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Studying disorders of vertebrate iron and heme metabolism using zebrafish

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Abstract

Iron is a crucial component of heme- and iron-sulfur clusters, involved in vital cellular functions such as oxygen transport, DNA synthesis, and respiration. Both excess and insufficient levels of iron and heme-precursors cause human disease, such as iron-deficiency anemia, hemochromatosis, and porphyrias. Hence, their levels must be tightly regulated, requiring a complex network of transporters and feedback mechanisms. The use of zebrafish to study these pathways and the underlying genetics offers many advantages, among others their optical transparency, ex-vivo development and high genetic and physiological conservations. This chapter first reviews well-established methods, such as large-scale mutagenesis screens that have led to the initial identification of a series of iron and heme transporters and the generation of a variety of mutant lines. Other widely used techniques are based on injection of RNA, including complementary morpholino knockdown and gene overexpression. In addition, we highlight several recently developed approaches, most notably endonuclease-based gene knockouts such as TALENs or the CRISPR/Cas9 system that have been used to study how loss of function can induce human disease phenocopies in zebrafish. Rescue by chemical complementation with iron-based compounds or small molecules can subsequently be used to confirm causality of the genetic defect for the observed phenotype. All together, zebrafish have proven to be – and will continue to serve as an ideal model to advance our understanding of the pathogenesis of human iron and heme-related diseases and to develop novel therapies to treat these conditions.

1. OVERVIEW OF VERTEBRATE CELLULAR IRON AND HEME METABOLISM

Iron is a trace metal essential for survival of almost all organisms. As a crucial component of heme- and iron sulfur (Fe-S) cluster-containing proteins, iron is involved in a number of vital cellular functions, such as oxygen transport, DNA synthesis, and respiration (Loreal et al., 2014; Muckenthaler & Lill, 2012). However, it can also have deleterious effects when present in excess by participation in the Fenton reaction, leading to the formation of reactive oxygen species (ROS) that result in lipid peroxidation and damage to DNA and proteins

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(Fenton, 1894). Fortunately, under physiological conditions, cellular iron levels are well regulated by a number of mechanisms that govern its uptake, mobilization, storage, and recycling (Andrews & Schmidt, 2007). The vast majority of iron is required for synthesis of the oxygen-carrier hemoglobin in red blood cells. This need is fulfilled mostly (90%) by recycled iron from degradation of senescent RBCs by macrophages in the spleen. Iron absorbed from the diet in the duodenum accounts for the remaining 10% (Zhang, Ghosh, & Rouault, 2014). Iron absorption is facilitated by a ferrireductase, whose exact identity remains controversial (Gunshin et al., 2005; McKie et al., 2001). After reduction of its ferric (Fe^{3+}) form to ferrous (Fe^{2+}), the iron is taken up into the enterocyte by the divalent metal transporter-1 (DMT-1/SLC11A2) (Fleming et al., 1997; Gunshin et al., 1997). Once inside the cell, iron-regulatory proteins (IRPs) and iron-responsive elements (IREs) control iron levels by regulating the expression of a series of proteins involved in uptake, storage, and utilization of iron (Muckenthaler, Galy, & Hentze, 2008). In the case of low intracellular iron, IRPs bind with high affinity to IREs to inhibit mRNA translation when in the 5'UTR, e.g., for ferritins, and simultaneously stabilize mRNA when in the 3'UTR, e.g., for the transferrin receptor 1 (*TfR1*), thereby facilitating uptake and utilization. Conversely, when iron excess occurs, ferritin translation will take place uninhibited and TfR1 expression is repressed (Torti & Torti, 2002; Zhang et al., 2014). Whereas this elegant IRE/IRP system is present in nonerythroid cells, different regulatory mechanisms may be in place in erythroid cells (Anderson, Shen, Eisenstein, & Leibold, 2012; Ponka, 1997). The iron demand of these cells is many orders of magnitude higher than for other mammalian tissues because iron is required not only for mitochondrial cytochromes but also as prosthetic groups for hemoglobin, which contains 70% of the total iron content of a normal adult (Ponka, 1997).

The only known cellular iron exporter is Ferroportin-1 (FPN1/SLC40A1) (Abboud & Haile, 2000; Donovan et al., 2000; McKie et al., 2000). Release of iron by FPN1 is controlled by the peptide hormone hepcidin (Ganz & Nemeth, 2012). Hepcidin regulates iron availability by binding to FPN1, promoting its internalization and degradation, thereby preventing duodenal iron uptake and release from stores into the circulation (Nemeth et al., 2004). Upon release by FPN1, Fe^{2+} is oxidized by multicopper ferroxidases [ceruloplasmin for macrophages (Mukhopadhyay, Attieh, & Fox, 1998) and hephaestin for enterocytes (Vulpe et al., 1999)] so that it can be bound by transferrin. These Fe^{3+} -transferrin (Tf) complexes then bind to Tf receptors on the cell surface to be endocytosed (Dautry-Varsat, Ciechanover, & Lodish, 1983). After reduction by STEAP3 ferrireductase (Ohgami et al., 2005) the Fe^{2+} enters the cytosol via the endosomal transporter DMT1 or ZIP14, which can take up both endosomal transferrin- and extracellular nontransferrin-bound iron (Liuzzi, Aydemir, Nam, Knutson, & Cousins, 2006; Zhao, Gao, Enns, & Knutson, 2010). The iron may then be transferred directly from endosomes to mitochondria via so-called kiss-and-run (Sheftel, Zhang, Brown, Shirihai, & Ponka, 2007; Zhang, Sheftel, & Ponka, 2005) or enters the cytosolic pool of metabolically active “labile” iron that is loosely coordinated by water, small molecules, and proteins (Shvartsman & Ioav Cabantchik, 2012). Most of the labile iron pool (LIP) is directed to mitochondria (Shvartsman & Ioav Cabantchik, 2012), where it can be used in the assembly of Fe–S clusters (Rouault, 2015) or inserted into protoporphyrin IX to form heme (Ajioka, Phillips, & Kushner, 2006).

In vertebrates, erythroid cells have by far the greatest need for iron, which is required for the synthesis of heme in the oxygen transport protein hemoglobin. Heme synthesis is a highly complex process involving eight nuclear-encoded enzymes, four of which are cytoplasmic and four of which localize to mitochondria (Ajioka et al., 2006). The first step, taking place in the mitochondrial matrix, comprises condensation of succinyl-CoA and glycine by aminolevulinic acid synthase (ALAS) to generate D-aminolevulinic acid (ALA). Of note, erythroid and nonerythroid cells express distinct isoforms of ALAS: ALAS1 is expressed ubiquitously, whereas ALAS2 is specific to differentiating erythroid cells. ALA is then exported across the mitochondrial outer membrane into the cytoplasm by an unknown mechanism, where it is converted in a series of four biosynthetic reactions to coproporphyrin III (CPIII). This intermediate is transported back into the mitochondrial intermembrane space, where it is converted to protoporphyrinogen III and then to protoporphyrin IX. Finally, heme is formed by metalation of protoporphyrin IX with Fe^{2+} by the enzyme ferrochelatase (FECH) (Ajioka et al., 2006; Chung, Chen, & Paw, 2012; Dailey & Meissner, 2013).

Cytosolic iron that is not directed to mitochondria may be used to metalate nonheme iron enzymes in the cytosol and nucleus, to assemble cytosolic Fe–S clusters, or to be stored in ferritin. The fate of cytosolic iron is likely determined by the activity of iron transporters, such as mitochondrial iron importers [mitoferrin 1 and 2 (Shaw et al., 2006)], and iron efflux pumps (e.g., ferroportin) (Abboud & Haile, 2000; Donovan et al., 2000; McKie et al., 2000), whose discovery by positional cloning will be discussed in this chapter. For more details on mechanisms of intracellular iron trafficking the reader is referred to this excellent review (Philpott & Ryu, 2014).

Although the regulation and mechanisms of iron metabolism and heme synthetic enzymes are well characterized, significant knowledge gaps remain regarding the pathways that govern transport and trafficking of iron and heme intermediates (Nilsson et al., 2009). The rest of this chapter presents an overview of past and present approaches and methodologies that employ zebrafish as a model to unravel new genes involved in cellular iron and heme synthesis and transport.

2. ADVANTAGEOUS PROPERTIES OF ZEBRAFISH TO STUDY GENETICS

The potential of the zebrafish as a vertebrate model system for genetic screening first became apparent over 2 decades ago by the work of George Streisinger (Streisinger, Walker, Dower, Knauber, & Singer, 1981). An important advantageous characteristic is that zebrafish development takes place externally. In addition, the transparency of the embryos allows for visualization of developmental processes from gastrulation to organogenesis and monitoring of gene expression in vivo without having to sacrifice the subjects. Furthermore, many genetic pathways underlying the development and the regulation thereof are fairly well conserved throughout evolution, with approximately 70% of human genes having at least one obvious zebrafish orthologue (Howe et al., 2013). Hence, similar phenotypes are often observed between zebrafish mutants and human disease (Shin & Fishman, 2002). From a practical perspective, compared to rodents and other mammalian systems, large numbers of zebrafish can be relatively easily maintained in the laboratory setting. Spawning, which can

be controlled by light, can take place frequently and does not depend on spawning season. Spawns are generally large (average 100 embryos) and embryos can be raised to (sexual) maturity within 2–3 months, which is less labor-intensive and cheaper than for other commonly used laboratory animals. Also, routine techniques used in genetic research, including knock-in, knockdown, and knockout, are well-established in zebrafish (Lin, Chiang, & Tsai, 2016). Hence, zebrafish are particularly suitable vertebrates for high-frequency mutagenesis and large-scale genetic screening studies.

Roughly, two approaches to screening can be distinguished. Genetic screens start with a phenotype and then attempts to identify the gene(s) responsible, whereas in candidate screens a known gene is disrupted, and subsequently, the phenotype is analyzed to determine its function. Both strategies will be discussed below in the light of their contribution to the study of iron and heme metabolism in zebrafish.

3. TOOLS TO STUDY IRON AND HEME METABOLISM USING ZEBRAFISH

3.1 GENETIC SCREENING

The approach of large-scale genetic screens starts with treating male fish with a mutagen, e.g., ethylnitrosurea (ENU) or gamma irradiation, which cause a large number of point mutations in the male germ cells (spermatogonia). Subsequently, these male F0 fish are crossed to wild-type females to produce the F1 generation. F1 fish are then in-crossed with each other to create the F2 generation, in which 50% of the fish will be heterozygous for a specific mutation and 50% will be wild-type. Finally, by in-crossing the heterozygous F2 siblings the F3 generation is created, in which 25% are wild-type, 50% heterozygous and 25% homozygous for recessive mutations (Patton & Zon, 2001). New developmental mutants are then characterized phenotypically, after which the responsible gene and its function can be identified.

Although other useful model systems (yeast, mouse, human) exist, zebrafish has contributed in especially meaningful ways to our understanding of iron and heme metabolism. The advantageous characteristics of zebrafish to study genetics led two labs in Tübingen (Germany) and Boston (USA) (Driever et al., 1996; Haffter et al., 1996) to initiate large-scale ENU mutagenesis screens. This resulted in the identification of ~2000 mutants affecting a large variety of processes such as early development, organ formation, behavior, and axonal pathfinding (Haffter et al., 1996). A number of the ~2000 mutated developmental genes that were identified in these two screens have been cloned, and by selecting those mutants with an anemic phenotype (identified as having low blood cell counts) several zebrafish lines with defects in iron or heme metabolism were established (see Table 1 for an overview).

For instance, positional cloning of the *weissherbst* (*weh*) mutant resulted in the identification of the, so far only known, cellular iron exporter FPN1 (Slc40A1) (Donovan et al., 2000). *Weissherbst* fish were found to suffer from hypochromic anemia, as evident from decreased hemoglobin levels, blocked erythroid maturation, and reduced numbers of erythrocytes (Donovan et al., 2000; Ransom et al., 1996). Erythroid cells of mutant embryo zebrafish had significantly lower iron concentrations compared to wild-type embryos. This suggests a

circulatory iron deficiency as the cause of the anemic phenotype, which was later confirmed by positional cloning, as discussed later in this chapter. Furthermore, the *FPN1* gene is highly conserved throughout evolution. In mammals, FPN1 is expressed mainly at sites of iron transport, such as the placenta, duodenum and liver. Murine FPN1 protein is predominantly present in the basolateral membrane of enterocytes, indicative of a role as an intestinal iron transporter (Donovan et al., 2000). This was subsequently confirmed by mouse knockout studies (Donovan et al., 2005). Taken together, these data led to the identification of FPN1 as the, to date only, iron exporter present in all eukaryotic organisms interrogated for this gene. Hence, these studies exemplify how genetic screens in zebrafish can lead to the identification of novel genes.

Positional cloning of *frascati* (*frs*) zebrafish mutants identified missense mutations in the mitoferrin *MFRN* (*Slc25A37*) gene. Previous studies in yeast (Foury & Roganti, 2002; Li & Kaplan, 2004; Mühlenhoff et al., 2003) had uncovered the role of the MRS3 and MRS4 yeast homologs in mitochondrial iron uptake, as they were found to facilitate both heme formation and Fe–S cluster biosynthesis, the two major iron-consuming mitochondrial processes (Mühlenhoff et al., 2003). Later, *frs* mutants were observed to develop hypochromic anemia and erythroid maturation arrest (Shaw et al., 2006). Positional cloning in zebrafish further supported that MFRN1 is the principal mitochondrial iron importer in vertebrate erythroblasts (Shaw et al., 2006), which was later validated by mouse knockouts (Chung et al., 2014; Troadec et al., 2011). The MFRN2 (*Slc25A28*) paralogue functions in mitochondrial iron import in nonerythroid tissues and erythroid progenitors (Shaw et al., 2006).

The protein DMT1 was originally isolated in the rat duodenum as a divalent ion transporter that is upregulated by dietary iron deficiency (Fleming et al., 1997; Gunshin et al., 1997). Several years later, the *chardonnay* (*cdy*) zebrafish mutation, which presents with reduced hemoglobin levels and delayed erythrocyte maturation, was found to have a mutation in *DMT1* (*Slc11A2*) (Donovan et al., 2002), leading to a severely truncated, nonfunctional protein. The DMT1 protein localizes to erythroid cells and the intestine, suggestive of its role in iron absorption. More direct evidence was obtained with overexpression experiments discussed under “Candidate gene approaches.” Of note, whereas the *weh* mutants die during early development (Donovan et al., 2000), anemic *cdy* homozygotes survive, which might be explained by yet-to be unraveled redundant modes of iron absorption (Donovan et al., 2002). In humans, mutations in DMT1 cause a phenotype of hypochromic microcytic anemia combined with iron overload (Mims et al., 2005). This supports the possible existence of alternative iron absorption mechanisms in the duodenum that can bypass DMT1 (Mims et al., 2005).

The zebrafish strain *gavi* (*gav*) was shown to have mutations in transferrin- α (*Tf- α*), which encodes the principal iron carrier in all vertebrates (Fraenkel et al., 2009). Mutants exhibit reduced Tf- α expression and impaired hemoglobin production, which causes hypochromic anemia and embryonic lethality by 14 days of development (Fraenkel et al., 2009). Interestingly, in humans, the phenotype of congenital hypotransferrinemia as a result of Tf mutations is highly similar to that of the *gav* mutants, including hypochromic anemia and embryonic death (Goldwurm et al., 2000; Hayashi, Wada, Suzuki, & Shimizu, 1993). The

gav mutant is thus an example of a zebrafish mutant that serves as an ideal model to study corresponding human pathologies.

Transferrin-bound iron is taken up into the cell by binding to the transferrin receptor 1 (TFR1). In four different zebrafish *chianti* (*cia*) mutants with varying degrees of hypochromic anemia and defective erythroid differentiation, positional cloning revealed missense and splicing mutations in the *TFR1a* gene (Ransom et al., 1996; Wingert et al., 2004). Remarkably, the cloning of *TFR1a* revealed a second *TFR1* gene, *TFR1b* (Wingert et al., 2004), whose presence probably resulted from the teleost whole genome duplication (Amores et al., 1998; Postlethwait et al., 1998). Whereas *TFR1a* is expressed in erythrocytes during early development and *cia* mutants are anemic, the *TFR1b* gene is expressed ubiquitously throughout embryogenesis and mutation leads to growth delay and brain necrosis. Together, TFR1a (*cia*) and TFR1b deficient zebrafish embryos recapitulate the phenotype of *TFR1*^{-/-} mice (Levy, Jin, Fujiwara, Kuo, & Andrews, 1999). Hence, the *cia* mutant allows study of the function of TFR1 in erythropoiesis unbiased by developmental abnormalities in other tissues.

Phenotypic analysis of *shiraz* (*sir*) mutant zebrafish revealed a novel connection between heme biosynthesis and Fe–S cluster formation, the two main iron-demanding processes in mitochondria that were previously believed to be independent processes in vertebrates. 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 (Wingert et al., 2005). *GRX5* is evolutionarily conserved, and studies in yeast (Rodriguez-Manzanique, Tamarit, Belli, Ros, & Herrero, 2002) revealed that GRX5 is required for the synthesis of Fe–S clusters in mitochondria, where GRX5 also localizes in zebrafish. Fe–S clusters are known to negatively regulate binding of IRP to IREs. Hence, the observed anemia in the absence of reduced mitochondrial iron levels was proposed to be caused by decreased Fe–S cluster assembly, leading to increased IRP1 activity, which in turn inhibits the expression of IRE-regulated target genes involved in heme biosynthesis. In these studies a role for GRX5 in heme synthesis regulation was confirmed in human patients carrying this mutation (Camaschella et al., 2007). These findings highlight the advantages of using zebrafish as a vertebrate model system for discovering novel biological pathways that might not be present, or may be different, in lower organisms.

In addition to iron deficiency or reduced Fe–S cluster assembly, defects in the availability of heme substrates or in the catalytic activity of enzymes involved in heme biosynthesis can cause human congenital anemias and/or porphyrias. Two well-characterized zebrafish mutants with porphyria are the *yquem* (*yqe*) and *freixenet* (*frx*) [also called *dracula* (*drc*)] mutants, which have mutations later in the heme synthesis pathway and therefore present with porphyria instead of congenital anemia. Both mutants have autofluorescent red blood cells that photoablate when exposed directly to light (Ransom et al., 1996). The gene mutated in *yqe* fish encodes the enzyme uroporphyrinogen decarboxylase (UROD), which converts uroporphyrinogen to coproporphyrinogen. Homozygous mutation of the *UROD* gene leads to two forms of porphyrias, porphyria cutanea tarda (PCT) and hepatoerythropoietic porphyria (HEP) in humans, similar to the phenotype in fish (Wang, Long, Marty, Sassa, & Lin, 1998). The most prevalent form of porphyria is the autosomal

dominant disease PCT. These patients have a slightly less severe phenotype, characterized by photosensitive skin and excessive excretion of protoheme biosynthesis intermediates, uroporphyrin and 7-decarboxylate porphyrin, in their urine. The homozygous *yqe* mutant represents the first true animal model for HEP (Wang et al., 1998), which has been proposed for studying the pathogenesis of HEP and for the development of new therapeutics.

The zebrafish mutant *fix/drc* was found to bear mutations in the gene encoding for FECH, the final enzyme that catalyzes metalation of protoporphyrin IX (PPIX) with Fe^{2+} . Mutation of FECH in humans presents as erythropoietic protoporphyria due to accumulation of light-sensitive, toxic porphyrins (Childs et al., 2000). Mutation of the mitochondrial ATPase inhibitory factor 1 (*ATPIF1*) gene, which regulates the catalytic efficiency of FECH, causes heme-deficient phenotype. As is the case in *fix* mutants with direct mutation of FECH, loss of *ATPIF1* in the anemic *pinotage* (*pnt*) mutants impairs heme synthesis as a consequence of diminished FECH activity combined with elevated mitochondrial pH. Thus, using cloning strategies and data from zebrafish genetic screens, the *pnt* mutant has provided insight into a novel role of *ATPIF1* as a regulator of heme synthesis, thereby advancing the understanding of mitochondrial heme homeostasis and erythropoiesis (Shah et al., 2012). Of note, in a mouse knockout of *ATPIF1*, no obvious phenotype was found (Nakamura, Fujikawa, & Yoshida, 2013); the explanation for this difference in phenotype between these two models is not clear.

The mutants discussed above are just a few examples of the many identified in the Boston and Tübingen screens that have significantly contributed to the general understanding of iron and heme metabolism. In addition, several candidate-screening techniques were used in many of the studies described above to obtain confirmation that the identified gene is indeed causative of the observed phenotype. These approaches will be discussed below and illustrated with exemplary studies performed on the zebrafish mutants introduced above.

3.2 CANDIDATE GENE APPROACHES

One of the major limitations of performing large-scale mutagenesis screens is that they are time-consuming and laborious. Recently, methods for targeted editing of specific candidate genomic loci in been vertebrates have developed to analyze gene and protein function in vivo. The workflow toward prioritization of candidate genes derived from expression studies, microarrays, or RNA sequencing starts with performing whole mount in-situ hybridization (ISH) studies to verify tissue-specific expression. Products of genes involved in hematopoiesis are expected to localize to the intermediate cell mass (ICM) of the zebrafish, the functional equivalent of mammalian yolk-sac blood islands (Detrich et al., 1995). See Fig. 1A–H for an example of screening by ISH for localization of gene products to the ICM (Nilsson et al., 2009). The candidate genes that pass this first “triage” are then screened for possible causality of the observed erythroid phenotype by a variety of techniques, of which the ones most commonly used to study heme and iron metabolism in zebrafish are discussed below.

3.2.1 Microinjection techniques—The ex-utero development of zebrafish enables researchers to manipulate gene expression by injection of antisense oligomers [morpholino

oligonucleotides (MOs) or cRNA]. This strategy can be employed to either block or drive overexpression of the candidate gene of interest.

3.2.1.1 Gene knockdown: The first viable sequence-specific gene inactivation method in zebrafish was knockdown using MOs (Ekker & Larson, 2001). MOs are synthetic oligonucleotides analogous to DNA, but with a morpholine ring instead of the ribose sugar moiety and a neutral charge backbone with phosphorodiamidate linkage resistant to phosphodiesterases. Their high affinity for RNA and no known cellular binding proteins or nucleases makes them very well suitable as antisense reagents (Summerton, 1999). The mechanism of gene targeting by MOs can be either alteration of transcriptional processing or inhibition of translation (Draper, Morcos, & Kimmel, 2001). Compared to conventional ENU mutagenesis, which targets only zygotic transcripts, MOs can potentially knock down both zygotic and maternal transcripts (Nasevicius & Ekker, 2000). Fig. 2A–G (Nilsson et al., 2009) shows clear examples in which MO disruption of mRNA translation of candidate genes identified through in situ (Fig. 1A–H) resulted in profoundly anemic zebrafish embryos. Here the read-out is qualitative, namely a lack of hemoglobinized cells after staining with α -dianisidine for hemoglobin. A quantitative read-out can be obtained by using flow cytometry (Kardon et al., 2015) on transgenic zebrafish lines that produce fluorescent erythrocytes, such as *Tg(globin-LCR:eGFP)* (Ganis et al., 2012).

Although MOs have been and are still commonly used to knock down gene expression during early development due to the relative ease of injecting developing embryos, they have some caveats that limit their use. Because MOs are short-lived, they only temporarily block translation or alter alternative splicing and therefore are unsuitable for analysis of late onset phenotypes. Moreover, gene knockdown by this approach has potential off-target effects, although they can be avoided with rigorous controls (Bill, Petzold, Clark, Schimmenti, & Ekker, 2009; Eisen & Smith, 2008). Nevertheless, controversy exists on the specificity of MO knockdowns compared to engineered knockouts using emerging methods, such as CRISPR/cas9 genome editing (Kok et al., 2015; Rossi et al., 2015).

3.2.1.2 Gene overexpression: As an example of microinjection of cRNA encoding the protein of interest can be employed to drive its overexpression. The correct timing and target of injection depends on the desired expression pattern: injection of the mRNA into the yolk of the one-cell stage embryo will drive protein expression in every cell of the developing embryo, whereas injection into a single blastomere of a multicell embryo generates mosaic expression in a subpopulation of cells (Rosen, Sweeney, & Mably, 2009). The injected cRNA transcript is commonly stabilized by regulatory mRNA regions, such as 3' untranslated regions (UTRs) (e.g., the poly-A-tail present in the beta-globin gene from *Xenopus laevis* (Brown, Zipkin, & Harland, 1993; Jiang, Xu, & Russell, 2006).

As a study using cRNA to verify hypothesized gene function, Shaw et al. (2006) introduced *MFRN* cRNA by microinjection into *frs* embryos at the one-cell stage and assessed whether hemoglobinization was restored. The injected embryos were allowed to develop to 72 hpf, stained with α -dianisidine to detect hemoglobinized cells, and genotyped. Half of the genotyped *frs* mutant embryos were rescued. Microinjection with a mutant *MFRN* cRNA

did not rescue any *frs* embryos. Together, these findings indicate that *MFRN* is the specific gene whose disruption causes the severe chronic anemia in *frs* embryos (Shaw et al., 2006).

Usage of knockdown and overexpression strategies provides compelling evidence that a candidate gene is indeed causative of an observed phenotype. For instance, MO knockdown of *TfR1a* in wild-type zebrafish embryos prevented erythrocyte hemoglobin synthesis, resulting in embryos that displayed hypochromic erythrocytes that were α -dianisidine negative. The ability of *TfR1a* to rescue hemoglobin production in *cia* mutants was then confirmed by overexpression. Wild-type *TfR1a* mRNA was injected into *cia* homozygous embryos, resulting in a partial rescue of α -dianisidine positive cells at the 36–40 hpf stage. These experiments demonstrated that mutations in *TfR1a* were causal for the *cia* phenotype (Wingert et al., 2004).

Whereas transient overexpression is sufficient to study protein localization and function during early zebrafish development (up to 3 days), it is less suitable to study later developmental stages or when the protein must be expressed in a specific tissue or cell type (Finckbeiner et al., 2011).

3.2.2 Emerging techniques

3.2.2.1 Gene knockout: The recent development of sequence-specific endonucleases, namely zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and RNA-guided nucleases (RGNs), has provided revolutionary approaches to editing the genome in situ. As ZFNs have not been used to study iron and heme metabolism in zebrafish, they are not further discussed here; readers are referred to (Amacher, 2008) and the ZFIN website (“Zinc Finger Consortium. Available from: zincfingers.org/default2.htm,”) for protocols and additional background. TALENs and RGNs are also widely used (Dahlem et al., 2012; Huang et al., 2011; Hwang et al., 2013; Jao, Wente, & Chen, 2013; Sung et al., 2014) in zebrafish for targeted mutagenesis. Readers are referred to these reviews (Hoban & Bauer, 2016; Hsu, Lander, & Zhang, 2014; Peng et al., 2014) for details on these techniques.

Genome-editing using TALENs has been demonstrated to be successful in a variety of model systems, including zebrafish (Joung & Sander, 2013). However, precise and efficient target-specific gene knock-in and knockout in vivo can be problematic. Both precision and efficiency are especially important for investigating endogenous gene expression or generating conditional gene knockouts in model organisms such as zebrafish, as gene targeting in embryonic stem cells is not available (Bedell et al., 2012). Recently, the clustered regularly interspaced palindromic repeats/CRISPR-associated (CRISPR/Cas) system has emerged as a promising alternative genome-editing approach. Site-specific cuts introduced by CRISPR/Cas9 can be used to engineer nonhomologous end-joining (NHEJ)-mediated deletions, discussed below, and introduction of small foreign DNA fragments via homology-directed repair (HDR) (Chang et al., 2013), which will be discussed in the next section on gene knock-in.

An exemplary study on the use of the CRISPR/Cas9 system to generate a zebrafish knockout line to study heme metabolism is the recently published study by Ablain, Durand, Yang, Zhou, and Zon (2015). A CRISPR-based vector system containing the *GATA-1* promoter

driving Cas9 expression was used to silence the gene encoding the UROD enzyme. UROD-deficient erythrocytes, like those present in the *yquem* mutant zebrafish, have a clear phenotype of red autofluorescent erythrocytes due to accumulation of porphyrin precursors. Indeed, UROD targeting by the sgRNA yielded red fluorescent erythrocytes in zebrafish embryos (Fig. 3A–D). Co-injection of mRNA encoding UROD partially rescued this phenotype, while injection of cRNA bearing the *yquem* mutation did not, demonstrating the specificity of the targeting. Importantly, whereas F0 embryos displayed mosaic gene disruption, the phenotype was highly penetrant in F1 fish. Hence, this seminal study demonstrates how the CRISPR/Cas9 vector system can be employed to spatially control tissue-restricted gene knockout and is thereby an example for other loss-of-function studies in zebrafish.

3.2.2.2 Gene knock-in: TALENs and the CRISPR/Cas9 system can also be used to insert small sequence stretches using single-stranded oligonucleotides (ssDNA) (Bedell et al., 2012; Chang et al., 2013; Hruscha et al., 2013; Hwang et al., 2013), bigger fragments like open reading frames (Zu et al., 2013), or even whole plasmid vectors (Auer, Duroure, De Cian, Concordet, & Del Bene, 2014). To generate a bioassay for potassium channel function in the heart, for example, Hoshijima, Jurynek, and Grunwald (2016) used TALENs to replace the coding sequences of the *KCNH6a* gene with GFP-coding sequences. The *KCNH6a*GFP/+ heterozygous embryos, which were fully viable, expressed the GFP reporter protein exclusively in the heart. Sequence analysis of the resulting *KCNH6a*GFP alleles from the genomes of F1 embryos confirmed that donor sequences had been perfectly integrated by HR (Hoshijima et al., 2016).

Nonhomologous end-joining (NHEJ) DNA repair mechanism, which is at least ten-fold more active than HR during early zebrafish development (Dai, Cui, Zhu, & Hu, 2010; Liu et al., 2012), can also be exploited to integrate DNA at specific target sites. Auer et al. (2014) used TALENs and RGNs to introduce the *KalTA4* sequence into the *Tg(neurod:eGFP)* locus via NHEJ. This approach relies on a bait sequence mimicking the endogenous cutting site, located upstream of a functional cassette. Both the endogenous locus and the donor plasmid are linearized by the (TALE or RG) nuclease. The double strand breaks in the genome and the donor plasmid are religated by DNA repair, resulting in targeted integration of the donor vector DNA (Auer & Del Bene, 2014).

3.2.2.3 Transplantation—Transplantation, which generates chimeric embryos (Lin, Long, Chen, & Hopkins, 1992), is a powerful technique that can be exploited for studies of blood formation, including heme and iron disorders. For a detailed description of this technique the reader is referred to Chapter: Transplantation in Zebrafish by Gansner, Dang, Ammerman, and Zon (2016) of this volume. An important issue addressed using transplantation is whether a gene function is cell-autonomous. To this end, mutant cells lacking the functional gene of interest are transplanted into a wild-type embryo and, conversely, wild-type cells are introduced into mutant embryos, after which the phenotypes of the transplanted cells and the host embryo cells are compared. Using transplantation, Parker and Stainier (1999) investigated the cell autonomy of the blood deficiency of *cloche*

mutants, and Liao et al. (2002) assessed the cell autonomy of the *bloodless* gene in hematopoiesis.

3.2.2.4 Rescue/chemical complementation: In-vivo complementation involves the selection of an enzyme or molecule based on its ability to complement an essential activity that has been deleted from the wild-type cell or organism (Baker et al., 2002). This strategy can be used in zebrafish to provide compelling evidence for the involvement of a specific protein in a biological pathway. For instance, Chen et al. (2013) reported that sorting nexin 3 (SNX3) regulates recycling of the TfR to the cell membrane in zebrafish embryos and is thus required for delivery of iron to erythroid progenitors. Silencing of *SNX3* with MO resulted in anemia and hemoglobin defects in zebrafish, attributable to impaired TfR-mediated iron uptake and its accumulation in early endosomes. The role of SNX3 in iron uptake was confirmed by treatment of *SNX3*-knockdown embryos with non-Tf iron chelate supplements followed by an assay using flow cytometry. Supplementation with the non-Tf iron chelate FeSIH was found to increase the number of erythrocytes in *SNX3*-knockdown *Tg(globin-LCR:eGFP)* embryos (Fig. 4B). These results strongly reinforced the proposed model that SNX3 specifically regulates TfR-dependent iron assimilation in red cell development (Chen et al., 2013).

Kardon et al. (2015) reported (patho)physiological insight regarding the role of the mitochondrial chaperone CLPX in heme metabolism by treating *CLPX* morphant *Tg(globin-LCR:eGFP)* embryos with ALA, the product of the ALAS enzyme (Fig. 5A–C). These morphants suffered from severe anemia, as shown by flow cytometry to quantify GFP + erythrocytes (Fig. 5A,B). Interestingly, supplementation with 2 mM ALA from 24 to 72 hpf rescued the anemia in ALAS2 mutant embryos (*sauternes*; Brownlie et al., 1998), but not in mitochondrial iron transporter (*MFRN1*) mutants (*frascati*; Shaw et al., 2006) (Fig. 5C), which indicates that ALA specifically rescues defects in ALA synthesis only; defects in later steps of the heme biosynthesis pathway are not rescued. Clearly, CLP stimulation of ALA synthesis is important for efficient heme synthesis during erythropoiesis (Kardon et al., 2015).

Because heme or iron metabolism pathways are closely linked in by Fe–S clusters, assignment of mutated genes to these two pathways can be problematic. Supplementation with FeSIH, a lipid soluble iron chelate, has been the “gold standard” for identifying genes exclusively involved in iron metabolism. However, high doses of FeSIH are also able to rescue embryos with heme-based TMEM14c deficiency (Y.Y. Yien, unpublished data), which indicates that FeSIH is not absolutely specific to iron metabolism. Recently, the small molecule-iron transporter, *hinokitiol* (CAS #499-44-5), was shown by Grillo et al. (2016) to selectively rescue zebrafish mutant embryos and cell lines with defects in transporters involved in iron, but not heme, metabolism. In *MFRN1*-deficient *frs* zebrafish, addition of hinokitiol rescued the anemic phenotype, as observed qualitatively by restored *o*-dianisidine staining for hemoglobin and quantitatively by the number of GFP + erythrocytes in *Mfrn1*-deficient *Tg(globinLCR:eGFP)* zebrafish. A derivative lacking the C2OH group, C2-deoxy hinokitiol (C2deOHino), required to chelate the iron could not rescue hemoglobinization, showing the specificity of hinokitiol. Furthermore, hinokitiol did not rescue *sauternes* (*sau*) zebrafish deficient in ALAS2, the initial enzyme in porphyrin biosynthesis. Hence, this

small molecule iron transporter opens up new possibilities to efficiently distinguish between causative genes involved in the iron versus heme metabolism pathways.

CONCLUSIONS AND FUTURE DIRECTIONS

As illustrated in this chapter, zebrafish provide an ideal molecular-genetic model for investigating diseases of iron and heme metabolism. New, revolutionary technologies, such as the CRISPR/Cas9 system, have greatly facilitated the generation of targeted loss-of-function fish mutants that affect these pathways, thereby augmenting our repertoire of experimental strategies. Hence, zebrafish allow one to study the functional consequences of genetic mutations that have been identified in humans and other mammals at the systemic level. Future studies in this field will likely focus on identifying and characterizing transporter proteins of iron and heme intermediates, which are to date largely unknown. Ultimately, the zebrafish provides an ideal research platform for developing new treatments for patients suffering from defects in iron and heme metabolism.

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FIGURE 1.

(A–H) Expression of candidate genes identified in a large-scale computational screen to identify mitochondrial proteins whose transcripts coexpress with the heme biosynthesis machinery. Whole embryo in-situ hybridization was performed on embryos at 24 hpf. (A) *Slc4a1* was used as control to delineate the intermediate cell mass (indicated by arrows). (B) *Cloche* (*clo*) embryos were used to show specificity of the hybridizations. (C–G) Candidates identified in this study.

Adapted with permission from Nilsson, R., Schultz, I. J., Pierce, E. L., Soltis, K. A., Naranuntarat, A., Ward, D. M., Mootha, V. K. (2009). Discovery of genes essential for heme biosynthesis through large-scale gene expression analysis. *Cell Metabolism*, 10(2), 119–130, Cell Press.

FIGURE 2.

(A–G) Morpholino knockdown of candidate genes in zebrafish results in profound anemia. Wild-type (WT) zebrafish embryos were injected at the 1-cell stage with the respective morpholino oligonucleotides (MOs) and stained at 48 hpf with *o*-dianisidine to detect hemoglobinized cells. (A) Uninjected (WT) embryos show normal hemoglobinization as indicated by dark-brown staining on the yolk sac (*arrow*). (B–G) MO-injected embryos. Accurate MO gene targeting was verified by RT-PCR (*slc25a39*, *slc22a4*, *slc22a5*, *tmem14c*, and *c1orf69*) or real-time quantitative RT-PCR (*isca1*) on cDNA from uninjected (WT) or morpholino-injected (MO) embryos. β -actin (*actb*) was used as a control for off-target effects in the RT-PCR. For RT-PCR, (ctrl) indicates no cDNA template control. For real-time quantitative RT-PCR, (ctrl MO) indicates embryos injected with a standard control morpholino.

Adapted with permission from Nilsson, R., Schultz, I. J., Pierce, E. L., Soltis, K. A., Naranuntarat, A., Ward, D. M., Mootha, V. K. (2009). Discovery of genes essential for heme biosynthesis through large-scale gene expression analysis. *Cell Metabolism*, 10(2), 119–130, Cell Press.

FIGURE 3.

(A–D) Fluorescent phenotype resulting from CRISPR targeting of *urod*. (A) Schematic representation of *urod* coding sequence. The position of the CRISPR target sequences at the beginning of exons three and five are indicated. (B) Outline of the experiment, *Cas9* mRNA is injected into one-cell stage embryos along with a gRNA targeting either *urod* or *p53* as a negative control (*ctrl*). Targeted mutation efficiency was assessed by sequencing, and the presence of red fluorescent erythrocytes by confocal microscopy. (C) T7E1 mutagenesis assay at the CRISPR target site in the *urod* gene. The assay was performed on genomic DNA from 2 dpf embryos injected at the one-cell stage with *Cas9* mRNA and either a gRNA against *p53* (negative control) or a gRNA against *urod*. Cleavage bands (*arrowheads*) indicate the presence of mutations at the target site. (D) Confocal images reveal the presence of fluorescent blood cells in *urod* mosaic knockout embryos at 30 hpf. The black and white insets show a 2× magnification of the fluorescent signal in the yolk region. Adapted with permission from Ablain, J., Durand, E. M., Yang, S., Zhou, Y., & Zon, L. I. (2015). A CRISPR/ Cas9 vector system for tissue-specific gene disruption in zebrafish. *Developmental Cell*, 32(6), 756–764, Cell Press.

FIGURE 4.

Complementation of hemoglobinization defects with non-Tf-bound iron supplements. Fe-SIH, and a chemically defined iron supplement increase hemoglobin production in *snx3*- and *mfrn*-silenced zebrafish morphants. Error bars represent SEM from three independent experiments. * $p < .001$ compared with the control clone; † $p < .05$ compared with the control, untreated embryos within the group.

Adapted with permission from Chen, C., Garcia-Santos, D., Ishikawa, Y., Seguin, A., Li, L., Fegan, K. H., ., Paw, B.H. (2013). Snx3 regulates recycling of the transferrin receptor and iron assimilation. *Cell Metabolism*, 17(3), 343–352, Cell Press.

FIGURE 5.

Morpholino knockdown of mtClpX can be rescued with aminolevulinic acid (ALA) supplementation. Embryos were grown from zygotes injected at the one- to two-cell stage with *clpxa*-targeting morpholinos (MOa and MOb) or uninjected zygotes (control). (A) Erythrocyte development at 72 hpf was quantified by flow cytometry, using dissociated cells from *Tg(globin-LCR:eGFP)* zebrafish. $p = .01$ for erythrocyte reduction by *clpxa* knockdown with either morpholino. (B) Rescue of *clpxa* MOb-induced anemia by ALA supplementation. *Tg(globin-LCR:eGFP)* zebrafish embryos were supplemented with 2-mM ALA from 24 to 72 hpf, upon which GFP + erythrocytes were quantified by flow cytometry. $p = .025$ for rescue of anemia in *clpxa* knockdown embryos by ALA supplementation. (C) Heterozygous *sauternes* (ALAS2 mutant) or *frascati* (MFRN mutant) zebrafish were crossed, and progeny were grown for 72 hpf, with or without ALA supplementation as in (B). Anemia was assayed by *o*-dianisidine staining. $p = .04$ for rescue of anemia in *sauternes* ± progeny by ALA. $n = 52$ for *sauternes* – ALA; $n = 43$ for *sauternes* + ALA; $n = 98$ for *frascati* – ALA; $n = 122$ for *frascati* + ALA. Error bars represent mean ± SD. Adapted with permission from Kardon, J. R., Yien, Y. Y., Huston, N. C., Branco, D. S., Hildick-Smith, G. J., Rhee, K. Y., ., Baker, T. A. (2015). Mitochondrial ClpX activates a key enzyme for heme biosynthesis and erythropoiesis. *Cell*, 161(4), 858–867, Cell Press.

Table 1
Overview of Zebrafish Mutants That Gave Insight Into Iron Metabolism and Heme Biosynthesis

	Name Mutant	Gene or Protein	Human Disease	Reference Zebrafish Mutant
Heme biosynthesis	<i>montalcino (mmo)</i>	<i>PPOX</i>	Variegate porphyria	Dooley et al. (2008)
	<i>sauternes (sau)</i>	<i>ALAS2</i>	X-linked dominant erythropoietic protoporphyria	Brownlie et al. (1998)
	<i>yquem (yqe)</i>	<i>UROD</i>	Porphyria cutanea tarda/hepato- erythropoietic porphyria	Wang et al. (1998)
Iron metabolism	<i>freixenet (frx)/dracula (drc)</i>	<i>FECH</i>	Erythropoietic protoporphyria	Ransom et al. (1996) and Childs et al. (2000)
	" <i>alad</i> "	<i>ALAD</i>	ALA dehydratase deficient porphyria	Sakamoto et al. (2004) (in medaka fish)
	" <i>cpox</i> "	<i>CPOX</i>	Hereditary coproporphyria <i>readers are referred to these porphyrias</i>	
	<i>gavi</i>	<i>TF</i>	Iron-deficiency anemia	Fraenkel et al. (2009)
	<i>shiraz (sir)</i>	<i>GRX5</i>	Nonketotic hyperglycinemia and severe neurodegeneration (Baker et al., 2014); Sideroblastic anemia (Camaschella et al., 2007)	Wingert et al. (2005)
	<i>frascati (frs)</i>	<i>MFRN1</i>	Erythropoietic protoporphyria associated with abnormal mitoferrin expression (Wang et al., 2011)	Shaw et al. (2006)
	<i>chardonnay (cdy)</i>	<i>DMT-1</i>	Hypochromic microcytic anemia, hepatic iron overload (Mims et al., 2005)	Donovan et al. (2002)
	<i>chianti (cia)</i>	<i>TFR1a</i>	Iron-deficiency anemia (experimentally induced by TFR antibody). (Larrick & Hyman, 1984). Combined immunodeficiency, intermittent neutropenia, thrombocytopenia, and mild anemia (rare phenotype) (Jabara et al., 2016)	Wingert et al. (2004)
	<i>weissherbst (wei)</i>	<i>FPN1</i>	Ferroportin disease (Zoller et al., 2005)	Donovan et al. (2000) and Fraenkel, Traver, Donovan, Zahrieh, and Zon (2005)
	<i>vlad tepes (vlt)</i> <i>cabernet (cab)</i> <i>grenache (gre)</i> <i>thunderbird (tbr)</i>	<i>GATA-1</i>	Familial dyserythropoietic anemia and thrombocytopenia (Nichols et al., 2000)	Lyons et al. (2002) and Ransom et al. (1996)
Erythropoiesis general	<i>retsina (ret)</i>	<i>band-3 (Slc4A1)</i>	<i>Readers are referred to this review</i> (Mohandas & Gallagher, 2008)	Paw et al. (2003)
	<i>chablis/merlot (mot)</i>	<i>protein 4.1</i>		Shafizadeh et al. (2002)
	<i>riesling (ris)</i>	<i>erythroid β spectrin</i>		Liao et al. (2000)
	<i>malbec</i>	<i>CDH5</i>		Anderson et al. (2015)
	<i>pinotage (pnt)</i>	<i>ATP1F1</i>		Shah et al. (2012)

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Name Mutant	Gene or Protein	Human Disease	Reference Zebrafish Mutant
<i>moonshine (mon)</i>	<i>TIF1 gamma</i>	Hemoglobinopathies (Cao & Galanello, 2010; Piel & Weatherall, 2014)	Ransom et al. (2004)
<i>zintfandel</i>	<i>globin</i>		Brownlie et al. (2003)
<i>cloche (clo)</i>	<i>bHLH-PAS transcription factor</i>		Stainier, Weinstein, Detrich, Zon, and Fishman (1995), Wang et al. (2007), Reischauer et al. (2016), Griffin, Amacher, Kimmel, and Kimmelman (1998) and Thompson et al. (1998)
<i>spadetail (spt) and TBX16</i>			Liao et al. (2002)
<i>bloodless (bls)</i>	<i>unknown</i>		