

# Haemochromatosis: novel gene discovery and the molecular pathophysiology of iron metabolism

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The application of molecular genetics to haemochromatosis and experimental mutagenesis in animals has transformed our capacity to investigate the unique physiology of iron homeostasis—a key problem in biology and medicine. The identification of *HFE*, the principal determinant of adult haemochromatosis (HFE1; OMIM 235200) and *TfR2*, recently implicated in a rarer form of the inherited disorder (HFE3; OMIM 604250), and the promise of candidate genes for juvenile haemochromatosis (HFE2; OMIM 602390) and neonatal haemochromatosis (OMIM 231100) provide the foundation for important studies into the control mechanism of iron balance in humans. The rare conditions atransferrinaemia (OMIM 209300) and acaeruloplasminaemia (OMIM 604290), each associated with tissue iron overload, have already implicated the iron transport ligand transferrin and the copper transporter caeruloplasmin in the control of iron homeostasis. Gene mapping studies in animal mutants with anaemia due to defects in the uptake or tissue transfer of iron have yielded novel proteins involved in iron transport: DMT1 (brush border transporter of ferrous iron) in the *mk/mk* mouse, hephaestin (basolateral multi-copper ferroxidase) in the sex-linked anaemic mouse (*sla*) and ferroportin1 (basolateral iron exporter) in zebrafish *weh* mutants. The discovery of genes that determine heritable defects of iron absorption and regulation in animals and humans thus holds promise for a complete mechanistic understanding of the molecular pathophysiology of iron metabolism.

## INTRODUCTION

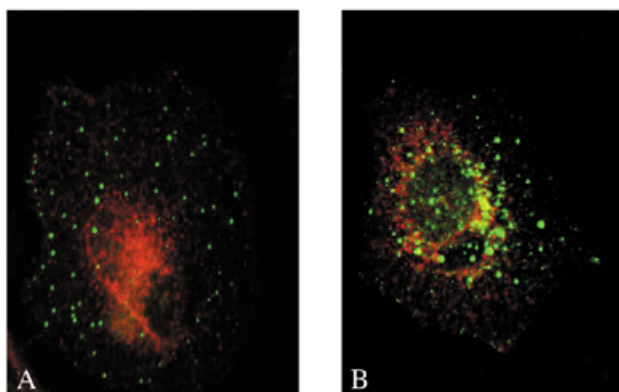
Iron is fastidiously conserved by living cells since it is required for aerobic respiration; however, in excess the metal is toxic. In mammals no effective excretory pathway exists and iron homeostasis is uniquely regulated by the intestinal mucosa, which controls the uptake and transfer of iron from the diet to meet the needs of growth and to recover obligatory losses. Human iron-storage disease (haemochromatosis) is an incompletely understood and progressive multi-system disorder that is reversible in its early phase. Within a few years, genetic studies in iron-overloaded patients and the characterization of novel phenotypes in experimental animals have revealed numerous components of previously unknown transport and metabolic pathways. The predisposing allele C282Y of the *HFE* gene, implicated in adult haemochromatosis (HFE1), exists at polymorphic frequency (~0.05) in most populations of European origin; its identification provides the basis for mass genetic screening of the population for a preventable disease. Striking progress has been made in genetic research, but uncertainties remain about key mechanistic aspects of the gene–environment interactions that mediate expression of haemochromatosis. Clarification of the natural course and penetrance of mutant forms of the *HFE* gene is required before the discoveries can be translated into improved outcomes in public health.

## GENETIC DETERMINANTS OF HUMAN IRON-STORAGE DISEASE

A major impetus has been provided by the isolation of *HFE* as the candidate gene for the most common genetic disorder of Caucasians, hereditary haemochromatosis (1). The disease is characterized by a slow accumulation of toxic iron from the diet with tissue injury and organ malfunction (2). Patients, typically men, develop symptoms in their fourth or fifth decade with a variable combination of liver cirrhosis and hepatoma, arthritis, hypogonadism, diabetes mellitus and cardiomyopathy. Removal of iron by repeated phlebotomy at early diagnosis may prevent disease progression and is associated with near-normal survival (3). Ninety per cent of northern Europeans with hereditary haemochromatosis are homozygous for a single missense mutation, C282Y, in exon 4 of the *HFE* gene on chromosome 6p21.3 (4). This discovery has facilitated DNA-based diagnosis and permitted genetic screening for affected families. A key issue to be resolved before the introduction of mass population screening for this disorder will be the penetrance and expressivity of the predisposing allele(s) (5,6).

Although intestinal uptake and transport of iron have long been known to determine body iron status, the pathophysiology of haemochromatosis is unknown. Clearly, the *HFE* gene product is implicated in the control of dietary iron capture by the intestine and its systemic distribution but, thus far, its molecular action has not been fully established. Nonetheless, the structural

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**Figure 1.** Localization of HFE (green) and TfR (red) was determined by double immunofluorescence and confocal microscopy in undifferentiated CaCo<sub>2</sub> cells. Cells in (A) were cultured in the absence of human transferrin but cells in (B) were grown in the presence of transferrin (5% human serum) for 24 h before fixation. All cells were double-stained using rabbit anti-HFE and mouse anti-TfR antibodies followed by FITC-conjugated anti-rabbit antibody (1:100; Jackson Laboratories, Bar Harbor, ME) with TRITC-conjugated anti-mouse antibody (Sigma, Poole, UK). Yellow staining in (B) confirms vesicular co-localization of HFE with TfR in the presence of iron-saturated transferrin.

properties and cellular localization of the HFE protein provide insights as to its possible function. HFE is a MHC class I-like molecule, which interacts with  $\beta_2$ -microglobulin for co-expression at the cell surface; the C282Y mutation destroys a critical disulphide bridge for the interaction with  $\beta_2$ -microglobulin and the mutant protein is retained within the Golgi complex (7).  $\beta_2$ -microglobulin-deficient mice are known to have iron overload in a pattern akin to haemochromatosis (8). Of possible functional importance, the HFE protein binds *in vitro* to homodimeric transferrin receptors and lowers the affinity for iron-saturated transferrin (9). This interaction occurs at the cell surface and along the endocytic pathway for transferrin iron uptake (10), where HFE and transferrin receptors co-localize (Fig. 1). Although a role for HFE in the modulation of receptor-mediated transferrin iron uptake has therefore been proposed, such a mechanism does not readily explain the effect of mutant HFE on intestinal transport of iron, as discussed below.

Lately, the genetic basis of a rare form of adult haemochromatosis (HFE3), in which *HFE*-linked mutations are not present, has been determined. Homozygosity mapping and pairwise linkage analysis in two Sicilian families narrowed the locus to an interval on chromosome 7q in which a second transferrin receptor (TfR2) has recently been mapped (11). A single nonsense mutation, Y250ter, accounted for all mutant alleles; this mutation was absent from 100 normal chromosomes (12). TfR2 shares 66% sequence identity with the transferrin receptor (TfR) and is expressed as two transcripts,  $\alpha$  and  $\beta$ : the former is a transmembrane protein expressed predominantly in the liver and the latter is a truncated intracellular protein of undetermined significance. TfR2 $\alpha$  has functional similarity to TfR: it has been shown to bind transferrin and stimulate uptake of transferrin-bound iron and is thus a true homologue of the ubiquitous receptor. Unlike adult patients with haemochromatosis in Northern Europe, most African-American

patients with iron-storage disease and one-third of Italian patients with haemochromatosis, do not harbour mutations in *HFE*. Clearly, a possible role for mutations at the *TfR2* locus merits further study in these and other variant forms of haemochromatosis.

Juvenile haemochromatosis (HFE2) is a severe iron-storage disease which affects both sexes before the age of 30 years; the early development of cardiomyopathy and hypogonadism also distinguishes it from the adult condition. The locus for this disorder has recently been mapped to chromosome 1q (13) but the gene has yet to be isolated.

The rare neonatal variant of haemochromatosis (NH), in which iron loading occurs *in utero*, is characterized by liver failure at or shortly after birth. There have been occasional survivors of the hepatic insult, some who have required orthotopic liver transplantation. Although NH may be a heterogeneous disorder, distinct subgroups with clear patterns of transmission, suggesting strong genetic predisposition, have been identified (A.L. Kelly *et al.*, submitted).

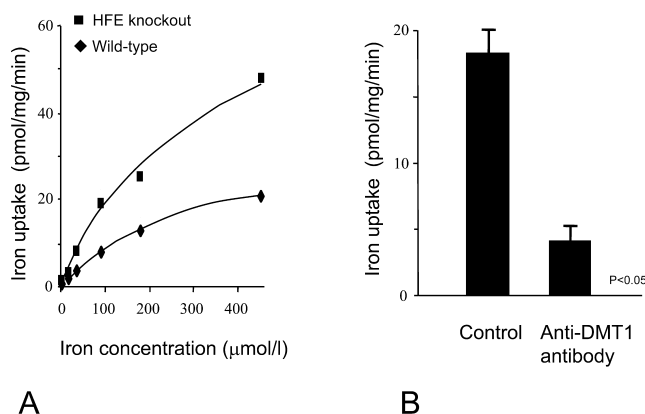
## INHERITED DISORDERS OF INTESTINAL IRON TRANSPORT IN ANIMALS

The study of human iron overload disorders has so far led to the discovery of novel proteins implicated in iron homeostasis. The recent identification of proteins involved directly in transport of iron across membranes has resulted principally from gene mapping studies in animals with inherited forms of iron deficiency, occurring either naturally or as a result of experimental mutagenesis (Table 1). The *mk/mk* mouse has defective intestinal iron absorption and incorporation of iron into developing red cells with an anaemia resembling that of severe iron deficiency. A single missense mutation (G185R) in the *Nramp2* gene, a member of the natural resistance-associated macrophage protein family, was identified by positional cloning (14). Contemporaneous functional cloning studies that examined iron uptake by oocytes, expressing mRNA from iron-deficient rat duodenum, led to the isolation of a single clone, *DCT1*, which increased uptake 200-fold (15). Renamed *DMT1* (divalent metal transporter 1), this gene was identical to *Nramp2*, thus accounting for the iron transport defect in the *mk/mk* mouse. Furthermore, the same mutation in *Nramp2* was responsible for the iron-deficient phenotype of the Belgrade rat, previously known to have impaired export of iron from endosomes participating in the transferrin cycle within erythroblasts (16).

DMT1 transports ferrous ions and other divalent metals; it is abundant in the small intestine where it is localized to the enterocyte brush border. Upregulation of *DMT1* occurs in iron deficiency; the mRNA contains a 3' iron response element (IRE) which, by the action of iron regulatory proteins (IRPs) when iron concentrations are low, enhances stability for translation. Duodenal *DMT1* mRNA is also increased in hereditary haemochromatosis (17) as well as in mice with targeted disruption of the *HFE* gene (18). We have shown by kinetic studies of ferrous iron uptake by mouse duodenum that there is a 2- to 3-fold increase in the maximum rates of absorption in *HFE*-knockout mice compared with wild-type controls (19). The inhibitory effect of DMT1 antibody blockade on iron uptake confirms DMT1 as the principal iron carrier in this process (Fig. 2). Ferric iron, at physiological concentrations, is largely reduced

**Table 1.** Genetic basis of disorders of iron transport and storage

Disorder	OMIM/strain	Locus	Gene/protein	Defect(s)
<b>Human</b>				
Atransferrinaemia	209300	3q21	Transferrin	Unknown
Acaeruloplasminaemia	604290	3q23–q25	Caeruloplasmin	W858ter; del2389G, etc.
Haemochromatosis (HFE 1) (adult)	235200	6p 21.3	HFE	C282Y; H63D; S65C
Haemochromatosis (HFE 2) (juvenile)	602390	1q	Unknown	–
Haemochromatosis (HFE 3) (adult)	604250	7q22	Transferrin receptor 2	Y250ter
Haemochromatosis (neonatal)	231100	Unknown	Unknown	–
<b>Murine</b>				
Hypotransferrinaemia	<i>hpx</i>	9	Transferrin	Splicing defect
$\beta_2$ -microglobulin-deficient (KO)	$\beta_2m^{-/-}$	2 (human 15q21–q22)	$\beta_2$ -microglobulin	<i>neo</i> gene disruption
Microcytic anaemia	<i>mk</i>	15 (human 12q13)	DMT-1 (Nramp 2)	G185R
Sex-linked anaemia	<i>sla</i>	X (human Xq11–q12)	Hephaestin	Del582bp
<b>Zebrafish</b>				
Weissherbst	<i>weh</i>	Linkage group 9 (human 2)	Ferroportin1	C361ter; L167F



**Figure 2.** Intestinal uptake of ferrous iron ( $^{59}\text{Fe}$ ) was compared between age and sex-matched pairs of *HFE*-knockout and control animals. For each animal the mean ( $\pm$  SE) uptake of four intestinal slices at each of six concentrations of iron was calculated. (A) A typical experiment in which uptake was increased at all concentrations in the knockout animal. The apparent  $V_{\text{max}}$  for ferrous iron uptake was significantly higher in the six knockout animals (data not shown) indicating an increase in the number of functional ferrous iron carriers. (B) Five duodenal slices from a single wild-type mouse were pre-incubated with avian antibodies raised against murine DMT1 peptide, with control samples exposed to pre-immune IgG. Mean ( $\pm$  SE) uptake of 18  $\mu\text{mol/l}$  ferrous iron was calculated; there was a significant inhibitory effect with antibody to DMT1, showing that DMT1 is the principal mediator of mucosal iron uptake in this system.

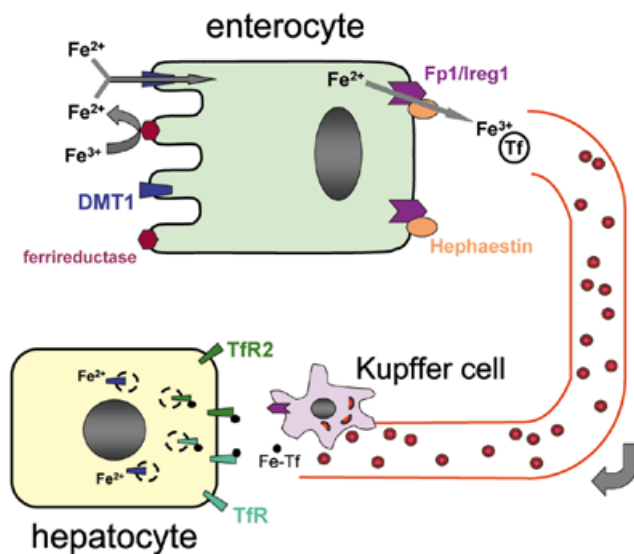
by brush border ferrireductases before uptake by DMT1 (20; W. Griffiths, W. Sly and T. Cox, unpublished data).

The experimental zebrafish mutant, *weissherbst*, has hypochromic anaemia caused by a defect in iron transfer from the yolk sac to the embryonic circulation. A positional cloning strategy identified a gene, *ferroportin1*, which is highly conserved across animal species, including humans (21). The gene encodes a multiple transmembrane domain protein which

functions as an iron exporter when expressed in *Xenopus* oocytes. Ferroportin1 is expressed on the basal surface of placental syncytiotrophoblasts in humans suggesting a similar role for transfer of iron from mother to embryo. Furthermore, expression in the small intestine is limited to the duodenum, the site of maximal iron absorption, and is immunolocalized to the basolateral surface of enterocytes where it is likely to export iron into the circulation. The same gene was simultaneously discovered by subtractive cloning of cDNA from homozygous hypotransferrinaemic (*hpx/hpx*) mice, which have high rates of iron absorption. As well as localizing to the basolateral enterocyte membrane, the alternately termed *Ireg1* was upregulated in iron-deficient mice and also in three humans with hereditary haemochromatosis (22). *Ireg1/ferroportin1* mRNA contains an IRE in the 5'-untranslated region which may allow post-transcriptional control of expression according to intracellular iron status.

Defective transfer of iron from the basolateral surface of enterocytes into the circulation had been described previously to account for iron deficiency in the sex-linked anaemic (*sla*) mouse (23). The gene identified recently does not, however, encode an iron transport protein *per se*, but instead encodes a caeruloplasmin-like multi-copper oxidase protein, hephaestin (24). In the rare human disorder acaeruloplasminaemia mutations in the caeruloplasmin gene are responsible for deficiency of the copper transport protein and associated tissue iron overload (25). Hephaestin is expressed on the basolateral surface of enterocytes where ferroxidation of iron is presumably required as part of the export process for transfer to circulating apotransferrin, which binds ferric iron. Coupling of ferroxidation and transport of iron is seen in the yeast, *Saccharomyces cerevisiae*, where the multi-copper oxidase protein Fet3 forms a transmembrane complex with the high-affinity iron uptake protein FTR1; both proteins are required for iron uptake (26).

Thus, distinct molecular genetic approaches have identified key mediators of uptake and transfer of inorganic iron by enterocytes (Fig. 3). It appears that expression of these



**Figure 3.** Molecular components of iron transport from intestine to plasma and sites of storage (liver). In the duodenum, luminal iron is captured by the transporter, DMT1, after reduction at the brush border of mature enterocytes by ferrireductases. Efflux of iron across the basolateral border is mediated by ferroportin1 (Fp1), also termed Ireg1, in conjunction with the ferroxidase, hephaestin. The expression of DMT1 and possibly ferroportin1 is increased in haemochromatosis caused by mutations in *HFE*; the nature of this regulation is unknown but may involve interactions with either TfR or the newly described TfR2 within the crypt cell where HFE is localized (data not shown). Ferric iron binds to apotransferrin and is transported in the plasma to systemic tissues including the liver where it is internalized by transferrin receptor-mediated endocytosis at cell membranes. TfR2 contributes significantly to hepatocyte iron entry particularly under iron loading conditions. Iron export from late endosomes for storage in the intracellular iron pool is mediated by DMT1. In the liver, HFE is expressed predominantly in Kupffer macrophages which release iron from the breakdown of senescent erythrocytes; this release of iron appears to be accelerated in hereditary haemochromatosis, suggesting a secondary role for HFE in the regulation of systemic iron delivery.

molecules is subject to regulation by the intracellular iron status of the cell by the IRE/IRP system which influences steady-state mRNA levels, such that each enterocyte, in its short life cycle, may have an individually tailored level of iron absorption. Upregulation of DMT1, ferroportin1 and hephaestin would account for the increased overall flux of iron through the intestine that occurs in hereditary haemochromatosis. Indeed evidence that the small intestine is the principal site of expression of the genetic defect in this disorder has been provided by the report that transplantation of intestine and liver from an *HFE* C282Y homozygote caused early iron loading in the recipient whereas transplantation of liver tissue alone from a C282Y homozygote does not (27).

### MOLECULAR CONTROL OF IRON TRANSPORT PATHWAYS

Hereditary haemochromatosis is also characterized by the peripheral deposition of excess iron in specific tissues, especially the liver. Although, within the liver, iron is sequestered predominantly in hepatocytes, HFE and also ferroportin1 are expressed mainly in the Kupffer cells (macrophages that

recycle iron from senescent erythrocytes); *in vitro* studies of monocytes from patients homozygous for the C282Y mutation show abnormal and more rapid release of iron (28). Conversely, the newly identified TfR2 is highly expressed in hepatocytes and unlike the homologous transferrin receptor lacks an iron-response element. Whereas hepatic TfR expression is reciprocally affected by body iron status, expression of TfR2 is unaltered in iron deficiency or in iron loading by diet or *HFE* gene disruption, implying that TfR2 is indeed insensitive to the level of iron (29). Furthermore, in normal or iron-loaded conditions expression of TfR2 exceeds that of the transferrin receptor, suggesting that TfR2 plays a significant role in hepatic iron loading and may confer a protective effect towards other parenchymal tissues from the damaging effects of iron toxicity. Also its role and distribution in endocrine and other tissues that show a predilection for iron storage and injury may well be informative. DMT1 is overexpressed in the hepatocytes of iron-loaded rats (30) where it may handle the delivery of excess iron to the intracellular iron pool (Fig. 3). HFE and DMT1 are reciprocally regulated by intracellular iron stores in CaCo2 cells (31); an interaction between the two is likely to be indirect, however, as HFE localizes to early endosomes in these cells distinct from DMT1, which occupies a late endosomal compartment where it presumably functions to release iron into the cell (32).

As excretion of iron from the body cannot be increased effectively to compensate for iron excess, iron balance in the adult is maintained by homeostatic feedback mechanisms operating within the main site of dietary iron absorption: the proximal small intestine. When these mechanisms break down, as in hereditary haemochromatosis, persistent iron loading occurs. HFE and TfR2 must be key regulators of iron homeostasis as homozygosity for point mutations in either gene encoding these proteins results in iron accumulation. A common role for the plasma iron transport ligand transferrin, common to TfR and TfR2, is also now established, since humans and mice with inherited loss of transferrin develop greatly increased tissue iron stores and their intestinal mucosa continues avidly to take up and transfer iron inappropriately from the lumen in the face of overall body iron excess (33,34). Within the small intestine HFE has been localized to the crypt cells but not to the mature absorptive enterocytes that derive from them (35); a perinuclear and basolateral pattern has been observed as well as co-localization with transferrin receptors (36). Interactions between HFE and TfR, or more likely TfR2, might alter intracellular iron concentrations and subsequent expression of the transport machinery of the mature enterocyte by the IRE/IRP system. It is noteworthy that in hereditary haemochromatosis the enterocytes stain poorly for ferritin and are paradoxically iron deficient (37). Since in the absence of functional HFE, a signal representative of low iron status is received, with consequent upregulation of transporters in the enterocytes, we would predict that wild-type HFE serves normally to stimulate, rather than decrease, iron uptake from transferrin. Clearly, the interaction between HFE and the newly discovered TfR2, now directly implicated in a haemochromatosis variant (12), needs further exploration, as this may hold the key to the functional programming of enterocytes in relation to body iron status.

## HEALTH IMPLICATIONS

At first glance, hereditary haemochromatosis is ideally suited for DNA-based genetic screening: a single gene disorder with a single mutation of diagnostic significance in target populations which is easily tested for, as well as a long incubation period without symptoms and treatment for pre-symptomatic disease which is deceptively facile and effective. However, the familiar problems of stigmatization, increased anxiety, life-insurance weighting and counselling—all points well-rehearsed in debate elsewhere—combined with the age-related penetrance and hitherto unknown gene–environment influences, are significant obstacles to the introduction of mass population screening. Clearly, homozygosity for the C282Y allele is not tantamount to a diagnosis of hereditary haemochromatosis. A difficulty also occurs evaluating the population burden of haemochromatosis because, although there are definitions of iron storage that reflect the biochemical phenotype, given the protean manifestations of the clinical disease, no internationally agreed case definition exists. This creates an additional problem for the institution of public health measures and policy review.

## FUTURE DIRECTIONS

Although startling progress has been made in identifying key components of the molecular pathways of iron transport, important questions still remain which merit further study. The eagerly sought after genes for the severe juvenile, neonatal and other variant forms of haemochromatosis may unleash novel regulators of iron metabolism. The effects of *TfR2* gene disruption in mice and the characterization of HFE–TfR2 interactions and downstream effects will be critical in unravelling the control mechanisms of intestinal iron absorption. From the medical viewpoint, a clear identification of the modifier genes and environmental co-factors that determine disease expression of iron storage will be a challenging requirement.

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