Knockout Mouse Models of Iron Homeostasis

Robert E. Fleming,[1] Qi Feng,[2] and Robert S. Britton[3]

[1]Departments of Pediatrics and Biochemistry & Molecular Biology, Saint Louis University School of Medicine, St. Louis, Missouri 63104; [2]Department of Pediatrics, Peking University First Hospital, Beijing 100034, China; [3]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Saint Louis University School of Medicine, St. Louis, Missouri 63110; email: flemingr@slu.edu, fengqizf@yahoo.com, brittonrs100@yahoo.com

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Abstract

Murine models have made valuable contributions to our understanding of iron metabolism. Investigation of mice with inherited forms of anemia has led to the discovery of novel proteins involved in iron homeostasis. A growing number of murine models are being developed to investigate mitochondrial iron metabolism. Mouse strains are available for the major forms of hereditary hemochromatosis. Findings in murine models support the concept that the pathogenesis of nearly all forms of hereditary hemochromatosis involves inappropriately low expression of hepcidin. The availability of mice with floxed iron-related genes allows the study of the in vivo consequences of cell-selective deletion of these genes.

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INTRODUCTION

Iron is a key micronutrient, and iron deficiency anemia is one of the most common nutritional conditions worldwide. Murine models have proven to be very useful in providing insight into human iron homeostasis and disorders of iron metabolism. Such models provide an integrated picture of the dynamics of iron metabolism in the whole animal, which is not possible in cell culture systems. Mice are

particularly useful as a model organism because of the availability of strains with spontaneous or induced mutations that affect iron metabolism, and because of the possibility of genetic manipulation (targeted mutagenesis and transgenic gene expression). This review describes the most extensively characterized murine models that have provided valuable information about the regulation of iron absorption, distribution, storage, and utilization. Several previous reviews of various aspects of this subject have been published (5–7, 60, 82, 106, 111).

INFORMATIVE MOUSE STRAINS WITH ANEMIA

Mice with inherited iron deficiency have been very valuable for learning about cellular iron transport. Because iron deficiency leads to anemia, pale mice were easily identified in inbred colonies, and the heritability of the trait could be established (11). With the advances in mouse genomics and DNA sequencing, the mutant genes responsible could be readily identified. The genetic defects and phenotypes for some of these informative mutant strains are summarized in **Table 1**.

Microcytic Anemia (*mk*) Mouse

Microcytic anemia (*mk*) mice were discovered in a colony at the Jackson Laboratory (86) and are characterized by severe hypochromic, microcytic anemia and poor viability. The *mk* trait was found to be autosomal recessive, and the mice have impaired intestinal iron absorption with decreased apical iron uptake by enterocytes (11). In addition, iron uptake by reticulocytes is also defective (32). In 1997, Fleming and coworkers (40) discovered that *mk* mice have a missense mutation in the gene for divalent metal transporter 1 (Dmt1). Dmt1 is a proton-coupled metal cation transporter (52), and selective deletion of Dmt1 expression in villus enterocytes demonstrates that it plays a major role in apical iron transport in these cells (51). The *mk* mutation results in the substitution of an arginine for a glycine (G185R) in transmembrane domain 4 (40), and this impairs Dmt1 localization and activity (19, 120). Dmt1 is also found in transferrin cycle endosomes, where it functions in exporting iron into the cytoplasm, and this explains the reticulocyte defect caused by the *mk* mutation (39, 50). Interestingly, Belgrade (*b*) rats contain the same G185R amino acid alteration in the *Dmt1* gene as that seen in the *mk* mouse (39). Belgrade rats also have autosomal recessively inherited hypochromic, microcytic anemia (14, 33, 118). Thus, both the *mk* mouse and the Belgrade rat demonstrate the functional consequences of impaired iron transport by Dmt1.

Dmt1: divalent metal transporter 1

Sex-Linked Anemia (*sla*) Mouse

The sex-linked anemia (*sla*) mouse arose in an irradiated mouse colony (34). The *sla* mouse develops moderate to severe microcytic,

Table 1 Informative mouse strains with anemia

Mouse	Genetic defect	Phenotype	Mechanism	References
mk mouse	Missense mutation in *Dmt1* gene	Microcytic anemia	Decrease in intestinal and endosomal iron transport	(40)
sla mouse	Deletion in hephaestin gene	Microcytic anemia, low iron stores	Decrease in enterocyte basolateral iron transport	(133)
hbd mouse	Deletion in *Sec15l1* gene	Microcytic hypochromic anemia	Impaired transferrin cycling in reticulocytes	(139)
nm1054 mouse	Deletion of *Steap3* gene	Microcytic hypochromic anemia	Impaired ferrireductase activity in transferrin endosome	(93, 94)
Steap3 knockout mouse		Similar to *nm1054* mouse	Impaired ferrireductase activity in transferrin endosome	(94)

Steap: 6-transmembrane epithelial antigen of the prostate

ALAS2: aminolevulinic acid synthase 2

XLSA: X-linked sideroblastic anemia

hypochromic anemia early in life (49). Although this mouse strain takes up iron normally from the intestinal lumen into mature epithelial cells, the subsequent egress of iron into the circulation is decreased (31, 75). Therefore, iron accumulates within enterocytes and is lost during turnover of these cells. Vulpe and colleagues identified the genetic defect in the *sla* mouse as being a large deletion in a gene they called hephaestin (*Heph*), named after the Greek god of metal-working (133). Hephaestin is a multicopper protein that shares 50% homology with ceruloplasmin, but unlike ceruloplasmin, it contains a predicted carboxy-terminal transmembrane domain (133). Like ceruloplasmin, hephaestin has ferroxidase activity, and it may act to facilitate iron egress from intestinal enterocytes (5, 7, 133).

Hemoglobin-Deficit (*hbd*) Mouse

The hemoglobin-deficit (*hbd*) mouse arose spontaneously in an inbred colony in Germany (114). This mouse strain is characterized by a hypochromic, microcytic anemia that is inherited in an autosomal, recessive manner (72). A deletion in the gene *Sec15l1* is responsible, and Sec15l1 is specific to hematopoietic cells and has homology to a yeast gene for vesicle docking (72, 139). Iron trafficking experiments in reticulocytes indicate that transferrin cycling is deficient, and it is proposed that the product of *Sec15l1* is directly involved in vesicular exocytosis (72), docking, fusing, or cargo delivery in erythroid precursors (142).

nm1054 Mouse and *Steap3* Knockout Mouse

The *nm1054* mouse appeared spontaneously in a colony at the Jackson Laboratory (93). This mouse has the characteristics of moderately severe, congenital, hypochromic, microcytic anemia, with an elevated red cell zinc protoporphyrin, consistent with functional erythroid iron deficiency (93). However, analysis of serum and tissue iron status indicates that *nm1054* mice are not systemically iron deficient. Fleming and colleagues (94) have identified that deletion of the gene for Steap3 (6-transmembrane epithelial antigen of the prostate 3) is responsible for this phenotype: *Steap3* knockout mice also share this phenotype. Steap3 is an endosomal ferrireductase required for efficient transferrin-dependent iron uptake in erythroid cells. However, both *nml054* and *Steap3*$^{-/-}$ erythroid precursors retain some residual ferrireductase as well as iron uptake activity, suggesting that there are other ways of reducing iron in the transferrin-cycle endosome (94). Steap2 and Steap4 are ferrireductases that are expressed in erythropoietic tissues, which makes them candidates for redundant ferrireductases in the erythroid transferrin-cycle endosome (95). Use of genetically manipulated mice should clarify the functions of Steap 2 and 4 in vivo.

DEFECTS IN MITOCHONDRIAL IRON METABOLISM

The sideroblastic anemias are a group of disorders characterized by a variable population of hypochromic red cells in the blood and by ringed sideroblasts in the bone marrow (60). The unifying characteristic of all sideroblastic anemias is the ring sideroblast, which is a pathological erythroid precursor containing iron-loaded mitochondria localized around the nucleus, thereby creating a ring-like appearance (116). Three forms of hereditary sideroblastic anemia are caused by defects in genes present on the X chromosome [mutations in the aminolevulinic acid synthase 2 (*ALAS2*), *ABCB7*, or *GRLX5* gene]: for the first two of these forms, mutant mouse models have been created. Mouse models have also been generated for erythropoietic protoporphyria, Friedreich ataxia, and mitoferrin 1 deficiency (**Table 2**).

Mouse Models of X-Linked Sideroblastic Anemia: *Alas2* Mutant Mice

The most frequent form of inherited sideroblastic anemia is X-linked sideroblastic anemia (XLSA), caused by mutations in the

Table 2 Murine models of altered mitochondrial iron metabolism

Protein	Mouse genotype	Phenotype	References
Erythroid 5-aminolevulinic acid synthase (ALAS2)	*Alas2* knockout mouse	Increased iron, but no ring sideroblasts, in primitive erythroid cells. Embryonic lethal by day 11.5	(141)
	Alas2 knockout mouse with transgenic rescue of low activity ALAS2	Ring sideroblasts in primitive erythroid cells	(85)
ATP-binding cassette transporter Abcb7	*Abcb7* knockout mouse	Mid-gestational death	(103)
	$Abcb7^{E433K}$ mouse	Siderocytosis	(104)
Ferrochelatase	$Fech^{m1Pas}$ mouse	Homozygous mouse has microcytic hypochromic anemia and severe porphyria	(73)
	Fech knockout mouse (exon 10 deletion)	Homozygous state is embryonic lethal. Heterozygotes have decreased ferrochelatase activity and mild porphyria	(74)
Frataxin	*Fxn* knockout mouse	Embryonic lethal	(107)
	Knock-in of GAA repeat mutation	No anomalies of motor coordination or iron metabolism	(80)
	Fxn knockout mice with human mutant *FXN* YAC constructs	Progressive neurodegeneration and cardiac pathology	(3)
Mitoferrin 1	*Mfrn1* knockout mouse	Embryonic lethal with profound anemia	(21)

erythroid-specific gene for *ALAS2* (60). ALAS2 catalyzes the first step of the heme biosynthetic pathway in erythroid cells. Hemizygous XLSA males have microcytic anemia with iron overload, indicating that XLSA belongs to the group of iron-loading anemias. Various mutations in *ALAS2* cause decreased ALAS2 activity in bone marrow erythroblasts, with resultant impairment of heme biosynthesis and insufficient protoporphyrin IX to use all the available iron (excess iron can be stored as mitochondrial ferritin) (16).

Knockout of the *Alas2* gene in mice results in arrest of erythroid differentiation, and an abnormal hematopoietic cell fraction emerges that accumulates a large amount of iron diffusely in the cytoplasm; typical ring sideroblasts are not found (141). Embryonic death occurs by day 11.5. However, when human ALAS2 is expressed in these mice at approximately 50% of normal activity, most of the primitive erythroid cells are transformed into ring sideroblasts while the majority of the circulating definitive erythroid cells become siderocytes (85). These results suggest that a partially depleted heme supply provokes ring sideroblast formation.

Mouse Models of X-Linked Sideroblastic Anemia with Ataxia: *Abcb7* Mutant Mice

XLSA with ataxia is a rare form of inherited sideroblastic anemia of early onset, associated with spinocerebellar ataxia and cerebellar hypoplasia (4). This form of sideroblastic anemia is due to partial loss-of-function mutations in the ATP-binding cassette transporter ABCB7, which cause mitochondrial accumulation of iron, elevated free erythrocyte protoporphyrin IX levels, and mild hypochromic microcytic anemia: Examination of bone marrow shows ringed sideroblasts (4, 60). ABCB7 is thought to export a mitochondrially derived metabolite required for cytosolic iron-sulfur cluster (ISC) biogenesis. It is an essential protein, as *Abcb7* knockout mice undergo mid-gestational death (103). To model the severe E433K mutation in humans, an $Abcb7^{E433K}$

ISC: iron-sulfur cluster

mutant mouse was created. It has siderocytosis but no ring sideroblasts (104). The absence of ring sideroblasts in these mice supports the suggestion that there is something biologically distinctive with respect to mitochondrial iron handling and/or toxicity between human and murine erythroid precursors (104).

Mouse Model of Erythropoietic Protoporphyria: *Fech*^{m1Pas} Mouse

Erythropoietic protoporphyria is caused by decreased activity of the mitochondrial enzyme ferrochelatase, the terminal enzyme of the heme biosynthetic pathway, that catalyzes the insertion of iron into protoporphyrin IX to form heme (60). Clinical symptoms result from an accumulation of protoporphyrin IX behind the partial enzyme block; they include life-long photosensitivity and, in about 2% of patients, severe liver disease. The inheritance of erythropoietic protoporphyria is usually described as an autosomal dominant disorder with incomplete penetrance: missense, nonsense, and splicing mutations have been identified in the *FECH* gene (60). Knockout of the murine *Fech* gene results in embryonic lethality, indicating the critical importance of ferrochelatase (74). Microcytic anemia occurs in 20% to 60% of patients with erythropoietic protoporphyria (58). This anemia is not dyserythropoietic, and there is no iron overload but rather iron deficiency. Therefore, ferrochelatase deficiency in erythropoietic protoporphyria appears to result in a steady state in which decreased erythropoiesis is matched by reduced iron absorption and supply (58).

A mouse model of erythropoietic protoporphyria, the homozygous *Fech*^{m1Pas} mouse, develops a similar microcytic anemia along with accumulation of protoporphyrin IX and liver injury (73). This mouse has a point mutation in the *Fech* gene resulting in about a 95% decrease in ferrochelatase activity. In this mouse model, there is increased expression of transferrin and redistribution of iron from peripheral tissues to the spleen while serum iron and ferritin levels are normal (73). Further investigation is needed to understand the cause of microcytic anemia in these mice and in patients with erythropoietic protoporphyria.

Mouse Models of Friedreich Ataxia: *Fxn* Mutant Mice

Friedreich ataxia, the most common hereditary ataxia, is an autosomal recessive neurodegenerative disease characterized by progressive ataxia associated with cardiomyopathy and increased incidence of diabetes (106). This condition is caused by reduced levels of frataxin, a highly conserved mitochondrial iron-chaperone involved in ISC biogenesis. Most patients are homozygous for a large GAA triplet expansion within the first intron of the frataxin (*FXN*) gene. The pathophysiologic consequences of frataxin deficiency are a disruption of ISC biosynthesis, mitochondrial iron overload coupled with cellular iron dysregulation, and increased sensitivity to oxidative stress (106).

Knockout of *Fxn* in the mouse leads to early embryonic lethality, demonstrating an important role for frataxin during mouse development (107). Heterozygous *Fxn*$^{+/-}$ mice express about 50% of wild-type frataxin levels but do not have a phenotype (106). Using a conditional gene-targeting approach, Puccio et al. (107) generated a striated muscle *Fxn*-deficient mouse and a neuron/cardiac muscle *Fxn*-deficient mouse, which together reproduce some features of the human disease: cardiac hypertrophy without skeletal muscle involvement, large sensory neuron dysfunction, and low activities of mitochondrial and extramitochondrial ISC proteins. Another mouse model contains a knock-in of the GAA repeat *Fxn* mutation (80). These GAA repeat knock-in mice were crossed with *Fxn* knockout mice to obtain double heterozygous mice expressing 25% to 36% of wild-type frataxin levels. However, these mice do not develop anomalies of motor coordination or iron metabolism (80), suggesting that their frataxin levels are sufficient to preserve ISC biogenesis. Using an innovative approach, Al-Mahdawi et al. (3) generated "humanized" GAA repeat expansion mice by

combining the constitutive *Fxn* knockout with the transgenic expression of a yeast artificial chromosome carrying the human *FXN* gene with GAA triplet expansion. These mice have coordination defects and progressive neuronal and cardiac pathology and should serve as a useful model to test potential therapeutic agents.

Mitoferrin 1 (*Mfrn1*) Knockout Mouse

Mitoferrin 1 is involved in the transport of iron into the mitochondrion (97, 115). It is located in the inner mitochondrial membrane and is a member of the mitochondrial solute carrier family (SLC25A37). Knockout of *Mfrn1* in mice results in embryonic lethality with profound anemia, demonstrating the essential role of mitoferrin 1 (21). Mitoferrin 1 is expressed mainly in erythroid cells, but its homolog mitoferrin 2 is expressed in nonerythroid tissues and may play a similar transport role there (97, 115).

MOUSE MODELS OF HEREDITARY HEMOCHROMATOSIS

Substantial progress has been made in developing mouse models of the major forms of hereditary hemochromatosis (HH). **Table 3** summarizes the currently available models and their phenotypes.

Hfe Mutant Mice: Model for HH Type 1

The positional cloning of the *HFE* gene, which is responsible for HH type 1, triggered intense interest in the function of HFE protein (35). HFE is a major histocompatibility complex (MHC) class I-like protein, and a single nucleotide change, resulting in the substitution of tyrosine for cysteine at amino acid 282 of the unprocessed protein (C282Y), is present in nearly all patients with HH type 1. In the original cloning study, a second mutation in *HFE* was also identified, which results in the substitution of aspartate for histidine at amino acid 63 (H63D) (35). Mutant mouse models have been developed to study the in vivo consequences of *Hfe* deletion or mutation.

Five different *Hfe* gene disruptions have been produced in the mouse: an exon 4 knockout (143), an exon 3 disruption/exon 4 knockout (71), an exon 2–3 knockout (10), a C282Y knock-in (71, 127), and an H63D knock-in (127). In each case, the mutant mice have increased hepatic iron concentrations (10, 71, 127, 143). In *Hfe* knockout mice, decreased

HH: hereditary hemochromatosis

MHC: major histocompatibility complex

Table 3 Murine models of hereditary hemochromatosis (HH)

Classification of human HH (protein involved)	Mouse genotype	Mouse phenotype	References
HH type 1 (HFE)	*Hfe*$^{-/-}$	Increased body iron, splenic iron sparing, decreased hepcidin	(1, 10, 15, 71, 143)
	Hfe$^{C282Y/C282Y}$	Milder phenotype than *Hfe*$^{-/-}$ mouse	(71)
	Hfe$^{H63D/H63D}$	Very mild phenotype compared to *Hfe*$^{-/-}$ mouse	(127)
HH type 2A (hemojuvelin)	*Hjv*$^{-/-}$	Increased body iron, splenic iron sparing, decreased hepcidin	(59, 92)
HH type 2B (hepcidin)	*Hamp1*$^{-/-}$	Increased body iron, splenic iron sparing, no hepcidin	(68, 89)
HH type 3 (transferrin receptor 2)	*Tfr2*$^{Y245X/Y245X}$	Increased body iron, splenic iron sparing, increased iron absorption, decreased hepcidin	(28, 41, 63)
	Tfr2$^{-/-}$	Similar phenotype to *Tfr2*$^{Y245X/Y245X}$ mouse	(136)
HH type 4 (ferroportin)	Missense mutation (H32R) in *Slc40a1* (flatiron mouse)	Heterozygous mice have iron loading of Kupffer cells, high serum ferritin, and low transferrin saturation	(144)

Bmp6: bone morphogenetic protein 6

hepcidin expression (1, 15), elevated transferrin saturation (143), and increased intestinal iron absorption (2, 10) have also been reported. Like patients with HH type 1, these mice demonstrate relative sparing of iron accumulation in macrophages (143), thought to be due to low hepcidin levels. Interestingly, mice that are homozygous for the C282Y mutation have less severe iron loading than do *Hfe* knockout mice, suggesting that the C282Y mutation is not a null allele (71). The H63D allele, when homozygous or combined with a more consequential mutation such as C282Y, leads to very mild hepatic iron accumulation (127). Iron overload in *Hfe* knockout mice provides proof-of-principle that loss of HFE function underlies HH type 1.

Hepatic expression of bone morphogenetic protein 6 (Bmp6) mRNA is higher in *Hfe* knockout mice, and the level of expression is appropriate for the increased hepatic iron concentrations in these mice (23, 62). However, levels of hepatic phosphorylated Smad 1/5/8 protein (a mediator of Bmp6 signaling) and Id1 mRNA (a target gene of Bmp6) are inappropriately low for hepatic iron concentration and Bmp6 mRNA levels in *Hfe* knockout mice (23, 62). These results suggest that Hfe acts to facilitate signal transduction to hepcidin induced by Bmp6.

Hfe knockout mice have been bred to mice carrying mutations in other genes involved in normal iron homeostasis (70). Studies using these compound mutant animals suggest that Dmt1, hephaestin, β2-microglobulin, and transferrin receptor can all modify the HH phenotype. A role for naturally occurring strain-dependent gene modifiers has been demonstrated by the variation in iron loading seen when the *Hfe* knockout allele is placed on different background strains (12, 24, 30, 42). Ongoing gene-mapping studies are anticipated to identify some of these modifiers in mice, and then it can be determined if the same modifier genes influence the phenotypic of C282Y homozygosity in humans.

Hfe knockout mice do not have progressive iron loading throughout their lifespan and do not develop hepatic fibrosis or cirrhosis (7, 143). Liver iron accumulation in these mice is rapid during the first weeks of life (1, 2). However, by about 10 weeks of age, the hepatic iron concentration reaches a plateau, accompanied in some strains by normalization of absolute levels of liver hepcidin expression (1) and iron absorption (2), although these remain excessive relative to iron stores.

Hemojuvelin (*Hjv*) Knockout Mouse: Model for HH Type 2A

Hjv knockout mice provide a model of HH type 2A. These mice manifest markedly increased iron deposition in liver, pancreas, and heart but sparing of iron accumulation in tissue macrophages (59, 92). Hepcidin mRNA expression is decreased in these mice (59, 92), and expression of ferroportin protein is increased in both intestinal enterocytes and macrophages (59). However, *Hjv* knockout mice do not develop diabetes or cardiomyopathy (92). In these mice, hepcidin expression is not responsive to high iron but does respond to the inflammatory agent, lipopolysaccharide (92), suggesting that hemojuvelin plays a key role in signaling from iron to hepcidin. Hemojuvelin is a coreceptor for BMPs (9), and BMP6 is now recognized as playing a key role in the iron-signaling pathway to hepcidin (8, 78).

Hepcidin Knockout Mouse: Model for HH Type 2B

Hepcidin acts to downregulate ferroportin expression, thereby decreasing intestinal iron absorption and causing iron retention in macrophages (87, 88). The first evidence that hepcidin is involved in iron homeostasis came from the observation that liver hepcidin mRNA expression is increased in mice with dietary iron loading (102). This was followed by the fortuitous discovery that coincidental deletion of the hepcidin genes (*Hamp1* and *Hamp2*) in *Usf2* knockout mice led to an HH-like phenotype (89), and this established the critical role of hepcidin as a negative regulator of intestinal iron

absorption. It was later discovered that hepcidin mutations are responsible for HH type 2B in several human pedigrees (109). Transgenic overexpression of hepcidin in mouse hepatocytes leads to a severe form of iron-deficiency anemia (90, 112). Additionally, liver hepcidin expression is also influenced by factors regulating intestinal iron absorption (iron stores, erythropoietic activity, hemoglobin, oxygen content, and inflammation) (91, 102).

To analyze the consequences of *Hamp1* deletion on iron metabolism without any disturbance due to *Usf2* deficiency, Lesbordes-Brion et al. (68) disrupted the *Hamp1* gene by targeting almost all of the coding region. *Hamp1* knockout mice develop early and severe multiorgan iron overload, with sparing of splenic macrophages.

Transferrin Receptor 2 Mutant Mice: Model for HH Type 3

HH type 3 is caused by mutations in transferrin receptor 2 (TFR2) (100). Human TFR2 is 45% identical with the classical transferrin receptor in the extracellular domain but contains no iron-responsive element (IRE) in its mRNA (64). The most common mutation found in human TFR2 is Y250X that introduces a stop codon into the mRNA, resulting in a truncated nonfunctional protein (17). TFR2 is expressed highly in the liver and is thought to influence iron metabolism by affecting the expression of hepcidin.

Two mouse models have been developed for HH type 3: a mouse that contains the Y245X mutation in *Tfr2* (murine ortholog of the human mutation Y250X) (41) and a *Tfr2* knockout mouse (136). Both mutant mice demonstrate hepatic iron overload and inappropriately low hepcidin levels (28, 63, 136). In additional studies, $Tfr2^{Y245X/Y245X}$ mice were shown to have increased iron absorption, elevated duodenal iron transport gene expression, and increased liver iron uptake (28). Targeted deletion of *Tfr2* in hepatocytes results in decreased hepcidin expression and hepatic iron overload, indicating that hepatocytes are the site of Tfr2's effects on hepcidin expression (137). Mice with combined deletion of both *Tfr2* and *Hfe* have a more pronounced decrease in hepcidin expression (relative to hepatic iron levels) than do those with either gene deletion alone (135).

TFR2: transferrin receptor 2

IRE: iron-responsive element

Ferroportin (*Fpn*) Mutant Mice: Model for HH Type 4

HH type 4 is autosomal dominant and is caused by missense mutations in the *SLC40A1* gene that encodes ferroportin. It is now considered that two categories of *SLC40A1* mutations exist (36, 96). The first category includes loss-of-function mutations that reduce the cell surface localization of ferroportin, reducing its ability to export iron. This causes iron deposition primarily in macrophages, and this disorder is sometimes termed ferroportin disease (99). The second category includes gain-of-function (or "loss-of-regulation") mutations that do not alter cell surface expression but rather abolish hepcidin-induced ferroportin internalization and degradation. In this case, cellular distribution of iron is similar to HH type 1, being primarily parenchymal. In both forms of HH type 4 (unlike other forms of HH), hepcidin expression is elevated rather than decreased (101).

Ferroportin has been targeted for deletion both globally and selectively in mice (27). Embryonic lethality of *Fpn* knockout animals indicates that ferroportin is essential early in development, especially in the extraembryonic visceral endoderm. Selective knockout of ferroportin in villus enterocytes results in severe iron-deficiency anemia, demonstrating that ferroportin plays a key role in iron absorption (27).

The discovery of the flatiron mouse has provided an interesting model for the first category of *SLC40A1* mutations (144). The flatiron mouse has a missense mutation (H32R) in *Slc40a1* that affects the localization and iron export activity of ferroportin. Similar to patients with ferroportin disease, these mice have iron loading of Kupffer cells, high serum ferritin levels, and low transferrin saturation. Studies in the flatiron mouse support the concept that mutations in ferroportin resulting in protein

Table 4 Murine models: Signaling to hepcidin

Gene	Genotype	Phenotype	References
Smad4	*Smad4*$^{-/-}$ (hepatocyte-specific)	Increased body iron, splenic iron sparing, decreased hepcidin	(138)
Bmp6	*Bmp6*$^{-/-}$	Increased body iron, splenic iron sparing, decreased hepcidin	(8, 78)
Tmprss6 (matriptase-2)	*Tmprss6*$^{-/-}$ *Tmprss6*$^{msk/msk}$	Microcytic anemia, low iron stores, low serum iron, increased hepcidin	(29, 43)

mislocalization act in a dominant-negative fashion, preventing wild-type ferroportin from reaching the cell surface and transporting iron (144). It is anticipated that new mouse models will also be developed to examine the in vivo effects of putative gain-of-function mutations of ferroportin.

MURINE MODELS: SIGNALING TO HEPCIDIN

Hepcidin is now considered to be the master iron-regulatory hormone (48), and several mouse models have been particularly informative regarding the intracellular signaling pathways that regulate hepcidin expression in the liver (**Table 4**).

Smad4 Knockout Mouse

The importance of a Smad4-dependent signaling pathway in regulating hepcidin expression is demonstrated by the phenotype of the hepatocyte-specific *Smad4* knockout mouse: It has low hepcidin expression and iron accumulation in liver, pancreas, and kidney (138). Of interest, hepcidin expression in this mouse is not responsive to injected interleukin-6 or iron-dextran (138), suggesting possible crosstalk between the inflammatory and iron signaling pathways to hepcidin at the level of Smad4 (or distal to it).

Bmp6 Knockout Mice

Although hemojuvelin was recognized as a coreceptor for BMPs, and multiple BMPs regulate hepcidin expression and iron metabolism (9, 87), it was uncertain which endogenous BMP(s) regulates hepcidin in vivo. This situation has been clarified by the demonstration that *Bmp6* knockout mice have an HH-like phenotype with low hepcidin levels and iron overload (8, 78). In wild-type mice, hepatic expression of Bmp6 mRNA is upregulated by iron loading. This and other evidence has implicated BMP6 as a major regulator of hepcidin expression in vivo.

Tmprss6 Mutant Mice

Characterization of an ethylnitrosourea-induced mutant mouse strain (called *mask*) with microcytic anemia led to the discovery that the protease Tmprss6 plays an important role in signaling to hepcidin (29). Tmprss6, also known as matriptase-2, is a type II plasma membrane protein whose major site of expression is the liver. Tmprss6 contains an extracellular C-terminal trypsin-like serine protease domain. The *mask* mutation, an A-to-G transition, eliminates a splice acceptor site, yielding two abnormal splice products that lack the proteolytic domain (29). Targeted deletion of the *Tmprss6* gene results in a similar high-hepcidin, iron-deficient phenotype (43). It is proposed that Tmprss6-mediated hepcidin suppression permits adequate absorption of iron from the diet, and that without hepcidin suppression, severe iron deficiency occurs (29, 43). A key substrate for the proteolytic activity of Tmprss6 is hemojuvelin, and this may explain the dampening effect of Tmprss6 on hepcidin expression (117). Interestingly, mutations in *TMPRSS6* cause iron-refractory iron deficiency anemia in humans (18).

MICE DEFICIENT IN IRON-RELATED PROTEINS

Table 5 summarizes the phenotype of several mouse lines that are deficient in certain

Table 5 Mice deficient in iron-related proteins

Protein	Mouse and genetic defect	Phenotype	References
Transferrin	Hypotransferrinemic ($Tf^{hpx/hpx}$) mouse, splicing defect	Severe anemia, iron overload	(13, 113, 128)
Transferrin receptor 1	*Tfrc* knockout mouse	Embryonic death with anemia and apoptosis of neuroepithelium	(69)
β2-microglobulin	*B2m* knockout mouse	Increased body iron, splenic iron sparing, decreased hepcidin	(26, 83, 110)
H-ferritin	*Fth* knockout mouse	Embryonic lethal in homozygotes, heterozygotes have normal brain iron levels with oxidative stress	(37, 123)
Irp1	*Irp1* knockout mouse	No overt abnormalities	(45, 79)
Irp2	*Irp2* knockout mouse	Microcytic anemia, increased duodenal and liver iron	(22, 46)
Ceruloplasmin	*Cp* knockout mouse	Hepatic and regional CNS iron overload, mild anemia	(56, 61, 98)
Ceruloplasmin/hephaestin	*Cp* and *Heph* double deficient mouse	Retinal iron accumulation and degeneration	(54, 55)
Haptoglobin	*Hp* knockout mouse	Increased duodenal ferroportin and iron transport, increased splenic and renal iron	(76)
Haptoglobin/Hfe	*Hp* and *Hfe* double knockout mouse	Milder phenotype than $Hfe^{-/-}$ mouse	(125)
Hemopexin	*Hx* knockout mouse	Increased regional CNS iron, increased renal injury after intravascular hemolysis	(81, 126, 130)
Duodenal cytochrome B	*Cybrd1* knockout mouse	Little impact on body iron stores	(53)
Heme oxygenase 1	*Hmox1* knockout mouse	Anemia, low serum iron, increased hepatic and renal iron	(105)
Flvcr (feline leukemia virus, subgroup C, receptor)	*Flvcr* knockout mouse	Macrocytic anemia with proerythroblast maturation arrest	(65)

iron-related proteins. These mice provide insight into the in vivo functions of these proteins.

Hypotransferrinemic ($Tf^{hpx/hpx}$) Mouse

Congenital hypotransferrinemia occurs rarely in humans, and a model of this condition is the hypotransferrinemic mouse. The *hpx* mutation in the transferrin gene (*Tf*) occurred spontaneously in an inbred mouse colony (13). The mice are born alive but die from severe anemia before weaning if they are not treated with exogenous transferrin or red blood cell transfusions (13). The *hpx* mutation is a point mutation that results in an error in mRNA splicing (128). Therefore, no normal *Tf* mRNA is made from the *hpx* allele, but a small amount of mRNA containing a 27-bp deletion is produced from the use of cryptic splice sites (128). Consequently, homozygous $Tf^{hpx/hpx}$ mice have less than 1% of normal levels of a shortened transferrin molecule containing a 9 amino acid deletion near the carboxy terminus (128). Despite their severe transferrin deficiency, $Tf^{hpx/hpx}$ mice initially given transferrin injections can survive after weaning without any further treatment. They develop massive iron overload in the liver, kidney, heart, and exocrine pancreas, whereas the spleen is spared (128). Hepcidin mRNA expression remains low (113), suggesting that the induction of hepcidin by iron is trumped by an inhibitory signal linked to erythropoietic drive. The hepatic iron concentration of $Tf^{hpx/hpx}$ mice is approximately 100-fold greater than that of wild-type mice and at least 15-fold higher than that

β2M: β2-microglobulin

of *Hfe* knockout mice (128). However, there is no histologically detectable fibrosis in the liver or pancreas in the $Tf^{hpx/hpx}$ mouse (128), suggesting that mice may be resistant to the profibrogenic effects of iron overload.

Transferrin Receptor (*Tfrc*) Knockout Mouse

Indicative of the key role of the transferrin receptor in early development, Levy and colleagues (69) demonstrated that *Tfrc* knockout mice undergo embryonic death with anemia and apoptosis of primitive neuroepithelium. It appears that inadequate iron uptake leads to neuronal apoptosis but that other tissues can obtain sufficient iron for development through mechanisms independent of the transferrin cycle. Haploinsufficiency for *Tfrc* results in microcytic, hypochromic erythrocytes, along with normal hemoglobin and hematocrit values (due to a compensatory increase in the number of red cells) (69). Although transferrin saturation is normal, *Tfrc* heterozygotes have lower levels of tissue iron (69).

β2-Microglobulin (*B2m*) Knockout Mice

β2-Microglobulin (β2M) forms a heterodimer with MHC class I molecules and with many atypical MHC class I-like proteins, including HFE. β2M is involved in the appropriate intracellular trafficking of its partner proteins, and targeted deletion of *B2m* in mice causes immune deficits and iron overload similar to HH type 1 (26, 110). Like *Hfe* knockout mice, *B2m* knockout mice have decreased hepcidin expression in the liver (83). The *HFE* C282Y mutation disrupts the binding of HFE to β2M, resulting in impaired intracellular transit, accelerated degradation, and failure of the C282Y protein to be presented normally at the cell surface (134). Therefore, the abrogation of the interaction with β2M provides a basis for the impaired function of the HFE C282Y protein in HH type 1.

Ceruloplasmin (*Cp*) Knockout Mice

Hereditary aceruloplasminemia is a rare autosomal recessive disorder characterized by iron overload, anemia, progressive neurodegeneration, diabetes, and retinal degeneration (56, 57, 60). This condition is caused by mutations in the ceruloplasmin (*CP*) gene, resulting in the absence of ceruloplasmin, a multicopper ferroxidase. *Cp* knockout mice have a progressive increase in iron levels within Kupffer cells and splenic macrophages, as well as in hepatocytes (56). Ferrokinetic studies in *Cp* knockout mice show no abnormalities in cellular iron uptake but a striking impairment in the egress of iron from macrophages and hepatocytes (56). These results indicate that ceruloplasmin plays an important role in determining the rate of iron efflux from cells with mobilizable iron stores. It is now appreciated that ceruloplasmin stabilizes ferroportin expression at the cell surface (25) and that glycosylphosphatidylinositol-linked ceruloplasmin is the predominant form expressed in brain (121).

Unlike patients with aceruloplasminemia, the original line of *Cp* knockout mice does not manifest significant brain iron overload, retinal degeneration, or neuropathy even at 24 months of age (55). Another line of *Cp* knockout mice shows increased iron deposition in several brain regions, including the cerebellum and brainstem; increased lipid peroxidation is also seen in some regions (98). Of interest, these mice have deficits in motor coordination that are thought to be associated with a loss of brainstem dopaminergic neurons (98). In the cerebellum of these mice, iron accumulation occurs mainly in astrocytes and is accompanied by a significant loss of these cells (61). In contrast, Purkinje neurons in *Cp* knockout mice do not accumulate iron but express high levels of Dmt1, suggesting that these cells may be iron deprived; there is also a significant reduction in the number of Purkinje neurons (61). It has been proposed that neuronal iron starvation with associated astrocyte and microglial iron overload may contribute to the neurodegeneration seen in aceruloplasminemia (121).

In order to examine the effect of combined deficiency of ceruloplasmin and hephaestin on the retina, the *Cp* knockout mouse was crossed with the *sla* mouse. The resulting compound mutant mouse has retinal iron accumulation with secondary increases in ferritin and, ultimately, retinal degeneration (55). Body iron status has not yet been reported for this mouse strain, but longevity is decreased (55, 54).

H-Ferritin (*Fth*) Knockout Mouse

Deletion of the H-ferritin gene (*Fth*) in mice results in early embryonic lethality (37, 123). Haploinsufficiency for *Fth* does not change brain iron levels, but the levels of H-ferritin are decreased by more than 50% (123). Interestingly, the brain expression of transferrin, transferrin receptor, L-ferritin, Dmt1, and ceruloplasmin are all increased in $Fth^{+/-}$ mice, suggestive of an iron-deficient state. There is also evidence of increased oxidative stress in the brain of these mice (123).

Haptoglobin (*Hp*) Knockout Mouse

Haptoglobin is the plasma protein with the highest binding affinity for hemoglobin. It delivers any hemoglobin in the plasma to the reticuloendothelial system, thus reducing loss of hemoglobin through the glomeruli and allowing heme-iron recycling (76). Analysis of *Hp* knockout mice reveals that they export significantly more iron from the duodenal mucosa to plasma. Increased iron export from the duodenum correlates with increased duodenal expression of ferroportin, at both the protein and mRNA levels, whereas hepatic hepcidin expression remains unchanged (76). Splenic and renal iron concentrations are increased. Marro and coworkers (76) suggest that haptoglobin, by controlling plasma levels of hemoglobin, participates in the regulation of ferroportin expression, thus influencing iron transfer from duodenal mucosa to plasma.

Interestingly, haptoglobin deficiency also influences the phenotype of *Hfe* knockout mice. *Hfe* and *Hp* compound-mutant mice accumulate significantly less hepatic iron than do *Hfe* knockout mice, suggesting that haptoglobin-mediated heme-iron recovery might contribute to iron loading in *HFE*-associated HH (125).

IRP: iron regulatory protein

Hemopexin (*Hx*) Knockout Mouse

Hemopexin is an acute-phase plasma glycoprotein produced mainly in the liver and released into plasma where it binds heme with high affinity and delivers it to the liver (124). The *Hx* knockout mouse was developed to evaluate the in vivo effect of hemopexin deficiency, and an interesting phenotype was observed in the brain (124). These mice have a two-fold increase in the number of iron-loaded oligodendrocytes in the basal ganglia and thalamus, but there is no increase in H- or L-ferritin expression in these regions (81). However, there is a substantial decrease in the number of ferritin-positive cells in the cerebral cortex of the knockout mouse (81). These results suggest that hemopexin may play a role in controlling iron distribution within brain. As anticipated from hemopexin's role in heme scavenging, the *Hx* knockout mouse has significantly more renal damage after phenylhydrazine-induced hemolysis (126), and increased endothelial activation and vascular permeability after heme overload (130).

Irp1 or *Irp2* Knockout Mice

The two iron regulatory proteins (IRPs), IRP1 and IRP2, bind to the mRNAs of ferritin, transferrin receptor, and other target genes to control the expression of these proteins at the posttranscriptional level (44, 60, 82, 111). In their native conformation, both IRPs have a high binding affinity for stem-loop structures (called IREs) present in the mRNAs of their target genes. IRP1 is an ISC protein that loses its RNA binding activity in iron-replete conditions, whereas IRP2 is degraded by the proteasome.

Genetic ablation of *Irp1* and *2* in mice has been informative about the in vivo functions of these proteins. Complete loss of both Irp1 and

FLVCR: feline leukemia virus (subgroup C) receptor

2 prevents viability of murine zygotes beyond the blastocyst stage of embryonic development (119). *Irp1* knockout mice develop no overt abnormalities (45, 79). *Irp2* knockout mice develop microcytic anemia (22, 46) and altered body iron distribution with duodenal and hepatic iron loading (46). In addition, the Ire/Irp system is essential to maintain the structural and functional integrity of the intestine (44). One line of *Irp2* knockout mice develops adult-onset neurodegeneration (66), whereas another line does not (47). Irp2 is sensitive to iron status and can compensate for the loss of Irp1 by increasing its binding activity (79). Therefore, accumulating evidence indicates that Irp2 may be the chief physiologic iron sensor in vivo (111).

Other Mice Deficient in Iron-Related Proteins

Duodenal cytochrome b (Dcytb, Cybrd1) is an iron-regulated ferric reductase that is highly expressed in duodenal enterocytes (77). Although knockout of *Dcytb* in mice has little effect on their body iron status (53), the potential role of Dcytb in iron absorption is still being evaluated (77).

Heme oxygenase 1 (Hmox1) has been targeted for gene deletion in mice to provide insight into a case of human HMOX1 deficiency that was characterized by growth retardation, hemolytic anemia, liver and kidney iron accumulation, and kidney injury (140). The *Hmox1* knockout mouse has anemia, low serum iron, and increased hepatic and renal iron (105). These results are consistent with an important role for Hmox1 in the reutilization of heme iron.

As its name connotes, the feline leukemia virus (subgroup C) receptor (FLVCR) was cloned as a viral receptor and was subsequently found to be a heme exporter (60, 108). *Flvcr* knockout mice develop macrocytic anemia with proerythroblast maturation arrest, which suggests that erythroid precursors must export excess heme to ensure survival (65).

MICE WITH FLOXED IRON-RELATED GENES

It is of great interest to be able to selectively delete iron-related genes in various cell types to assess their function and impact. This can be accomplished using the Cre-loxP system, and this approach has already been applied to several iron-related genes (**Table 6**). The Cre-loxP system utilizes the ability of Cre recombinase to catalyze recombination between two loxP sites in DNA (67, 84). To accomplish cell-selective gene deletion, transgenic mice containing an iron-related gene flanked by loxP sites are crossed with transgenic mice containing a *Cre* gene construct with a cell-selective promoter. The resulting mice contain both the *Cre* gene construct and the loxP-flanked iron-related gene (floxed gene). In cells where Cre recombinase is expressed, the floxed iron-related gene will be deleted. There are now a substantial number of available mouse strains that already contain the *Cre* gene driven by either ubiquitous or cell-selective promoters. When using the Cre-loxP system, it is important to evaluate the specificity and efficiency of gene deletion (67).

Using the Cre-loxP approach, studies have found that deletion of either *Dmt1* or *Fpn* in villus enterocytes results in iron-deficiency anemia, highlighting the importance of these transporters in dietary iron absorption (27, 51). The developing hippocampus may be particularly susceptible to iron deficiency, and selective knockout of *Dmt1* in hippocampal neurons provides the first conditionally targeted model of iron uptake in the brain (20). Deletion of *Dmt1* in these mice disrupts hippocampal neuronal development and spatial memory behavior. Hepatocyte-selective deletion of *Hfe* (131) or *Tfr2* (137) results in an HH phenotype, suggesting that Hfe and Tfr2 act in hepatocytes to regulate hepcidin expression. Deletion of the gene for H-ferritin (*Fth*) in the intestine results in increased ferroportin expression, enhanced iron absorption, and iron overload (129). Cell-selective deletion of *Irp2* (with or without *Irp1*) has been used to investigate its action in the

Table 6 Mice with floxed iron-related genes

Gene	Observations	References
Dmt1 (Slc11a2)	Selective knockout in intestine produces iron-deficiency anemia	(51)
	Selective knockout in hippocampus disrupts hippocampal neuronal development and spatial memory behavior	(20)
Fpn (*Slc40a1*)	Selective knockout in intestine produces iron-deficiency anemia	(27)
Hfe	Selective knockout in hepatocytes (but not in villus enterocytes) produces HH phenotype	(131, 132)
Tfr2	Selective knockout in hepatocytes produces HH phenotype	(137)
Fth	Selective knockout in intestine results in increased iron absorption and iron overload	(129)
Irp2	Selective knockout in liver or intestine causes tissue-specific iron loading	(38, 45)
Irp1 and 2	Selective knockout of both *Irp1* and *Irp2* in intestine causes malabsorption and death	(44)
Abcb7	Selective knockout in hepatocytes impairs cytosolic iron-sulfur cluster assembly	(103)
Fxn	Selective knockout in neurons/cardiac muscle, liver, or striated muscle is useful to model different aspects of Friedreich ataxia	(122)

intestine and liver (38, 44). Some mouse models of Friedreich ataxia have used cell-selective deletion of the frataxin gene (107). In the future, additional mouse lines with floxed iron-related genes will be generated, thus allowing investigation of the in vivo consequences of cell-selective deletion of these genes.

SUMMARY AND CONCLUSIONS

Murine models continue to make valuable contributions to our understanding of iron metabolism. Investigation of mice with inherited forms of anemia has led to the discovery of novel proteins involved in iron homeostasis (e.g., hephaestin, Steap3). Murine models are now available for the major forms of HH, and findings in these mice support the concept that the pathogenesis of HH types 1, 2, and 3 involves inappropriately low expression of hepcidin. Altered hepcidin expression in mice deficient in Bmp6, Tmprss6, or Smad4 has focused attention on the role that these molecules play in iron signaling to hepcidin. A growing number of murine models are being developed to investigate mitochondrial iron metabolism. In the future, the production of mice with floxed iron-related genes will accelerate, thus allowing the study of the in vivo consequences of cell-selective deletion of these genes.

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Errata

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