J. Membrane Biol. 206, 3–7 (2005)
DOI: 10.1007/s00232-005-0768-1

The Journal of
Membrane
Biology

Impaired Iron Transport Activity of Ferroportin 1 in Hereditary Iron Overload

J.A. McGregor[1], M. Shayeghi[1], C.D. Vulpe[2], G.J. Anderson[3], A. Pietrangelo[4], R.J. Simpson[1], A.T. Mckie[1]
[1]Division of Nutritional Sciences, King's College London, London, UK
[2]Department of Nutritional Sciences & Toxicology, University of California, Berkeley, USA
[3]Iron Metabolism Laboratory, Queensland Institute of Medical Research, Brisbane, Australia
[4]Department of Internal Medicine, University of Modena, Modena, Italy

Received: 14 September 2005

Abstract. To investigate the functional significance of mutations in Ferroportin that cause hereditary iron overload, we directly measured the iron efflux activity of the proteins expressed in *Xenopus* oocytes. We found that wild type and mutant Ferroportin molecules (A77D, N144H, Q248H and V162Δ) were all expressed at the plasma membrane at similar levels. All mutations caused significant reductions in ^{59}Fe efflux compared to wild type but all retained some residual transport activity. A77D had the strongest effect on ^{59}Fe efflux (remaining activity 9% of wild-type control), whereas the N144H mutation retained the highest efflux activity (42% of control). The Q248H and V162Δ mutations were intermediate between these values. Co-injection of mutant and wild-type mRNAs revealed that the A77D and N144H mutations had a dominant negative effect on the function of the WT protein.

Key words: Ferroportin — Ireg1 — Iron — Efflux — Oocyte — *Xenopus* — Hemochromatosis

Introduction

Ferroportin-associated hereditary iron overload (also referred to as Type 4 hemochromatosis or Ferroportin disease), is an autosomal dominant disease of aberrant tissue iron distribution clinically distinct from classical (*HFE*-associated) hemochromatosis (Pietrangelo, 2004a). Patients present with early elevated serum ferritin levels and a characteristic accumulation of iron in the reticuloendothelial cells, particularly Kupffer cells and splenic macrophages (for a review on Ferroportin disease *see* Pietrangelo, 2004b). A paradoxical feature of this disease is that, although the macrophages are loaded with iron, transferrin saturation levels are in the low to normal range, suggesting that the patients are not systemically iron-loaded. Moreover, patients have a poor tolerance to phlebotomy and may present with marginal hypochromic microcytic anemia.

The gene responsible, Slc40A1 (Ireg1/Ferroportin1/MTP1), is highly expressed on the basolateral membrane of duodenal enterocytes and the plasma membrane of reticuloendothelial cells (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000) where it plays a key role in absorption of dietary iron and in the release of iron derived from the breakdown of hemoglobin following phagocytosis of senescent erythrocytes. The Ferroportin protein sequence is unique and does not share any homology with other transport proteins (McKie & Barlow, 2003) and appears to be the only route by which iron can exit cells, as mice lacking Ferroportin accumulate iron in cells, particularly duodenal enterocytes and macrophages (Donovan et al., 2005). Iron efflux from duodenal enterocytes and macrophages is a major determinant of plasma iron levels and recently Ferroportin has been shown to be the target of the iron regulatory peptide, hepcidin, which binds to Ferroportin, causing internalization and degradation of the protein (Nemeth et al., 2004). Thus, iron efflux via Ferroportin is a major point of regulation in control of both dietary iron absorption and iron recycling through RE cells.

Several mutations in Ferroportin have been reported to cause Ferroportin disease (Montosi et al., 2001; Njajou et al., 2001; Cazzola et al., 2002; Devalia et al., 2002; Wallace et al., 2002). In addition, a variant of Ferroportin present in individuals of African decent has been associated with African iron overload or Bantu siderosis (Barton et al., 2003;

Correspondence to: A.T. Mckie; email: andrew.t.mckie@kcl.ac.uk

Beutler et al., 2003; Gordeuk et al., 2003). The clinical symptoms of these conditions would suggest a loss of function in Ferroportin but, to date, there are few studies to support this hypothesis (Drakesmith et al., 2005; Schimanski et al., 2005). Additionally, since the disease is autosomal dominant, it is not clear whether the mutant protein exerts a dominant negative effect on the wild-type protein or is simply inactive with haplo-insufficiency accounting for the phenotype. Recent studies in cultured cells have provided evidence that some mutations resulted in incorrect membrane targeting while others were correctly expressed in the membrane and maintained full activity but were apparently resistant to internalization caused by hepcidin (Drakesmith et al., 2005; Schimanski et al., 2005). Those studies were unable to identify any functional consequence of the Q248H mutation.

Here we show, by direct measurement of iron transport activity using microinjection of ^{59}Fe into *Xenopus* oocytes expressing Ferroportin proteins, that disease-causing mutations as well as the Q248H variant present in African populations result in reduced iron transport activity.

Materials and Methods

Iron Efflux Assay

Mutations were introduced into a plasmid containing the wild-type human Ferroportin cDNA using site-directed mutagenesis (Quickchange, Stratagene). All mutants were fully sequenced to confirm the correct sequence. Wild-type (WT) and mutant Ferroportin cRNA was transcribed *in vitro* from the T7 promoter using the mMessage mMachine High Yield Capped RNA Transcription Kit (Ambion). Oocytes were prepared as previously described (McKie et al., 2000) and microinjected with 30 ng (30 nl) of either WT, mutant or mixtures of WT and mutant Ferroportin cRNA or 30 nl of water as a control. The oocytes were left for 3 days to express the protein and then microinjected with freshly prepared 59Fe ascorbate (1 mM ascorbate) at pH 7. Individual oocytes were each placed into 100 μl efflux buffer (in mM: 88 NaCl, 1 KCl, 0.82 MgSO4, 2.4 $NaHCO_3$, 2.5 sodium pyruvate, 10 HEPES, 0.41 $CaCl_2$, 2 mg/ml BSA [pH 7]). Efflux was determined by measuring radioactivity in the efflux buffer after 24 h. Radioactivity remaining in each oocyte was also measured and efflux was expressed as a percentage of the total counts injected. Radioactivity was determined by gamma counting. For the titration experiments, cRNA solutions of 1 μg/μl were made for each cRNA WT, A77D and N114H. The WT cRNA was then mixed with the mutant cRNA in the following way 3:1 (75% WT); 1:1 (50% WT) and 1:3 (25% WT). Synthetic Hepcidin (Hep25) was a kind gift from T Ganz.

Immunocytochemistry

Oocytes were fixed in 100% methanol at −20°C for 4 h, rehydrated with 2 × 15 min washes in 50% methanol/ 50% ethanol, followed by a wash with PBS, and bi-sectioned. The samples were blocked (10% donkey serum, 2% BSA in PBS) for 90 min, then washed in PBS followed by 3 × washes in TBSN (Tris-buffered saline, 0.1% Nonidet P-40). The samples were incubated for 16 h with rabbit anti-Ferroportin primary antibody (McKie et al., 2000) in TBSN containing 2% BSA, followed by a 24 h wash with TBSN and a 24 h incubation with an FITC-conjugated anti-rabbit secondary antibody (Dako; 1:100 dilution in TBSN containing 2% BSA). The samples were then finally washed with TBSN for 24 h and dehydrated with 50% methanol/50%PBS for 15 min. The samples were then mounted onto cavity slides with Murray's clear mounting medium (benzyl benzoate: benzyl alcohol 1:2 [v/v]), sealed, and visualized on a confocal microscope (Biorad MRC 1024).

Statistical Analysis

Data were analyzed by one-way analysis of variance with post-hoc analysis by Student's *t*-test.

Results

We tested the iron efflux activity of three published Ferroportin mutants: A77D, N144H, V162Δ (Montosi et al., 2001; Njajou et al., 2001; Devalia et al., 2002; Wallace et al., 2002; Beutler et al., 2003), which lead to Ferroportin disease, and one mutation (Q248H), which is associated with African iron overload. Oocytes expressing mutants all demonstrated significantly reduced iron efflux to varying degrees compared to wild-type (WT) Ferroportin ($P < 0.02$) (Fig. 1*A*). A77D had the greatest effect on efflux (9% of WT), whilst the Q248H, V162Δ and N144H mutants showed 25%, 36% and 46% of wild-type efflux respectively. Mutants showed efflux ranging from 2- to 10-fold above background ($P \leq 0.02$), indicating that all proteins retained some function (A77D, 2-fold higher; $P = 0.014$; Q248H, 6-fold higher, $P < 0.0001$; V162Δ, 8-fold higher, $P = 0.02$; and N144H, 10-fold higher, $P < 0.0001$).

In heterozygotes expressing autosomal dominant Ferroportin disease, it would be predicted that 50% of Ferroportin mRNA would be derived from the normal allele and 50% from the mutant allele. We tested the efflux activity of WT Ferroportin in the presence of two mutant cRNAs (A77D and N144H) by titrating WT against mutant cRNA. As the proportion of WT to A77D and N144H cRNA increased, there was a significant decrease in ^{59}Fe efflux. At 75% WT (1:4 mutant:WT) there was a significant decease in efflux with N144H ($P = 0.01$), with A77D just failing to reach significance ($P = 0.09$). However, at 50% WT (1:1 ratio mutant:WT) efflux was significantly reduced to approximately half that of 100% WT for both A77D and N144H (Fig. 1*B* A77D $P < 0.0001$ and N144H $P = 0.001$). We also titrated wild-type Ferroportin and mutants A77D and N144H individually against water. Over a four-fold dilution range there was no significant decrease in transport activity of the respective cRNAs (Fig. 1*B*; A77D and N144H *not shown*) or in the levels of protein expression in the oocyte membrane by immunostaining (*not shown*), indicating the amount of

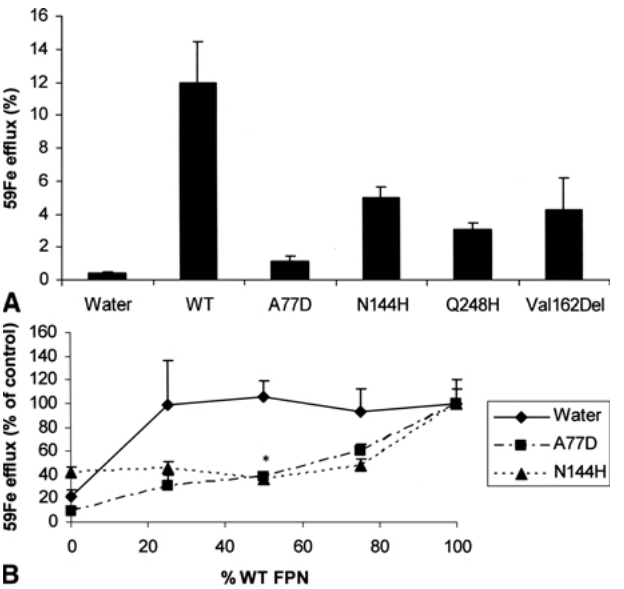

Fig. 1. (*A*) Effect of mutations on ferroportin-mediated efflux of iron. Oocytes were injected with WT or mutant cRNA or water as controls. Seventy two hours post injection of cRNA, radioactive ferrous ascorbate was injected and efflux was allowed to take place over 24 h. The amount of radioactivity in the buffer was measured and expressed as a percentage of the amount of radioactivity injected. [a]$P < 0.01$ compared to water-injected controls, [b]$P < 0.02$ compared to WT-injected oocytes. Experiment shown is representative of 3 similar experiments in oocytes harvested from different toads. (*B*) Titration of WT Ferroportin cRNA with mutant (A77D and N144H) cRNA. Oocytes were injected with various amounts of WT cRNA mixed with mutant cRNA or water. Seventy two hours post injection of cRNA, radioactive ferrous ascorbate was injected and efflux was allowed to take place over 24 h. The amount of radioactivity in the buffer was measured and expressed as a percentage of the amount of radioactivity injected. * $P \leq 0.001$

protein produced from the different cRNAs over four-fold dilution was approximately equal.

Discussion

We found that all the ferroportin mutants tested (A77D, N144H, V162Δ and Q248H) had reduced iron efflux activity compared to WT. However, all had at least some iron efflux activity. A77D seemed to have the most dramatic effect, but even this mutant exhibited iron efflux, which was 2-fold higher than background. In the N144H, iron efflux was approximately 10-fold above background and V162Δ and Q248H mutants' iron efflux was 8- and 6-fold above background. Our experiments where we injected mixtures of WT and either A77D or N144H suggest that expression of these mutant Ferroportin proteins exerted a dominant negative effect on wild-type Ferroportin function. This suggests that ferroportin may function as a dimer or multimer. Schimanski et al. could not detect any interaction between A77D and WT Ferroportin in 293T cells, however, these cells were able to separate A77D from WT Ferroportin to distinct cellular compartments (Schimanski et al., 2005). In contrast, we found both proteins reach the plasma membrane in *Xenopus* oocytes, hence an interaction could happen there. Using the same cell line (293T) and similar techniques to Schimanski et al. (2005) and De Domenico et al. (2005) were able to show functional interaction between WT and mutant ferroportin proteins. At the present time we do not know what the relative levels of the mutant vs the wild-type proteins are in duodenum or macrophages of patients with Ferroportin disease, however, our model for the heterozygote (1:1 co-injection of WT and mutant cRNAs) results in 50% efflux activity of Ferroportin. In the absence of other compensatory mechanisms (such as hepcidin resistance), this would be sufficient to account for approximately 0.5 mg of iron to be absorbed through the intestine from diet and approximately 15 mg of iron to be recycled daily via the macrophages. Clearly, the residual activity appears to be sufficient for survival and accounts for the phenotype of the disease.

Support for the dominant negative model has come from mice where Ferroportin was knocked out (Donovan et al., 2005). Mice homozygous for the null ferroportin allele died before birth; however, heterozygotes survived and appeared normal. If mutations caused a loss of function of Ferroportin through haplo-insufficiency then heterozygous mice would show a similar phenotype to patients with ferroportin disease. However heterozygote mice did not appear to reproduce the human disease even after a year, implying that haplo-insufficiency is not the cause of the disease.

Mutations in iron transport membrane proteins, such as DMT1, can result in inefficient trafficking to the membrane or mis-folding, leading to retention of the protein within cytoplasmic compartments (Canonne-Hergaux et al., 2000; Touret et al., 2004). In oocytes we observed that the WT and all mutant proteins were efficiently translated and targeted to the plasma membrane of the oocytes (Fig. 2). This is in contrast to a recent report in 293T cells that suggested the A77D and V162Δ mutants had no iron efflux activity cells due to a defect in membrane targeting (Schimanski et al., 2005). The V162Δ was also found to have a membrane-targeting defect in a similar study using cultured cells, although the A77D mutant was not examined (De Domenico et al., 2005). However, in another study it was found that all mutated ferroportins were expressed at the surface to some extent in cultured COS1 cells (Liu, Yang & Halle, 2005). It is possible that targeting of membrane proteins may be different between oocytes and mammalian cells (and also between different mammalian cells) and this fact enables us to use oocytes to study the iron efflux activity of the mutant proteins.

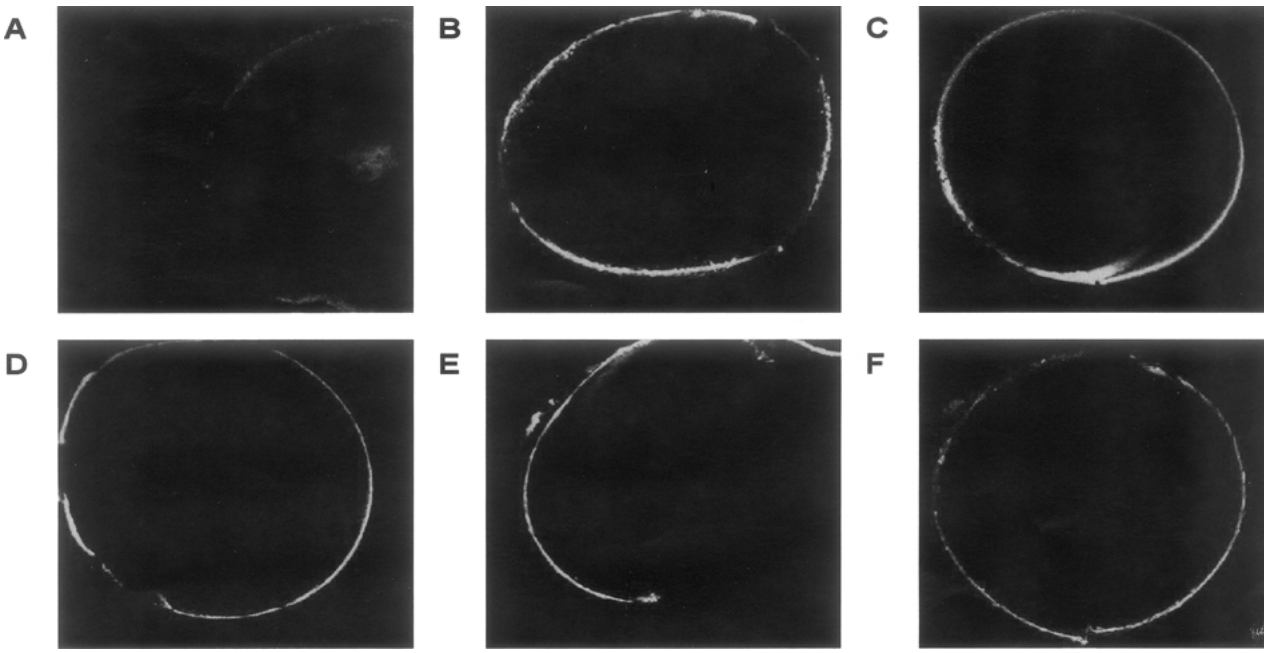

Fig. 2. Subcellular localization of Ferroportin mutant proteins in *Xenopus* oocytes. Seventy two hours post-injection with cRNA, oocytes were bi-sectioned and immunostained with ferroportin antisera. (A) Water, (B) WT, (C) A77D, (D) N144H, (E) Q248H, (F) V162Δ.

The iron regulatory peptide hepcidin has been shown to regulate efflux of iron from cells by binding to ferroportin on the membrane and causing internalization and degradation of the protein (Nemeth et al., 2004). Several recent studies have shown that the internalization of Ferroportin by hepcidin is affected by the various disease mutations. In the first of these studies, ferroportin internalization occurred normally for some mutations (e.g., Q248H), while for others (e.g., C326Y, N144H) no internalization was evident, thus leading to hepcidin resistance (Drakesmith et al., 2005). Two further reports also found that N144H was resistant to hepcidin-mediated internalization (De Domenico et al., 2005; Liu et al., 2005). In oocytes we were unable to detect any effect of hepcidin (25-mer) on the iron efflux activity of wild-type Ferroportin (*data not shown*), suggesting that the mechanism leading to Ferroportin internalization may be absent in *Xenopus* oocytes and hepcidin does not directly block Ferroportin-mediated iron efflux. The latter is supported by De Domenico who showed that hepcidin resistant mutants were still able to bind hepcidin, suggesting that hepcidin binding does not block iron transport directly (De Domenico et al., 2005).

The polymorphism Q248H associated with African iron overload appears to have a milder phenotype than the other mutations, with patients having a trend towards raised serum ferritin, with macrophage iron loading and mild anemia (Barton et al., 2003; Beutler et al., 2003). In our hands, the Q248H mutation gave a significantly higher efflux activity than A77D. The mutation appears to be common within the African/African-American population (8% in African-Americans). It is interesting to note that the phenotype of Ferroportin disease is reminiscent of the anemia of chronic disease or anemia of inflammation and the high frequency of the Q248H mutation in African populations suggests positive selection has occurred, perhaps as a protection against pathogens (e.g., malaria).

In conclusion, our data show that several disease-causing mutations in Ferroportin lead to reduced iron transport activity of the protein. For two of the mutations (A77D and N144H), addition of equimolar levels of mutant cRNAs to WT resulted in an inhibition of iron transport activity to approximately 50% of wild type, suggesting that these mutations exert a dominant negative effect on the function of the wild-type protein. These findings likely account for accumulation of iron in RE cells and the low to normal transferrin saturation levels observed in patients with these mutations. The lack of internalization of WT ferroportin by hepcidin in oocytes meant we could not examine hepcidin resistance in oocytes; however, as shown recently, some mutations render ferroportin hepcidin-resistant and hence would lead to a gain of function with increased iron absorption and increased iron release through the RE cells (Drakesmith et al., 2005; De Domenico et al., 2005; Liu et al., 2005). This adds another layer of complexity to the regulation of iron efflux from cells in Ferroportin disease. Iron absorption tests in patients with or without hepcidin treatment could provide

further insight into the nature of the iron transport defect in this disease.

The authors thank the Medical Research Council for funding (ATM is a Career Development Fellow), Kings College London for a studentship award (JAM) and the NIH and HFSP for additional support.

References

Abboud, S., Haile, D.J. 2000. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *J. Biol. Chem.* **275:**19906–19912

Barton, J.C., Acton, R.T., Rivers, C.A., Bertoli, L.F., Gelbart, T., West, C., Beutler, E. 2003. Genotypic and phenotypic heterogeneity of African Americans with primary iron overload. *Blood Cells Mol. Dis.* **31:**310–319

Beutler, E., Barton, J.C., Felitti, V.J., Gelbart, J., West, C., Lee, P.L., Waalen, J., Vulpe, C. 2003. Ferroportin 1 (SCL40A1) variant associated with iron overload in African-Americans. *Blood Cells Mol. Dis.* **31:**305–309

Canonne-Hergaux, F., Fleming, M.D., Levy, J.E., Gauthier, S., Ralph, J., Picard, V., Andrews, N.C., Gros, P. 2000. The Nramp2/DMT1 iron transporter is induced in the duodenum of microcytic anemia mk mice but is not properly targeted to the intestinal brush border. *Blood* **96:**3964–3970

Cazzola, M., Cremonesi, L., Papaioannou, M., Soriani, N., Kioumi, A., Charalambidou, A., Paroni, R., Romtsou, K., Levi, S., Ferrari, M., Arosio, P., Christakis, J. 2002. Genetic hyperferritinaemia and reticuloendothelial iron overload associated with a three base pair deletion in the coding region of the ferroportin gene (SLC11A3). *Br. J. Haematol.* **119:**539–546

De Domenico, I., Ward, D.M., Nemeth, E., Vaughn, M.B., Musci, G., Ganz, T., Kaplan, J. 2005. The molecular basis of ferroportin-linked hemochromatosis. *Proc. Natl. Acad. Sci. USA* **102:**8955–8960

Devalia, V., Carter, K., Walker, A.P., Perkins, S.J., Worwood, M., May, A., Dooley, J.S. 2002. Autosomal dominant reticuloendothelial iron overload associated with a 3-base pair deletion in the ferroportin 1 gene (SLC11A3). *Blood* **100:**695–697

Donovan, A., Brownlie, A., Zhou, Y., Shepard, J., Pratt, S.J., Moynihan, J., Paw, B.H., Drejer, A., Barut, B., Zapata, A., Law, T.C., Brugnara, C., Lux, S.E., Pinkus, G.S., Pinkus, J.L, Kingsley, P.D., Palis, J., Fleming, M.D., Andrews, N.C., Zon, L.I. 2000. Positional cloning of zebrafish ferroportin 1 identifies a conserved vertebrate iron exporter. *Nature* **403:**776–781

Donovan, A., Lima, C.A., Pinkus, J.L., Pinkus, G.S., Zon, L.I., Robine, S., Andrews, N.C. 2005. The iron exporter ferroportin/SIc40a1 is essential for iron homeostasis. *Cell Metabolism* **1:**191–200

Drakesmith H., Schimanski L.M., Ormerod E., Merryweather-Clarke A.T., Viprakasit V., Edwards J.P., Sweetland E., Bastin J.M., Cowley D., Chinthammitr Y., Robson K.J., Townsend A.R., 2005. Resistance to hepcidin is conferred by hemochromatosis-associated mutations of ferroportin. *Blood.* **106:**1092–1097

Gordeuk, V.R., Caleffi, A., Corradini, E., Ferrara, F., Jones, R.A., Castro, O., Onyekwere, O., Kittles, R., Pignatti, E., Montosi, G., Garuti, C., Gangaidzo, I.T., Gomo, Z.A., Moyo, V.M., Rouault, T.A., MacPhail, P., Pietrangelo, A. 2003. Iron overload in Africans and African-Americans and a common mutation in the SCL40A1 (ferroportin 1) gene. *Blood Cells Mol. Dis.* **31:**299–304

Liu, X.B., Yang, F., Haile, D.J. 2005. Functional consequences of ferroportin 1 mutations. *Blood Cells Mol. Dis.* **35:**33–46

McKie, A.T., Barlow, D.J. 2004. The SLC40 basolateral iron transporter family (IREG1/ferroportin/MTP1). *Pfluegers Arch.* **447**:801–806

McKie, A.T., Marciani, P., Rolfs, A., Brennan, K., Wehr, K., Barrow, D., Miret, S., Bomford, A., Peters, T.J., Farzaneh, F., Hediger, M.A., Hentze, M.W., Simpson, R.J. 2000. A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. *Mol Cell* **5:**299–309

Montosi, G., Donovan, A., Totaro, A., Garuti, C., Pignatti, E., Cassanelli, S., Trenor, C.C., Gasparini, P., Andrews, N.C., Pietrangelo, A. 2001. Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC1 1A3) gene. *J. Clin. Invest* **108:**619–623

Nemeth, E., Tuttle, M.S., Powelson, J., Vaughn, M.B., Donovan, A., Ward, D.M., Ganz, T., Kaplan, J. 2004. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* **306:**2090–2093

Njajou, O.T., Vaessen, N., Joosse, M., Berghuis, B., van Dongen, J.W., Breuning, M.H., Snijders, P.J., Rutten, W.P., Sandkuijl, L.A., Oostra, B.A., van Duijn, C.M., Heutink, P. 2001. A mutation in SLC11A3 is associated with autosomal dominant hemochromatosis. *Nat. Genet.* **28:**213–214

Pietrangelo, A. 2004a. Hereditary hemochromatosis—a new look at an old disease. *N. Engl. J Med.* **350:**2383–2397

Pietrangelo, A. 2004b. The ferroportin disease. *Blood Cells Mol. Dis.* **32:**131–138

Schimanski L.M., Drakesmith H., Merryweather-Clarke A.T., Viprakasit V., Edwards J.P., Sweetland E., Bastin J.M., Cowley D., Chinthammitr Y., Robson K.J., Townsend A.R., 2005. In vitro functional analysis of human ferroportin (FPN) and hemochromatosis-associated FPN mutations. *Blood.* **105:**4096–4102

Touret, N., Martin-Orozco, N., Paroutis, P., Furuya, W., Lam-Yuk-Tseung, S., Forbes, J., Gros, P., Grinstein, S. 2004. Molecular and cellular mechanisms underlying iron transport deficiency in microcytic anemia. *Blood* **104:**1526–1533

Wallace, D.F., Pedersen, P., Dixon, J.L., Stephenson, P., Searle, J.W., Powell, L.W., Subramaniam, V.N. 2002. Novel mutation in ferroportin1 is associated with autosomal dominant hemochromatosis. *Blood* **100:**692–694