissues [59, 60]. *C. elegans* MRP-5 (CeMRP-5) localizes to the basolateral membrane of intestinal cells and loss of MRP-5 results in accumulation of ZnMP in intestinal cells and growth retardation. Overexpression of CeMRP −5 and human MRP5 in yeast retards growth owing to heme depletion, which can be rescued by co-expressing *C. elegans* heme importers. Interestingly, overexpression of CeMRP-5 and human MPR5 causes heme levels to increase in the secretory pathway in both yeast and mammalian cells, as measured by a secretory pathway hemoprotein reporter. However, the physiological role of MRP5 in vertebrates and its involvement in erythropoiesis is unclear.

Heme carrier protein 1 (HCP1, SLC46A1) is a membrane protein expressed in enterocytes and was proposed to be an intestinal heme transporter [61]. However, subsequent studies revealed that HCP1 is a proton-coupled folate transporter (PCFT) rather than a heme transporter [62]. Erythroblasts from HCP1 knockout mice showed deficiency in differentiation and high apoptosis rate resulting in severe macrocytic normochromic anemia, which was ascribed to folate but not heme deficiency [63]. Moreover, knockdown of HCP1

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by shRNA in Caco-2 cells attenuated both heme and folate uptake but increased heme oxygenase expression, suggesting HCP1 could potentially function as a low affinity heme importer [62, 64].

The Heme Responsive Gene −1 (HRG1, SLC48A1) was identified as a heme importer in the intestine of *C. elegans* [65]. Four HRG1 homologs, CeHRG-1, CeHRG-4, CeHRG-5 and CeHRG-6 were found in the *C. elegans* genome. Significant heme-induced inward currents were observed in *Xenopus* oocytes injected with *Cehrg-1*, *Cehrg-4*, and human *HRG1* mRNA, indicating heme-dependent transport across cell membranes [65]. Human *HRG1* (*SLC48A1*) mRNA is abundant in the brain, kidney, heart, skeletal muscle, in addition to cell lines derived from duodenum and bone marrow [65]. HRG1 localizes to acidic endosomal and lysosomal organelles in HEK293 cells, and its binding affinity to heme increases as pH decreases. Human HRG1 interacts with the C subunit of the vacuolar proton ATPase (V-ATPase) pump and enhances endosomal acidification [66]. These studies collectively suggest that HRG1 transports heme from the exoplasmic space or the lumen of acidic endosome–lysosomal compartments into the cytoplasm. Global transcriptomic expression profiling shows that *Hrg1* mRNA is expressed during erythroblast maturation [67]. Why a heme importer would be expressed during erythropoiesis since developing RBCs are capable of de novo heme synthesis is puzzling.

5. Zebrafish as a genetic model to study heme and iron metabolism

The complete sequencing of the zebrafish genome makes it a powerful tool for gene discovery research as the zebrafish genome largely resembles the human genome [68]. Large-scale forward genetic studies have been carried-out in some invertebrate model organisms, particularly in the nematode and fruit fly. However, it is extremely expensive to perform this approach in vertebrate animal model like mice. Zebrafish was the first vertebrate organism established for large-scale forward genetic screening. Chemical mutagenesis is achieved by exposing adult male fish to N-ethyl-N-nitrosourea (ENU) for several days which induces point mutations in the spermatogonia. These male fish are then mated with wildtype (WT) female fish to propagate the mutation to F1 progenies [4, 69]. Screening methods such as antibody staining, whole mount *in situ* hybridization (WISH) and behavioral analysis can be employed to identify morphological or genetic phenotypes. By contrast, gene-specific knockdown mediated by morpholino-injection serves as an efficient tool for reverse genetic study [70]. Mutagenesis and targeted gene disruptions have contributed to uncovering the genes involved in heme and iron metabolism in zebrafish. Recent development of gene-editing tools such as TALENs and CRISPR/cas9 for targeted gene disruption complements transient morpholino gene knockdowns that were typically used for reverse genetic studies [71, 72].

Several key genes which are involved in heme synthesis and metabolism have been elucidated in zebrafish by both forward and reverse genetic manipulation. In Table 1, we summarize the genes and corresponding mutants identified in zebrafish together with related disease in humans. We have also compiled a list of mammalian heme-iron metabolism genes and performed Bulk-BLAST homology alignments to identify potential orthologs in the zebrafish genome [73] (Supplemental Table 1). With the advent of current gene-editing tools

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for targeted gene disruptions, generating new zebrafish mutants and alleles will be a powerful approach to model human disorders of heme and iron metabolism in a facile vertebrate model.

5. 1 ALAS2

Alas2 is the erythroid-specific enzyme for heme synthesis, while Alas1 is found in all other tissues. Zebrafish mutant *sauternes (sau)* was identified from ENU mutagenesis and positional cloning revealed that *sau* contains mutation in *alas2* [74]. The *sau* mutants have a microcytic, hypochromic anemia, delayed erythroid maturation, and abnormal globin gene expression, suggesting that defects in heme synthesis can affect globin protein production. Interestingly, *sau* mutants have normal RBC numbers and show anemia around 33 hpf, possibly because maternal heme may permit early RBC development or genetic compensation by Alas1. As mutations in *alas2* cause congenital sideroblastic anemia (CSA) in humans, *sau* represents the first animal model of this disease in zebrafish [74]. Most interestingly, *sau* zebrafish mutants are viable even though it has only one-tenth of overall heme content compared to WT zebrafish. *Sau* mutants can be fully rescued by supplementing developing embryos with ALA, which further enhances its value as a model to study the pathology and possible evaluate the cure methods for CSA in humans [75].

5. 2 ALAD and CPOX

Unlike ALAS, only one form of ALAD and CPOX was found in both human and zebrafish. By using targeted TALEN and CRISPR/Cas9, zebrafish $alad^{-/-}$ and $cpox^{-/-}$ were recently generated to model the ALA-dehydratase-deficient porphyria (ADP) and hereditary coproporphyria (HCP) [76]. $alad^{-/-}$ and $cpox^{-/-}$ mutants suffer from hypochromic anemia, owing to deficiency in heme synthesis, with accumulation of ALA and coproporphyrinogen-III. The abnormal morphology of early RBCs was possibly due by accumulated globin without heme, representing sickle-cell anemia in humans with globin protein aggregations.

5. 3 UROD

Zebrafish mutant *yqe* contains a nonsense mutation in the gene encoding UROD, which converts uroporphyrinogen to coproporphyrinogen. Homozygous mutation in *urod* leads to two forms of porphyrias, porphyria cutanea tarda (PCT) and hepatoerythropoietic porphyria (HEP) in human, similar to the phenotypes in zebrafish with photosensitive porphyria syndrome [77]. Excessive amounts of uroporphyrinogens and 7-carboxylate porphyrin accumulate in *yqe* embryos, representing human HEP patients characterized by photosensitive skin and excessive excretion of heme biosynthesis byproducts, uroporphyrin and 7-decarboxylate porphyrin in their urine. The zebrafish *yqe* mutant phenocopies human patients facilitating studying of HEP pathogenesis and development of new therapeutics.

5. 4 PPOX

Zebrafish porphyria mutant, *montalcino* (*mno*), contains mutation in *ppox*, which catalyzes the oxidation of protoporphyrinogen, representing human variegate porphyria [78]. Initially, at the onset of circulation, *mno* displays normal numbers of RBCs but are *o-dianisidine* negative. A visible decrease in circulating erythrocytes can be observed after 36 hpf and the

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RBCs in the mutant embryos are fluorescent. The *mno* mutant zebrafish could survive to approximately 25 dpf. Accordingly, human *PPOX* could partially rescue the hypochromia in homozygous mutants, revealing functional conservation. The zebrafish *mno* mutant will be very useful for further elucidating the pathophysiology of variegate porphyria and identifying chemical modifiers of this disease.

5. 5 FECH

Two different mutant alleles of *fech* have been identified in zebrafish, *freixenet* (*frx*) and *dracula* (*drc*) [4, 79]. Protoporphyrin IX accumulates in *fech* mutant embryos owing to a deficiency in the activity of ferrochelatase, the terminal enzyme in the pathway for heme biosynthesis. The mutants show light-dependent hemolysis and liver diseases, similar to that seen in humans with heredity erythropoietic protoporphyria (HEP) resulting from a disorder of ferrochelatase.

5. 6 GRX5

Phenotypic analysis of *shiraz* (*sir*) mutant zebrafish revealed an intimate connection between heme biosynthesis and [4Fe-4S] formation, connecting two main uses for iron in the mitochondria which are previously usually thought to be independent processes [80]. In *sir* mutants, hypochromic anemia, in the context of normal mitochondrial iron and oxidative stress levels, was shown to be caused by a deletion in the glutaredoxin 5 (*grx5*) gene. GRX5 is required for the synthesis of Fe-S clusters in the mitochondria [81]. The zebrafish protein also localizes to the mitochondria and is capable of rescuing *grx5*-deficient yeast strain. Fe-S clusters are known to negatively regulate binding of IRP1 to IREs. Decreased Fe-S cluster assembly in *sir* mutant leads to increased IRP1 activity, which inhibits the expression of IRE-regulated target genes involved in heme biosynthesis, such as *alas2*. Indeed, *alas2* expression is absent in *sir* mutants. Deletion of IREs in the *alas2* mRNA rescued the anemic phenotype while overexpression of full-length *alas2* mRNA did not, suggesting that the function of GRX5 in regulating ALAS2 expression is through Fe-S clusters and IRP/IRE activity. An evolutionary conserved role for GRX5 in regulating heme synthesis was confirmed in human patients with *GRX5* mutation [82]. Thus, *shiraz* mutant will be a good tool for searching effective therapeutics for diseases related to Fe-S deficiencies.

5. 7 MFRN1

Two mitoferrin homologs are found in the zebrafish genome, Mfrn1 and Mfrn2. Positional cloning of *frascati* (*frs*) zebrafish mutants identified a missense mutation in the *mfrn1* (*slc25A37*) gene, an erythroid-specific form [27]. *Frs* mutants develop hypochromic anemia and show defects in erythroid maturation. Mouse *Mfrn1* rescues the phenotypes in zebrafish *frs* mutants. The same anemic phenotype was observed in a mouse model with *Mfrn1*-knockout [28, 83]. The MFRN2 (Slc25A28) paralog functions in mitochondrial iron import in non-erythroid tissues.

5. 8 DMT1

DMT1 is upregulated by dietary iron deficiency and the Belgrade rat carries a mutation in *dmt1* suffer from iron deficient anemia [23, 84]. Zebrafish mutants *chardonnay* (*cdy*) carry a

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nonsense mutation in *dmt1* with reduced hemoglobin levels and delayed erythrocyte maturation [85]. The Dmt1 protein is expressed in erythroid cells and the duodenum suggesting its role in erythropoiesis and intestinal iron absorption. Cells with overexpression of WT zebrafish *dmt1* uptake nearly ten-times the amount of iron as non-transfected control cells, whereas the *cdy* mutant protein is not functional. However, *cdy* mutants can survive to adulthood despite severe anemia, again suggesting an alternate path for iron absorption in zebrafish. In humans, mutations in *DMT1* cause a phenotype of hypochromic microcytic anemia combined with iron overload, further supporting the possible existence of alternative mechanisms for duodenal iron absorption that bypasses DMT1 [86].

5. 9 FPN1

The first identified iron exporter Fpn1 (Slc40A1) was found by position-cloning of zebrafish *weissherbst* (*weh*) mutant [87]. *weh* mutant embryos show hypochromic anemia with decreased hemoglobin levels, blocked erythroid maturation, and reduced numbers of erythrocytes. Erythroid cells of mutant embryos have significantly lower iron concentrations compared to WT embryos, suggesting iron deficiency. Microinjection of iron-dextran rescues the anemic phenotype of *weh* mutants and continued injection of iron-dextran rescues the embryonic lethality. These rescued fish, however, are only normal until 6 months of age and eventually develop hypochromic anemia by 12 months, suggesting that Fpn1 is also involved in adult red cell function, specifically iron-recycling in adult zebrafish. Compared with iron injected WT fish, the rescued mutants had increased iron staining in kidney macrophages, as well as increased staining in intestinal villi, suggesting that *fpn1* mutation impairs iron export in these tissues. The iron-rescued *weh* mutants also have hepatic iron overload, with particularly high iron levels in the liver [88]. FPN1 also localizes to the yolk-syncytial layer (YSL) during embryonic development, suggesting that FPN1 may transport maternal iron from the yolk for embryogenesis. Both mice and humans have homologs of FPN1with high similarities to zebrafish Fpn1. Mammalian *FPN1* is robustly expressed in the placenta, duodenum, and liver. At the protein level, human FPN1 is concentrated on the basal surface of syncytiotrophoblasts in the placenta, an organ that is functionally like zebrafish YSL, indicating that human FPN1 plays a role in maternal-fetal iron export. In mice, FPN1 is expressed on the basolateral surface of enterocytes, suggesting a role in intestinal iron transport [89].

5. 10 TF

The zebrafish mutant *gavi* (*gav*) was shown to have mutations in transferrin-a (Tf-a), which encodes the principal serum iron carrier [90]. *Gav* mutant embryos exhibit reduced *tf-a* expression and impaired hemoglobin production with hypochromic anemia and embryonic lethality by 14 dpf. In humans, phenotype of congenital hypotransferrinemia caused by *TF* mutation is highly similar to those of *gav* mutants, including hypochromic anemia and embryonic death [91]. The *gav* mutant is thus an ideal whole vertebrate anemia model for studying symptoms corresponding to human pathologies related to Tf.

5. 11 TFR1

Transferrin-bound iron is taken up into cells by binding to the transferrin receptor 1 (TFR1). Four different zebrafish *chianti* (*cia*) mutants with varying degrees of hypochromic anemia

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and defective erythroid differentiation were ascribed to mutations in *tfr1a* gene [92]. During early development, *tfr1a* transcripts are expressed specifically in erythrocytes. Importantly, cytoplasmic delivery of iron by microinjection at one-cell stage - but not intravenous iron injections rescue the hypochromia in *cia* mutants, indicating that *tfr1a* mutation prevents erythrocytes from taking-up and utilizing circulating iron. Intriguingly, a second *tfr1* gene, *tfr1b* was identified together with *tfr1a*, a typical feature in zebrafish genome which has undergone whole genome duplication [93, 94]. Whereas *tfr1a* is expressed in erythrocytes during early development and *cia* mutants are anemic, *tfr1b* is ubiquitously expressed throughout embryogenesis and knockdown of *tfr1b* by morpholinos do not affect hemoglobinization. *Tfr1b* morphants have retarded growth and develop brain necrosis, a phenotype that is similar to the neurologic defects observed in the mouse model [95], indicating that *tfr1b* may be involved in iron uptake in non-erythroid tissues. *Tfr1a* (*cia*) and *tfr1b* deficient zebrafish embryos recapitulate the phenotype of $TFR1^{-/-}$ mice [92].

5. 12 FLVCR and HRG1

Currently no zebrafish mutants have been reported with perturbation of Flvcr. However, the function of zebrafish Flvcr is related to erythroid differentiation and maturation as determined by morpholino gene knockdowns [96]. Two splicing isoforms have been identified: *flvcr1a* and *flvcr1b*. Flvcr1a is required for the expansion of committed erythroid progenitors but cannot drive their terminal differentiation, while Flvcr1b contributes to the expansion phase and is required for differentiation [96]. The coordinated expression of *flvcr1a* and *flvcr1b* contributes to controlling the cytosolic heme pool required to sustain regulation of erythroid progenitors and hemoglobin synthesis for hemoglobinization during terminal maturation. Interestingly, treatment with succinylacetone (SA), an inhibitor of heme synthesis, rescues the phenotype of *flvcr1a* morphants while heme supplementation restores hemoglobinzation of *flvcr1b* morphants, suggesting that the intracellular heme pool during erythropoiesis is tightly regulated. As $Flvcr^{-/-}$mice are embryonically lethal, zebrafish *flvcr* mutants will be useful to dissect the embryonic functions of FLVCR.

Recently, zebrafish mutants for *hrg1* (*Slc48a1*), a heme transporter with homologs in mammals, was generated by CRISPR/cas9 [73, 97]. Zebrafish genome contains two *hrg1* paralogs - *hrg1a* (*slc48a1b*) and *hrg1b* (*slc48a1a*). Both are ubiquitously expressed throughout developing embryos and in the one-cell embryo. More importantly, *hrg1a;hrg1b* double mutants show accumulation of heme in the kidney macrophages due to defects in heme-iron recycling from damaged RBCs. RNAseq results show significant perturbation in heme-iron metabolism and immune-related genes in the absence of *hrg1*. Altogether, the fish studies affirm that Hrg1 is essential for recycling RBCs and that this function is performed by the kidney marrow, which is the adult hematopoietic organ in zebrafish [73].

6. Conclusions

Zebrafish has proven to be an indispensable vertebrate model to study heme and iron metabolism with unique genetic advantages not afforded by mammalian models. The advantages of *ex utero* genetic manipulation and embryonic transparency allow convenient functional validation of both zebrafish and human genes. Moreover, chemical-genetics drug

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screens in zebrafish provide a more comprehensive whole animal in-a-dish analyses for drug tests than typical cell-culture based assays [98]. Given the high degree of conservation in the heme and iron acquisition pathways in zebrafish and humans, it would be prudent to develop diseased models of humanized zebrafish for targeted high throughput screens to identify more efficacious therapeutic drugs against rare diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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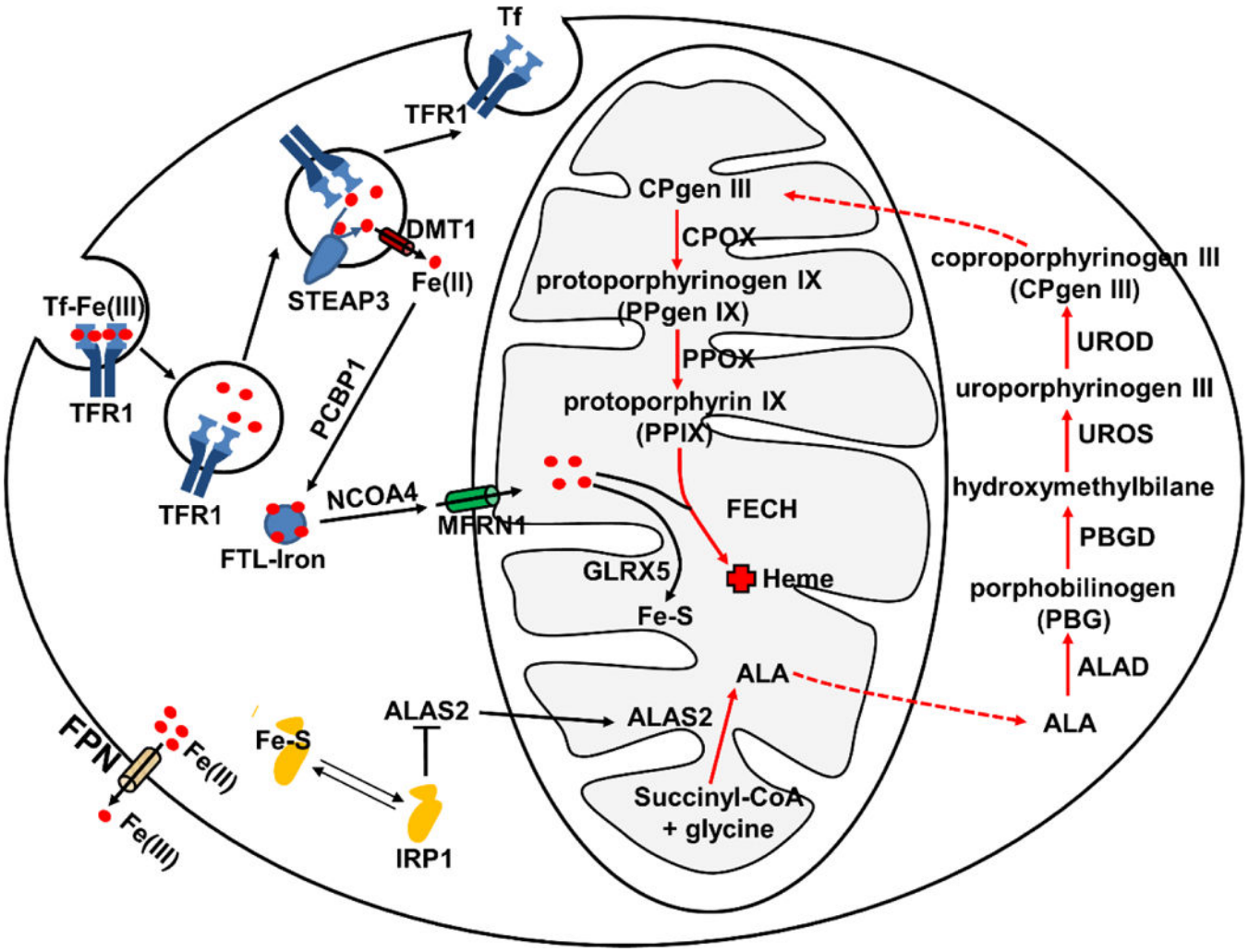

Fig.1. Hene synthesis and iron metabolism in erythroid cells

Iron acquisition in erythroid cells is dependent on endocytosis of Tf-bound Fe (Tf-Fe(III)) via the transferrin receptor (TFR1). Iron (Fe(III)) is imported into the cytoplasm by DMT1 after reduction by STEAP3. Iron can be stored in the ferritin (FTL) (FTL-Iron) mediated by PCBP1 and can be released from FTL promoted by NCOA4. MFRN1 is responsible for bringing iron to the mitochondria for heme synthesis. The red arrow represents overall heme synthetic pathway. Heme biosynthesis initiates in the mitochondrial matrix and is catalyzed by δ-aminolevulinic acid synthase (ALAS2) to synthesize δ-aminolevulinic acid (ALA) from glycine and succinyl-coenzyme A. ALA is subsequently transported out of the mitochondria to the cytosol for the following four enzymatic reaction steps: ALA to porphobilinogen (PBG) catalyzed by ALA dehydratase (ALAD); PBG to an unstable polymer hydroxymethylbilane by porphobilinogen deaminase (PBGD); hydroxymethylbilane to uroporphyrinogen III (UROgen III) by uroporphyrinogen synthase (UROS); and UROgen III to coproporphyrinogen III (CPgen III) by uroporphyrinogen decarboxylase (UROD). CPgen III is then transported into the mitochondria where coproporphyrinogen oxidase (CPOX), a mitochondrial intermembrane space enzyme, catalyzes the formation of protoporphyrinogen IX (PPgen IX). The inner mitochondrial

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membrane enzyme protoporphyrinogen oxidase (PPOX) catalyzes the formation of protoporphyrin IX (PPIX) from PPgen IX in the mitochondrial matrix. On the last step, ferrochelatase (FECH) catalyzes the insertion of ferrous iron (Fe^{2+}) into PPIX to form heme. Iron is also used for Fe-S cluster synthesis with involvement of GLRX5. IRP1 binding can inhibit translation of ALAS2 to prevent the accumulation of toxic heme intermediates. Cellular iron efflux is mediated by FPN and requires iron oxidation on the extracellular side.

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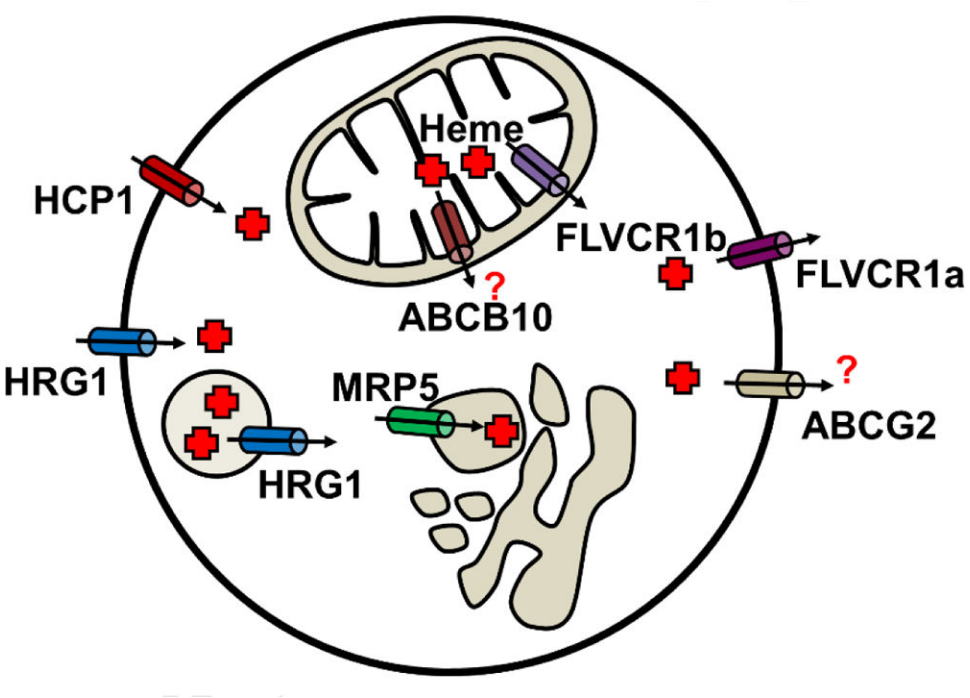

Fig.2. The ins and outs of heme transport

Mitochondrial isoform FLVCR1b transports heme into the cytosol. ABCB10 is reported to forms complex with FECH and MFRN1. It is not clear whether ABCB10 transport heme. The cell surface FLVCR1a and the ABC transporter ABCG2 have been implicated in heme e xport. MPR5 is a heme exporter which can transport heme from cytosol to the secretory pathway. HCP1 is a folate importer as well as a low-affinity heme importer. HRG-1 is a heme importer that localizes to endosomal/lysosomal compartments, but also traffics to the plasma membrane.

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Table 1

Summary of zebrafish mutants related to heme -iron metabolism

Genes	Mutant Name or Morpholino Knockdown	Potential Human Disease	References
ALAS2	*sauternes (sau)*	Congenital Sideroblastic Anemia (CSA)	[74]
ALAD	*Alad*$^{-/-}$	ALA dehydratase deficient porphyria (ADP)	[76]
CPOX	*Cpox*$^{-/-}$	Hereditary coproporphyria (HCP)	[76]
UROD	*yquem (yqe)*	Porphyria cutanea tarda (PCT) / hepato-erythropoietic porphyria (HEP)	[77]
PPOX	*montalcino (mno)*	Human variegate porphyria	[78]
FECH	*Freixenet (frx) / dracula (drc)*	Erythropoietic protoporphyria	[4] [78]
GRX5	*shiraz (sir)*	Sideroblastic anemia	[80] [81]
MFRN1	*frascati (frs)*	Erythropoietic protoporphyria	[27]
DMT-1	*chardonnay (cdy)*	Hypochromic microcytic anemia	[85] [86]
FPN1	*weissherbst (wei)*	Iron-deficient anemia	[87] [88]
TF	*gavi*	congenital hypotransferrinemia	[90]
TFR1	*chianti (cia)*	hypochromic anemia	[92]
HRG1	*hrg1a;hrg1b (CRISPR)*	unknown	[73]
FLVCR	Morpholino Knockdown	Heme toxicity	[96]