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Review

The NRAMP family of metal-ion transporters

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Abstract

The family of NRAMP metal ion transporters functions in diverse organisms from bacteria to human. NRAMP1 functions in metal transport across the phagosomal membrane of macrophages, and defective NRAMP1 causes sensitivity to several intracellular pathogens. DCT1 (NRAMP2) transport metal ions at the plasma membrane of cells of both the duodenum and in peripheral tissues, and defective DCT1 cause anemia. The driving force for the metal-ion transport is proton gradient (protonmotive force). In DCT1 the stoichiometry between metal ion and proton varied at different conditions due to a mechanistic proton slip. Though the metal ion transport by Smf1p, the yeast homolog of DCT1, is also a protonmotive force, a slippage of sodium ions was observed. The mechanism of the above phenomena could be explained by a combination between transporter and channel mechanisms.

Keywords: NRAMP; Metal-ions; Transport; Transporters; Homeostasis

1. Introduction

Metal ions are vital life elements that participate in many metabolic processes in every living cell. On the other hand, these essential nutrients are toxic at elevated levels. Therefore, shortage or excess in metal ion, as a result of genetic disorders as well as malnutrition, might cause death or severe diseases. For example, abnormal iron uptake has been implicated in the most common hereditary disease hemochromatosis, as well as in anemia and atherosclerosis, and in neurological diseases such as Parkinson's, Alzheimer's, Huntington's, Friedreich's ataxia and Pica [1–9]. To prevent such an illness, cells must maintain metal ion homeostasis, and this is carried out through highly regulated processes of uptake, storage and secretion.

Factors controlling metal ion transport across cellular membranes, intracellular homeostasis and regulatory responses of cells to changing environmental supply of metal ions have been subjects of many studies in recent years [for review, see 10]. Of particular interest were divalent trace metals like Cu^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+} because of their important role in cell metabolism, especially as cofactors of many enzymes. Usually their intracellular concentration is kept at a low, rather constant physiological level. Toxic metals like Cd^{2+}, Co^{2+} and Ni^{2+} can be a major threat to the health of mammals and could suppress plant growth, mostly because they interfere in various ways with transport, homeostasis or function of the essential metals. To minimize their deteriorating effects, cells have developed various strategies, among which transport or sequestration into organelles and binding by thiols are most prominent [10–14].

The different metal ions may be grouped into redox-active ions such as Fe^{2+}, Cu^{2+}, Co^{2+} and, to a lesser extent, Mn^{2+}, and non-redox-active ions such as Ca^{2+} and Zn^{2+}. Zinc and calcium are suitable for transcription factors and other enzymes involved in DNA metabolism, because the presence of redox-active metal ions in those proteins can lead to radical formation that might result in nucleic acid damage. The redox-active ions normally function in enzymes that participate in redox reactions and the conversion of active oxygen-containing components.

A specific set of transporters functions in each cellular compartment to provide a delicate balance of transport activities across their membranes [8,15,16]. In some of the cellular organelles and the plasma membrane, low and high affinity transporters act in concert to maintain the right balance of metal ion concentrations. Moreover, highly specific transport systems function along with a wide range of metal ion transporters to

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achieve the right concentration balance [10–12,17]. Only in recent years has research focused on genes encoding metal ion transporters in eukaryotes and on the genetic disorders associated with them [6–8]. As a result, a large number of candidate genes were discovered and some of the transporters, particularly those for Ca^{2+}, Cu^{2+} and Fe^{2+}, have been described in detail. Yet, several observations indicate that many more proteins, involved in transport and homeostasis of metal ions, await their identification.

In the last few years, it has become apparent that, from bacteria to man, the family of natural resistance-associated macrophage protein (NRAMP) metal ion transporters plays a major role in metal ion homeostasis [13,18–21]. The family members function as general metal ion transporters and can transport Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, Ni^{2+}, and Co^{2+}[22–26]. Members of the NRAMP family share remarkable protein sequence identity of 28% (yeast), 40% (plant), and 55% (fly) with the mammalian proteins (46%, 58%, and 73% similarity, respectively) [27]. This family is represented in yeast by three genes (*SMF1, SMF2*, and *SMF3*), in fly by *MVL* (*malvolio*) and in mammals by *NRAMP1* and *NRAMP2* . DCT1 (*d*ivalent *c*ation *t*ransporter 1, also called DMT1 for *d*ivalent *m*etal *t*ransporter) is probably the rat isoform of the human NRAMP2, with which it shares 92% identity and is 73% identical to human NRAMP1 [28].

1.1. Discovery of the Nramp family

The yeast genes *SMF1* and *SMF2* (*s*uppressor of *mif1*) were cloned in 1992 as a high copy number suppressor of a temperature-sensitive *mif1-1* mutant [29]. *MIF1 (MAS1)* and *MAS2 (MIF2)* encode the processing enhancing protein and the matrix processing peptidase, respectively. The two proteins function as a heterodimer to form the active holoenzyme of mitochondrial processing peptidase, which is vital for cell growth [30–32]. The activity of the purified peptidase is inhibited by chelators, such as EDTA or orthophenantroline, and is stimulated by Mn^{2+}, Zn^{2+}, or Co^{2+}[33]. But the function of the proteins Smf1p and Smf2p encoded by the suppressive genes was unknown.

The mouse Nramp1 (natural resistance-associated macrophage protein, encoded by *Nramp1*, OMIM No. 600266; now classified as solute carrier family 11 member 1, *Slc11a1*) was cloned in 1993 as a gene responsible for mouse resistance to infection by mycobacteria [34]. *Nramp1* was found to be identical to the *Ity* and the *Lsh* gene conferring resistance to infections by *Salmonella typhimurium* and *Leishmania donovani*, respectively [35]. It was proposed that *Nramp1* encodes an integral membrane protein that has structural homology with known prokaryotic and eukaryotic transport systems [34]. But yet the function of this transporter was unknown. The seminal discovery of *Nramp1* thrived the interest of this membrane protein [36–45].

In 1995 it was found that MVL (encoded by *mvl*, *malvolio*), the *Drosophila melanogaster* homologue of NRAMP, is expressed in macrophages and the nervous system, and is required for normal taste behavior [46]. Because of a limited sequence identity between Nramp1 and malvolio proteins with nitrate transporter from *Aspergillus*, it was suggested that the *Drosophila* and mammalian proteins also function in NO^{2-} or NO^{3-} transport [34,46]. The identification of another member of the family, Nramp2 in mouse, and the human orthologs NRAMP2 (encoded by *NRAMP2*, OMIM No. 600523; now designated *SLC11A2*) followed soon afterwards [27,47–51] but the molecular actions of these proteins still remained unclear.

Soon thereafter, the *SMF1* gene was identified as a suppressor of a *csp2 (cdc1-1)* mutant phenotype, that was sensitive to the presence of EGTA in the medium [22]. The Chelator Sensitive Phenotype (Csp^{-}) of the *csp2* mutant was caused by a mutation in the *CDC1* gene, in which Gly^{149} was substituted by arginine. The *cdc1-1* mutant exhibited very similar complementation characteristics to the *mas1* (or *mif1-1*) mutant, with the exception of their growth inhibiting conditions (which are EGTA and 37 °C, respectively). Re-examination of the *mif1-1* mutant revealed that its temperature-sensitivity could be alleviated by the addition of 1 mM Mn^{2+} to the medium or by overexpression of Smf1p [22]. Further investigation of the *SMF1* null mutant showed that cells exhibited a significant decrease in Mn^{2+} uptake. On the other hand, the presence of Smf1p at multiple copies stimulated Mn^{2+} uptake (>5-fold) that was inhibited by Zn^{2+}. Using Smf1p specific antibodies, it was also shown that Smf1p is located in the yeast plasma membrane [22]. These observations led us to propose that Smf1p is a high affinity Mn^{2+} and/or Zn^{2+} transporter. In both cases, the *mif1-1* and the *cdc1-1*, the mutation could be relieved by supplementing the media with Mn^{2+} or overexpressing Smf1p that stimulates the Mn^{2+} transport from the medium and elevates its concentration in the cytoplasm [22,24]. The temperature sensitivity of *mif1-1* mutant may result from reduced stability of the mutated processing peptidase, which needed higher manganese concentrations for its function at higher temperature. Cdc1p may be a Mn^{2+}-dependent cell division cycle protein that is vital for cell growth [52,53], but the G149R mutation rendered it less stable and cell-sensitive to low Mn^{2+} concentration in the medium. It is remarkable that, prior to these studies, manganese was not considered to be an essential element for yeast growth. Only by discovering that mutations in Cdc1p and Mas1p can be complemented by the addition of Mn^{2+} did it become apparent that this metal-ion is vital for yeast [22,52,53].

The discovery that Smf1p is the yeast Nramp homologue and a metal-ion transporter [22] paved the way for the advancement of our knowledge about the substrate of the mammalian NRAMP. We then proposed the role of Nramp1 in macrophage defense against microbial invasion. Its function as a possible scavenger of metal-ions from the lumen of bacteria-containing phagosomes [22,54] will be discussed later on.

The ~30% identity in the amino acid sequence between Smf1p and MVL raised the possibility that the *Drosophila* proteins also have a similar function [22]. According to this hypothesis, metal ion homeostasis is impaired in the *mvl* mutant, resulting in a loss of taste perception for sugars. To test this hypothesis, *mvl* mutant flies were allowed to develop from eggs to adulthood on a medium containing elevated concentrations of metals. Mutant flies that were reared in the presence of 10 mM $MnCl_2$ or $FeCl_2$ developed into adults with recovered taste behavior [55]. Furthermore, exposure of adult mutant flies to these ions in the testing plate for only 2 h was sufficient to restore normal taste behavior. The

suppression of defective taste behavior suggests that the MVL protein functions as an Mn^{2+} and Fe^{2+} transporter and that Mn^{2+} and/or Fe^{2+} are involved in the signal transduction of taste perception in *Drosophila* adults [55]. The role of Mn^{2+} and Fe^{2+} in neurotransmission and taste perception is not clear. Their possible involvement in neural development was ruled out, since Mn^{2+} and Fe^{2+} suppressed the mutant phenotype even when supplied to flies only at the adult stage, when the nervous system had already been developed. A recent, very interesting, study was based on these findings and on the fact that sucrose responsiveness is related to division of labor in honeybee colonies [56]. Honeybee foragers are more responsive to sucrose in the laboratory than are younger nurse bees, and pollen foragers are more responsive to sucrose than nectar foragers. The study showed that levels of *mvl* mRNA in the brain and manganese in the head were higher in pollen foragers compared with nurses, with nectar foragers intermediate. Manganese treatment increased honeybee sucrose responsiveness and caused precocious foraging. Manganese levels showed a similar pattern to *mvl* mRNA but manganese treatment did not increase pollen foraging [56]. Alterations in metal ion homeostasis are known to result in several neurological diseases, and the *Drosophila* mutants may provide a useful genetic tool to study their molecular mechanism.

The gene *Nramp2* (now designated *Slc11a2*) encoding the protein DCT1 (*d*ivalent *c*ation *t*ransporter, also called DMT1), the rat ortholog of NRAMP2, was cloned in 1997 by functional screening of a rat cDNA that induced iron transport activity in *Xenopus laevis* oocytes [28]. At around the same time and on nearly the same location, Andrews' group used a positional cloning strategy to identify the defective *Nramp2* gene in an inbred mouse strain (*mk*) with microcytic anemia [1,57]. The DCT1 [28] and the following Smf1p [25] expression in *Xenopus laevis* oocytes led to functional characterization of these proteins as a general divalent metal-ion transporter. These findings explained the *mk* mouse phenotype and also supported our proposed mechanisms, by which Nramp1 conferred resistance to bacterial infection [22].

2. Functional properties and physiological roles of the Nramp family

Recent high-resolution structures of a few transporters shed light on the mechanism of action of ion driven transport [58]. The structure of the proton-driven lactose permease of *E. coli* clearly demonstrated that conformational changes are a major part of the transport mechanism [59]. However, the mechanism of metal-ion transport by eukaryotic cells is largely obscure.

The large number of transporters occupying the plasma membrane of a typical eukaryotic cell makes the study into the mechanism of a single transporter very difficult. Therefore, most of the information about those transporters has come from electrophysiological studies on DCT1 and Smf1p that were expressed in *Xenopus* oocytes [25,26,28,60,61]. One of the main advantages of oocytes as a heterologous expression system is their self-sufficiency in terms of nutrition and therefore providing a system with very low background for foreign transporters expressed in their plasma membrane. Nevertheless, there are compelling studies using mammalian cell lines and tissues that contributed to our knowledge about the function of transporters of the NRAMP family [62–65].

2.1. DCT1

DCT1 transports a wide range of divalent metal-ions [28,62,63]. It operates as a symporter of metals and H^+, while the proton gradient is the driving force for the metal transport [25,28,60,64,65]. Electrophysiology experiments showed that Fe^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, and, to a lesser extent, Ni^{2+} and Pb^{2+} are all evoke currents in oocytes expressing DCT1, suggesting that each of these are ligands (elements that binds to the transporter and initiates a process) [28]. However, direct measurement was required to confirm that each of the ligands is actually transported. So radioactive uptake experiments in oocytes using $^{55}Fe^{2+}$, $^{54}Mn^{2+}$, $^{60}Co^{2+}$, $^{65}Zn^{2+}$ and $^{109}Cd^{2+}$ revealed that these metals are transported by DCT1 [26,28,60,66,67]. pH-dependent Fe^{2+},Mn^{2+}, and Co^{2+} transport has also been demonstrated in DCT1-transfected, but not non-transfected, Chinese hamster ovary (CHO) cells and pig kidney (LLC-PK_1) cells using a fluorescent and radiotracer assay in vitro [68,69]. Studies using Caco-2 cells showed that DCT1 knockdown significantly inhibits $^{55}Fe^{2+}$ and $^{109}Cd^{2+}$ uptake, with no effect on uptake of lead [64]. On the other hand, overexpression of DCT1 in yeast and human fibroblasts resulted in increased transport of lead, which was greater at an acidic pH and inhibited by Fe^{2+}[70,71]. A possible explanation for those disparate observations can be that there is more than one transporter for lead in Caco-2 cells. Another study showed that treatment of Caco-2 cells with a DCT1 antisense oligonucleotide resulted in 80% and 48% inhibition of $^{55}Fe^{2+}$ and $^{64}Cu^{2+}$ uptake. This study also suggested that copper is transported in the reduced (Cu^{1+}) state by DCT1 [36].

The $^{55}Fe^{2+}$ uptake by DCT1 was not affected by substitution of chloride in the medium with other anions, such as NO_3^- or SCN^-[28]. Since it seems surprising that the transport of a divalent cation such as Fe^{2+} will be driven solely by additional cation H^+, we further investigated the influence of anions on the currents generated by DCT1 [13,60]. In the presence of chloride, the $^{55}Fe^{2+}$ uptake was insensitive to imposed membrane potentials from −75 to +50 mV. Substitution of chloride anions by gluconate or isethionate drastically reduced the $^{55}Fe^{2+}$ uptake into the oocytes and rendered it sensitive to membrane potential [13]. Therefore, the metal ion transport is dependent on the presence of Cl^- or other small anions (such as NO_3^- or SCN^-) but not gluconate or SO_4^{2-}. The role of Cl^- in the mechanism of DCT1 remains unknown.

It is proposed that DCT1 plays a crucial role in iron absorption from the duodenum as well as transport of the low pH released iron in the endosomes [for reviews, see 72,73]. DCT1 mRNA is found in many different tissues, including the brain, proximal intestine, kidney, bone marrow and reticulocytes [28,48,74,75]. The protein and its mRNA are most abundant in the proximal duodenum, consistent with a function in intestinal iron absorption [28,76]. DCT1 was also colocalized with transferrin in the recycling endosomes and with late endosomal and lysosomal proteins of many cell types, including reticulocytes and transfected cells in

vitro [48,63,75,77–81]. These findings are consistent with a function in transport iron from the acidified lumen of the endosomes into the cytoplasm [1,82,83]. In macrophages, DCT1 is localized to the recycling endosomes [84], but is also localized to erythrocyte-containing phagosomes of macrophages and sperm-containing phagosomes of Sertoli cells [84]. These results suggest that DCT1 may have a role in the recycling of iron from dying erythrocytes and in iron recycling from degenerating spermatozoids.

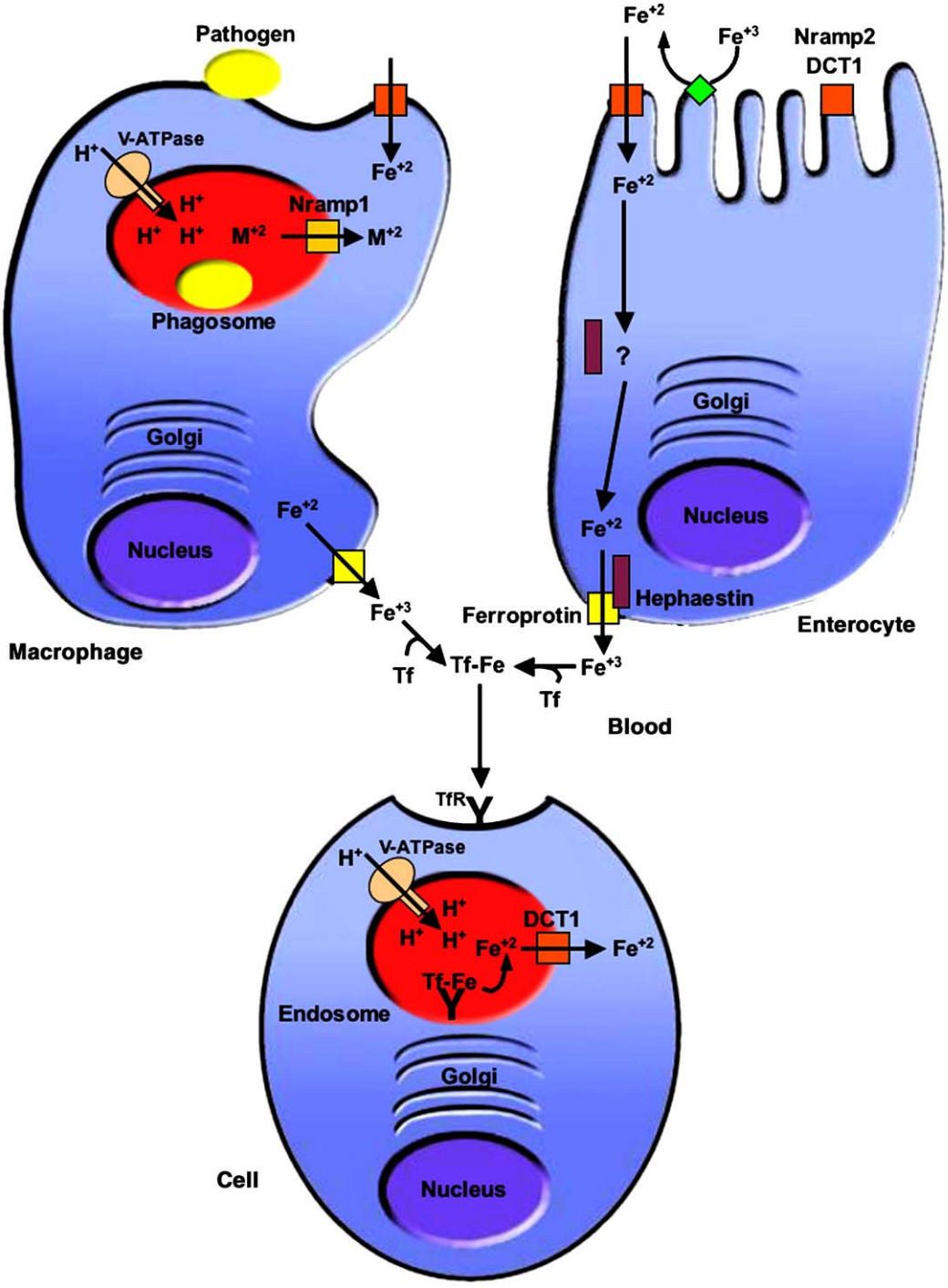

Fig. 1. Schematic representation of key elements in iron transport. The different tissues are represented by a duodenal cell (enterocyte), a blood cell (macrophage) and a parenchymal cell such as a hepatocyte (cell). It is proposed that DCT1 (Nramp2) (orange square) is the main port of iron entry in the duodenum. The reduced iron is provided directly from the food (meat) or is enzymatically (green diamond) reduced on site, probably by the duodenal cytochrome b (Dcytb). Iron is competing for transport with other metal ions. The mechanism by which iron is transferred to the basolateral membrane is not known, but involves the intracellular ferroxidase, Hephaestin (purple rectangle). The basolateral transfer of Fe2+ is likely to be mediated by the membrane protein IREG1, also called ferroportin (Yellow Square). IREG1 is highly expressed in duodenal enterocytes and in macrophages. Hephaestin could be in direct contact with IREG1 and may oxidize the ferrous iron. In the blood stream, the iron binds to transferrin, and the transferrin receptor of different cells is taken up by endocytosis, liberated in the endosomes by the acidic pH, generated by V-ATPase, and transported to the cytoplasm by Nramp2 or Nramp1 in macrophages. Tf, transferrin; Y, transferrin receptor.

The importance of DCT1 is illustrated by some compelling examples: while *Slc11a2*$^{-/-}$ mice died within the first few days of life with severe systemic iron deficiency and consequent anemia [85] a single mutation in DCT1 (G216R) (Fig. 4), which dramatically reduces the transporter uptake activity [1,86], impairs both of these aspects of iron homeostasis (intestinal iron absorption and transport of iron from the endosomes) and causes microcytic anemia in *mk* mice and *Belgrade* rats [1,57,63]. Another example is a mutation of DCT1 that was identified in a human female with severe hypochromic microcytic anemia and iron overload. This homozygous mutation, in the ultimate nucleotide of exon 12, severely impairs splicing, thus causing skipping of exon 12, and introduces the mutation E430D (Fig. 4) in to the protein encoded by the remaining, properly spliced transcript found in the patient [87,88].

Fig. 1 depicts a balanced view of the function of key elements in transporters and receptor-mediated iron uptake. Since oxidized iron is more abundant outside the mammalian cells and DCT1 transports iron only in its reduced form, Fe^{3+} reduction to Fe^{2+} is the first step in iron transport [8]. At the enterocyte membrane, this step could be mediated by duodenal cytochrome *b* (Dcytb), a ferric reductase enzyme resident on the luminal surface of intestinal absorptive cells [2,3,89]. However, in a very recent work, Gunshin et al. demonstrated that loss of Cybrd1 (Dcytb) had little or no impact on body iron stores in *Cybrd1*-null mice [90]. Hence, there might be other mechanisms available for the reduction of dietary iron. The mechanism by which iron is transferred to the basolateral membrane is not known, but involves the intracellular ferroxidase, Hephaestin [91]. The basolateral transfer of Fe^{2+} is likely to be mediated by the membrane protein IREG1, also called Ferroportin [92–94]. Ferroportin is the only mammalian iron exporter identified to date and is highly expressed in duodenal enterocytes and in macrophages [95]. Hephaestin could be in direct contact with IREG1 and may oxidize the ferrous iron (Fe^{2+} to Fe^{3+}) to enable its binding by transferrin for transportation to the body tissues [for a review on iron absorption, see 96 97]. Subsequently, Fe-transferrin binds to the transferrin receptor and is internalized by endocytosis to the endosomes. Acidification of the endosomal lumen by the vacuolar (V)-ATPase permits the dissociation of Fe^{3+} from transferrin and provides the H^+ gradient to energize DCT1-mediated Fe^{2+} transport to the cytoplasm [1,23,78].

2.2. The slip phenomenon of DCT1 and Smf1p

It has been demonstrated that DCT1 cotransports Fe^{2+} together with H^+ with a stoichiometry of 1:1 [28]. The metal ion transport is therefore dependent on proton concentration in the external side of the membrane, as protons are cotransported with iron [65], but the nature of the driving force for transport is not apparent. At physiological membrane potentials of −90 to −30 mV, the apparent affinity constant for H^+ was about 1 μM, suggesting that at neutral pH, proton binding is the rate-limiting step in the transport process. At low pH, DCT1 expressed in *Xenopus* oocytes exhibited a metal-ion induced, uncoupled proton current into the oocyte (at membrane potential of −50 mV and pH 5.5 the number of H^+ ions transported with one Fe^{2+} ion increased to 10), and under certain conditions the transporter operates as an H^+ uniporter [28]. We defined this phenomenon as a mechanistic slip

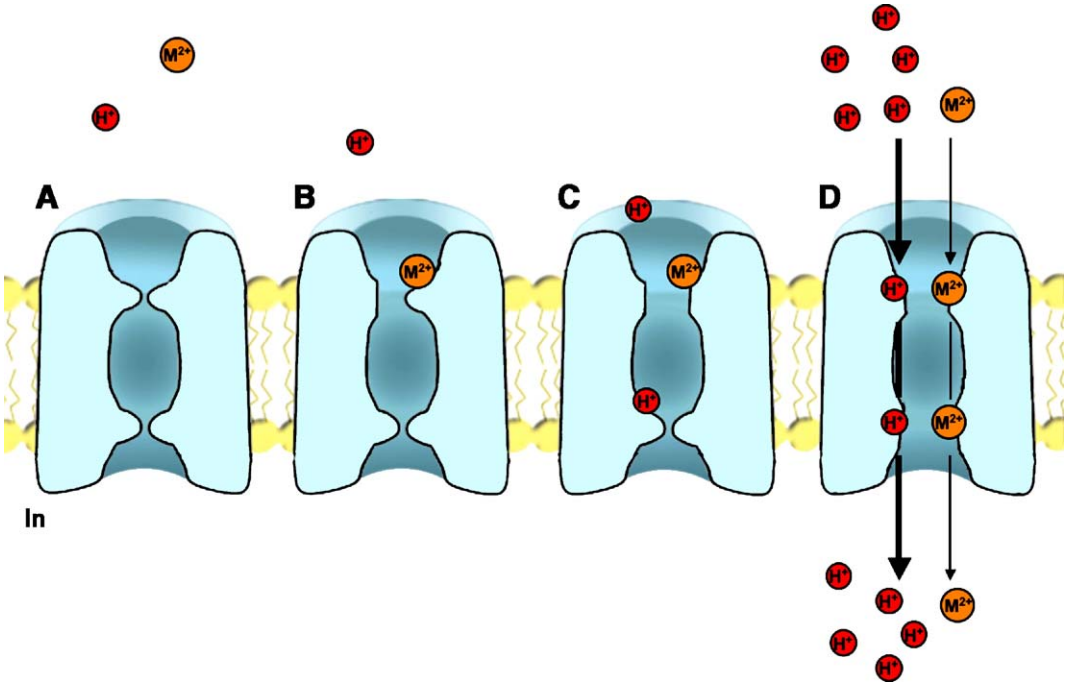

Fig. 2. Schematic representation of DCT1 conformations involved in the transport of protons and metal-ions as well as proton slippage. It is proposed that binding of a metal-ion (M^{2+}) (B) promotes movement of a proton into an internal proton binding site (C). Binding of this proton allows the movement of the metal-ion into its internal site. This step confers a semi-channel conformation that functions in a proton-dependent metal-ion transport and, at the same time, allows a channel-like proton slippage that is dependent on the presence of metal-ions (D). According to this concept, a transport kinetic scheme has no meaning.

that is an integral part of the transporter's mechanism of action [98]. The proton slip is dependent on the presence of metal ions in the medium, and is influenced by the membrane potential. It increases as the imposed potentials become more negative (on changing the membrane potential from +10 to −80 mV at pH 5.5, the number of H^+ ions transported with one Fe^{2+} ion increased from 3 to ~18) [25,28,60]. To illustrate the slip mechanism, a scheme of semi-channel conformation was drawn in Fig. 2.

The mechanism of metal ion transport by the yeast Smf1p is closely related to that of the mammalian DCT1. Smf1p can also transport Mn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, Ni^{2+} and Co^{2+}[22–25] and exhibits similar affinities towards the various metal ions [60,99]. Yeast cells contain two additional genes of this family, and *SMF3*, and indirect evidence indicates that they also exhibit broad range metal-ion specificity, which differs from Smf1p [99]. The triple null mutant *smf1,2,3*Δ cannot grow on medium with reduced divalent metal ions' concentration (containing EGTA) or medium buffered at pH 7.5. The null mutant *smf1Δ* is unable to grow in the presence of EGTA [22,99]. The close relation between Smf1p and DCT1 is best demonstrated by the fact that the both suppress the above phenotype and permit growth in the presence of EGTA or at pH 7.5 [100].

Although H^+ is the driving force in both the most striking difference between DCT1 and Smf1p is in the uncoupled slip that was shown to be H^+ in DCT1 and Na^+ in Smf1p [25,98]. The sodium slip in Smf1p is not dependent on the presence of metal ions and increases with elevation of the pH. Sodium is unlikely to be bound to the metal-ion transport site, because metal ions do not compete with sodium on the slip current and elevation of the metal concentration did not affect the inhibition of their transport by sodium [60]. Therefore, sodium is likely to compete with protons on the proton-binding site and to generate a sodium slippage through the proton transport pathway [98].

It is clear that such an uncoupled slip imposes an energetic penalty on the cell. One can argue that the low metal ion to proton transport of DCT1, resulting from a proton slippage, can be explained as a necessity of the transport mechanism in which positively charged protons are driving two positive charges of the metal ion in the same direction. Recently, by the use of site-directed mutagenesis of DCT1, we generated a single mutation – F227I – that almost abolished the proton slip, without any significant changes in the metal ion transport activity and expression levels of the transporter [26]. Moreover, the DCT1 mutant retained the capability to suppress the growth arrest of the null mutant *smf1Δ* in the presence of EGTA [26]. This observation suggests that the proton slippage is not a mechanistic necessity. It supports the idea that the proton slip has a physiological advantage, and that it was positively selected during the evolution of DCT1. A minor modification in terms of evolutionary changes could eliminate the energy wasting. The evolutionary and physiological significance of this phenomenon could be explained in terms of a protection mechanism against overloading the cell with metal ions [13]. Apparently, the evolution of the system could not provide an alternative driving force for the proton electrochemical gradient.

Considering that excess metal-ions are toxic, a protection mechanism against too much transport of these elements had to be developed in DCT1 as well as in Smf1p. Several kinds of food products are highly enriched in iron, and eating too much of them can also cause heartburn. The excess acid may reach the duodenum together with high iron concentrations, and this combination of very high driving force and substrate abundance may be deleterious. Uncoupling by a built-in proton slip could protect the organism from too much metal-ions' intake. It was suggested that a similar protection might function in the sodium slip through the yeast Smf1p [25]. In this case, the yeast cells may be protected against excessive influxes of toxic metal ions by evolving a sodium slippage that competes with the metal ion uptake under conditions of increasing salt concentrations in the medium.

2.3. NRAMP1

It has long been recognized that the pathogenicity of a broad range of intracellular parasites is dependent on the availability of

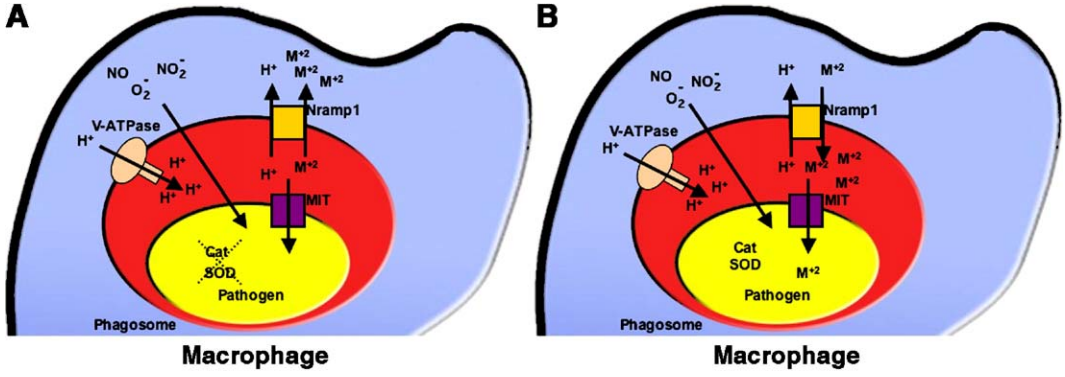

Fig. 3. Two hypotheses for the function of Nramp1 in macrophage–pathogen interaction. The infecting bacterium is taken into the macrophage phagosome where it is challenged by reactive oxygen components. The bacterium protects itself by producing metalloenzymes, such as catalase (Cat) and superoxide dismutase (SOD) that can neutralize the toxic agents. Two hypotheses describe Nramp1′s contribution to the macrophage attack on the pathogen: (A) Nramp1 operates as a symporter that cotransports metal-ions and protons from the phagosomal lumen into the cytoplasm. The metal-ion depletion of the phagosomal lumen restricts the pathogen ability to produce and activate the protective enzymes (such as catalase and superoxide dismutase), and prevents the propagation of the ingested microorganisms. (B) Nramp1 operates as an antiporter of protons and metal-ions. It transports metal-ions from the cytoplasm into the phagosomal lumen. The accumulated metal-ions in the phagosome generate highly reactive hydroxyl radicals, which contribute to the bacteriostatic effect of the macrophage. MIT—metal ion transporter; M^{2+}—divalent metal-ion.

transition metal ions [for reviews, see 101 102]. The discovery of a macrophage protein known to confer resistance to intracellular parasites Nramp1 (Natural resistance associated macrophage protein) and its recognition as a metal ion transporter substantiated it [22,34,103].

We proposed that the role of Nramp1 in macrophage defense against microbial invasion is to reduce the metal-ion concentrations inside the bacteria-containing phagosomes (Fig. 3A) [22,23]. Consequently, it limits the production and function of the engulfed bacterial metalo-enzymes required for their defense against reactive oxygen and/or nitrogen toxic intermediates that are poured upon them by the phagocytes [104,105]. It was proposed that Nramp1, like its yeast homologue, transports metal-ions from the phagosomal lumen into the cytoplasm. Thus, the metal-ion depletion of the phagosomal lumen becomes a rate-limiting step in metalloenzymes' function of the engulfed bacteria. This restricts the mycobacterial ability to produce and activate enzymes such as SOD, and prevents the propagation of the ingested microorganisms (Fig. 3A). Conversely, an increased concentration of metal-ions in the phagosome caused by a defective Nramp1 transporter (Bcgs) may promote the growth of the mycobacteria and render the invaded organism sensitive to the pathogen [22,27,103].

The above hypothesis was addressed by several studies, proving that the transporter indeed transports divalent metal ions (including Mn^{2+}, Fe^{2+}, and Co^{2+}) [68,84]. However, in respect to the directionality of the metal-ion pumping by Nramp1 in macrophages, scientists differ. One group of scientists proposes that Nramp1 pumps metal ions into the macrophage phagosomes and facilitates accumulation of metal ions in their lumen (Fig. 3B). Addition of Fe^{2+} to Nramp1 expressing macrophages in tissue culture was shown to further inhibit the growth of *Mycobacterium avium*, and that this effect could be reversed by the addition of hydroxyl radicals scavengers [106]. Moreover, Nramp1 expressing macrophages grown with $^{55}Fe^{2+}$ accumulated four times more Fe^{2+} in their phagosomes than the cells lacking Nramp1; the same was true for import into isolated phagosomes. There was a burst of hydroxyl radicals after infection in the Nramp1-expressing but not in the non-expressing cells [107]. The iron uptake was shown to be dependent on pH gradient and with its disruption by lysomotropic agents like chloroquine and ammonium chloride, the amount of iron import to isolated Nramp1-expressing phagosomes decreased. This iron uptake could also be inhibited by treatment of the phagosomes with an antibody against the putative outer fourth loop [108]. In another work, expression of Nramp1 in *Xenopus* oocytes, demonstrated that it could transport Fe^{2+}, Zn^{2+} and Mn^{2+} in exchange with H^+[109]. Although large metal-ion induced currents were recorded, the metal ion transport was extremely low. The activity of *Nramp1* mRNA injected oocytes was only up to 2-fold in comparison with water-injected oocytes, whereas with *Nramp2* (DCT1) injected oocytes, rates that are more than 1000-fold in the background are frequently obtained. This makes one wonder what the source of the large metal-ion induced currents is. These findings led this group to believe that Nramp1 functions as a pH-dependent antiporter, importing divalent cations into the bacteria-containing phagosome. The accumulated cations in the phagosome generate highly reactive hydroxyl radicals by the Haber–Weiss reaction, which contributes to the bacteriostatic effect of the macrophage on the engulfed bacteria [106–109].

The other group of scientists proposes that the transporter pumps out the metal ions from the phagosome into the cytoplasm (Fig. 3A). They showed that, in Nramp1 expressing macrophage, 28% of the iron was bound to ferritin and 60% was in a soluble fraction, namely in the cytoplasm [110]. Upon induction of the macrophage, the iron in the cytoplasm was increased to 82% at the expense of iron bound to ferritin that was less than 5%. This suggests that Nramp1 is required for releasing iron from phagosomes and transporting it into the cytoplasm [110]. Using calcein as a marker, it was demonstrated that Nramp1 could transport iron from vesicles to the cytoplasm in relaxed macrophage [111]. Monitoring zymosan-FF6 as a phagocytosed indicator, it was shown that phagosomes from wild type mice transported Mn^{2+} out of the phagosome significantly faster as compared to knockout mice. This transport was dependent on the pH gradient generated by V-ATPase [112]. In analogy, *Nramp2* (DCT1) was found to associate with erythrocyte-containing phagosomes, suggesting a role in recycling of iron from dying erythrocytes, which are the main source of iron in our body. *Nramp2* was also found in sperm containing phagosomes of Sertoli cells, suggesting a role in iron recycling from degenerating spermatozoids [84]. This also suggests that the transporter works by exporting metal ions from the phagosome into the cytoplasm. These investigators argue that the transport of divalent cations out of the microenvironment of the bacteria (phagosome) by Nramp1 results in an enhanced bacteriostatic activity. Recently, it was demonstrated that Nramp1 behaves just like DCT1, when this protein is expressed at the plasma membrane of transfected CHO cells [68]. Nramp1 modified by insertion of a hemagglutinin epitope into the predicted TM (transmembrane) 7/8 loop was expressed at the plasma membrane (Nramp1HA). The detection of the HA tag by immunofluorescence in non-permeabilized cells confirmed that the loop is extracellular, as previously demonstrated for DCT1 [68,113]. Additionally, Nramp1 detection by antibodies directed against the amino terminus of the protein in Triton X-100 permeabilized cells, but not in intact cells, demonstrated that the N-terminus of Nramp1 was intracellular. Together, these results indicate that Nramp1 has a membrane topology similar to that of DCT1. Experiments using radioisotopic $^{55}Fe^{2+}$ and $^{54}Mn^{2+}$ as well as metal-sensitive fluorophores calcein and Fura2, demonstrated that both Nramp1 and DCT1 mediate the cellular uptake of Fe^{2+}, Mn^{2+} and Co^{2+}, and that the transport occurs down the proton gradient [68]. These experiments also suggested that Nramp1 may be a more efficient transporter of Mn^{2+} compared to Fe^{2+}, and a more efficient Mn^{2+} transporter than DCT1. The membrane topology and transport properties of Nramp1 and DCT1 presented in this study were indistinguishable. This suggests that Nramp1 divalent-metal transport at the phagosomal membrane is mechanistically similar to that of DCT1, i.e. that is acts as a H^+/metal-ion cotransporter.

Regardless of the directionality of the transport, there is no dispute about the necessity of transport of divalent cations by this transporter for defense against bacterial infection [54]. However, as mentioned above, we favor the metal ion depletion hypothesis, where the reduction in metal ion concentration in the phagosomal

lumen becomes a rate-limiting step in metalloenzymes' production by the engulfed bacteria (Fig. 3A).

3. Structure–function analysis

Alignment of the amino acid sequences of the NRAMP family members identifies a common and conserved hydrophobic core of 10 TM domains and either one or two non-conserved, highly hydrophobic TM domains. The TM domains are predicted from hydropathy profiling, calculations of hydrophobic moment, and other computer-assisted analyses [114], and from direct epitope-mapping studies. These mapping studies demonstrated that bout proteins, Nramp1 and DCT1, have 12 TM domains, the location of the loop between TM7 and TM8 is extracellular and the location of the N and C-termini is intracellular (Fig. 4) [48,50,68,113]. The topology mapping of MntH, the *E. coli* Nramp Ortholog, revealed an 11 TM domain protein with intracellular N-terminus and extracellular C-terminus [115].

There are four known major mammalian DCT1 isoforms, resulting from alternative splicing at the 5′ and 3′ ends of the pre–messenger RNA (Fig. 4) [116]. Isoform I has an iron responsive element (IRE) in the 3′ untranslated region, whereas isoform II has an alternative 3′ untranslated region, lacking the IRE. In addition, isoforms I and II have a different C-terminus, of 18 and 25 amino-acids, respectively [114]. The two isomers differ by levels of expression in the various tissues [117].

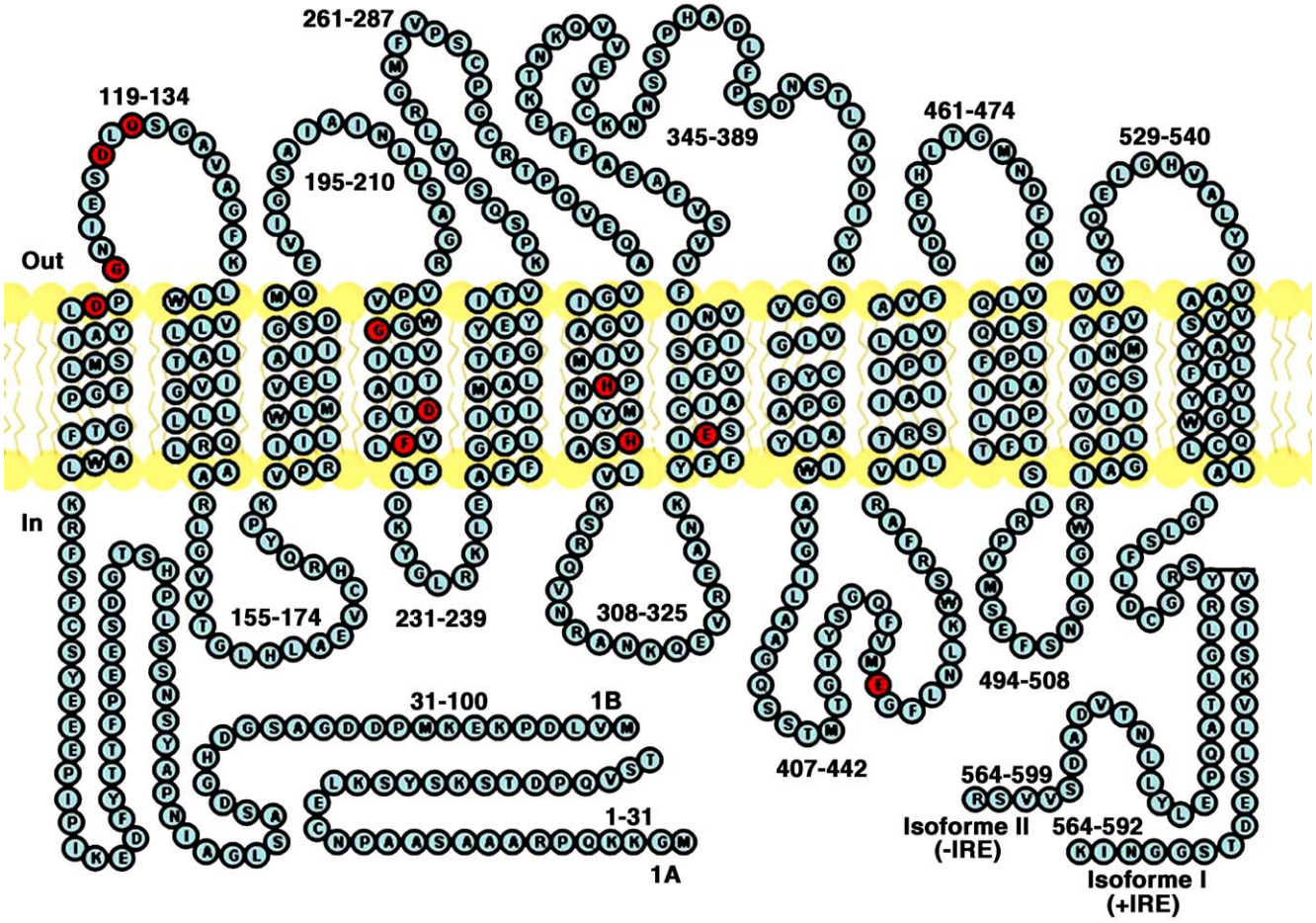

Fig. 4. Schematic representation of DCT1. The predicted 12 transmembranal (TM) domains and individual intracellular and extracellular segments are identified, along with their position within the primary sequence. The N and C-termini are intracellular, and the loop between TM7 and TM8 is extracellular. There are four known major mammalian DCT1 isoforms, resulting from alternative splicing at the 5′ and 3′ ends of the pre–messenger RNA. Isoform I has an iron responsive element (IRE) in the 3′ untranslated region, whereas isoform II has an alternative 3′ untranslated region, lacking the IRE. In addition, isoforms I and II have different C-terminus, of 18 and 25 amino-acids, respectively. Alternative 5′ exons designated 1A and 1B have also been described. The exon 1A isoform adds an in-frame AUG translation initiation codon, extending the DCT1 ORF by a conserved sequence of 31 amino acids. The amino acid sequence counting starts from the methionine of isoform 1A. In red: amino acid residues whose replacement led to significant alterations in DCT1′s properties. The mutations G119A, D124Q, Q126D in the first external loop, and the different combinations of the three, led to changes in uptake activity, in specificity to the various substrates and in the affinity to protons of DCT1. The mutation G216R in TM4 causes microcytic anemia in mk−/− mice and Belgrade rats. The mutation F227I in TM4 almost abolished the proton slip generated by DCT1, without any significant changes in the metal ion transport activity and expression levels of the transporter. Three negatively charged and highly conserved residues — D117, D223, E330 in TM1, 4, and 7, respectively, are essential for metal transport. The mutations H267A and H272A in TM6 demonstrated decreased uptake activity as compared to that of wt DCT1. The metal uptake by H272A became independent of pHo. In addition, H272A generated an approximate 10-fold increase in the leak current and made it sensitive to the presence of Fe2+ in the medium. The mutation E430D was identified in a human female with severe hypochromic microcytic anemia and iron overload. This mutation severely impairs splicing and introduces the mutation E430D into the fourth internal loop of the protein encoded by the remaining, properly spliced transcript, found in the patient (membrane—yellow; intracellular—In; extracellular—Out).

Alternative 5′ exons designated 1A and 1B have also been described [116]. The exon 1A isoform adds an in-frame AUG translation initiation codon, extending the DCT1 ORF by a conserved sequence of 29–31 amino acids. The expression of this 5′ exon is probably part of the iron regulation of DCT1, and is tissue-specific (particularly prevalent in the duodenum and kidney) [116].

As the first step in structure–function analysis, site-directed mutagenesis was employed. A concerted approach for the study of Smf1p and DCT1 expressed in yeast cells and *Xenopus* oocytes was developed [26,61,100]. It uses yeast null mutants lacking the SMF metal ion transporters, and *Xenopus* oocytes, where those transporters are absent, as an expression medium for Smf1p and DCT1 in appropriate plasmids [60]. As mentioned above, the null mutant *smf1Δ* is unable to grow in the presence of EGTA [22,99]. Expression of Smf1p or the mammalian transporter DCT1 suppresses the above-mentioned phenotype and allows growth of the yeast mutant in the presence of EGTA. Expression of the same genes in parallel in *Xenopus* oocytes are ideal heterologous expression systems for metal ion transporters [118], which can reveal retained partial activities of the transporter that we are unable to measure in the yeast system.

Mutations in the first external loop of DCT1 were generated and the mutated transporter was expressed in both yeast and oocytes. Almost every mutation changed the properties of the transporter. The residues to which their replacement gave the most significant changes were G119, D124, Q126 and the different combination of the three (Fig. 4). Changes in uptake activity, in specificity to the various substrates and in the affinity to protons, were observed [61]. These results suggested that loop 1 is involved in the metal ion binding and coupling of the metal-ion transport to the proton-driving force.

By use of the same approach, residues in the putative TM4 of DCT1 were mutated. The mutation G216R in TM4 causes microcytic anemia in $mk^{-/-}$ mice and Belgrade rats (Fig. 4) [57]. The G216R mutation was subsequently shown to result in loss of Fe^{2+} transport ability [1]. This suggests that the TM4 may have an important biological function. A mutation F227I in TM4 (Fig. 4) was generated and resulted in an increase of up to 14-fold in the ratio between metal ions to protons transported without any significant changes in the metal ion transport activity and expression levels of the transporter [26]. This result supports the suggested importance of TM4 in the uptake mechanism and also supports the idea that the slippage, which was almost abolished by this mutation, is not a mechanistic necessity. Proton slippage probably has a physiological advantage, and this property was positively selected during the evolution of DCT1.A similar approach to study structure–function relationships in the NRAMP family was employed [68]. Mutant cDNAs are expressed in yeast and were tested for their ability to complement the phenotypes of the *smf1,2Δ* mutant (impaired growth on metal chelators and impaired growth at alkaline pH). Mutants showing partial or complete loss of function are subsequently expressed in CHO cells and tested for Fe^{2+} and Co^{2+} transport at the plasma membrane. These studies identified 3 negatively charged and highly conserved residues — D117A, D223A, E330A in TM 1, 4, and 7 respectively, as essential for metal transport by DCT1 (Fig. 4). The mutation G216R caused a partial or complete loss of function in mammalian and yeast cells, respectively. A pair of mutation-sensitive and highly conserved histidines (His267, His272) was identified in TM6 (Fig. 4). Inactive His267 and His272 mutants could be rescued by lowering the pH of the transport assay. This indicates that His267/His272 are not directly involved in metal binding, but rather play an important role in pH regulation of metal transport by NRAMP proteins [68]. Subsequently, expression of DCT1 mutants H267A and H272A in oocytes demonstrated a decrease of more than 75% in $^{55}Fe^{2+}$ uptake activity as compared to that of wt DCT1 [119]. However, unlike H267A that, except for the decrease in the activity, relatively maintained the transporter properties of wt DCT1, H272A generated an approximate 10-fold increase in the leak current. In contrast to wt DCT1, in which the leak current depends on the presence of the metal, Fe^{2+} inhibited the leak current mediated by H272A. The $^{55}Fe^{2+}$ uptake mediated by H272A was independent of pH_o (7.0, 6.1, or 5.2), whereas wt DCT1 mediated $^{55}Fe^{2+}$ uptake is markedly stimulated at low pH_o. This increase in leak current can explain the decrease in metal ion uptake by H272 (Fig. 2) [98].

The location of the mutation in DCT1 reviewed above (the first external loop, TM 4 and 6) signifies the importance of these regions in the transporter activity. The first loop probably takes part in the metal ion and protons, binding and coupling, and TM4 and 6 probably participate in the formation of the selective pathway through which the protons and metal ion pass. Other domains in DCT1, which are involved in the transport process, are yet to be identified. Only high-resolution structure of one or more of the NRAMP family members may provide a detailed description of the unusual mechanism of operation by this important family of metal ion transporters.

References

[1] M.D. Fleming, M.A. Romano, M.A. Su, L.M. Garrick, M.D. Garrick, N.C. Andrews, Nramp2 is mutated in the anemic Belgrade (b) rat: evidence of a role for Nramp2 in endosomal iron transport, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 1148–1153.

[2] T. Herrmann, M. Muckenthaler, F. van der Hoeven, K. Brennan, S.G. Gehrke, N. Hubert, C. Sergi, H.J. Grone, I. Kaiser, I. Gosch, M. Volkmann, H.D. Riedel, M.W. Hentze, A.F. Stewart, W. Stremmel, Iron overload in adult Hfe-deficient mice independent of changes in the steady-state expression of the duodenal iron transporters DMT1 and Ireg1/ferroportin, J. Mol. Med. 82 (2004) 39–48.

[3] M. Muckenthaler, C.N. Roy, A.O. Custodio, B. Minana, J. deGraaf, L.K. Montross, N.C. Andrews, M.W. Hentze, Regulatory defects in liver and intestine implicate abnormal hepcidin and Cybrd1 expression in mouse hemochromatosis, Nat. Genet. 34 (2003) 102–107.

[4] P. Fergelot, M. Orhant, A. Thenie, P. Loyer, M. Ropert-Bouchet, S. Lohyer, J.Y. Le Gall, J. Mosser, Over-expression of wild-type and mutant HFE in a human melanocytic cell line reveals an intracellular bridge between MHC class I pathway and transferrin iron uptake, Biol. Cell 95 (5) (2003) 243–255.

[5] W. Li, A. Hellsten, J.D. Nyhalah, X.M. Yuan, Enhanced expression of natural resistance-associated macrophage protein 1 in atherosclerotic lesions may be associated with oxidized lipid-induced apoptosis, Ann. N. Y. Acad. Sci. 1030 (2004) 202–207.

[6] M. Babcock, D. de Silva, R. Oaks, S. Davis-Kaplan, S. Jiralerspong, L. Montermini, M. Pandolfo, J. Kaplan, Regulation of mitochondrial iron accumulation by Yfh1p, a putative homolog of frataxin, Science 276 (1997) 1709–1712.

[7] N.C. Andrews, J.E. Levy, Iron is hot: an update on the pathophysiology of hemochromatosis, Blood 92 (1998) 1845–1851.
[8] C. Askwith, J. Kaplan, Iron copper transport in yeast and its relevance to human disease, Trends Biochem. Sci. 23 (1998) 135–138.
[9] T. Moos, E.H. Morgan, The metabolism of neuronal iron and its pathogenic role in neurological disease: review annals. NYAS, Online 1012 (2004) 14–26.
[10] D. Radisky, J. Kaplan, Regulation of transition metal transport across the yeast plasma membrane, J. Biol. Chem. 27 (48) (1999) 4481–4484.
[11] M.A. Hediger, Membrane permeability. The diversity of transmembrane transport processes, Curr. Opin. Cell Biol. 9 (4) (1997) 543–546.
[12] D.J. Eide, The molecular biology of metal ion transport in *Saccharomyces cerevisiae*, Annu. Rev. Nutr. 18 (1998) 441–469.
[13] N. Nelson, Metal ion transporters and homeostasis, EMBO J. 18 (16) (1999) 4361–4371.
[14] S.R. Davis-Kaplan, D.M. Ward, S.L. Shiflett, J. Kaplan, Genome-wide analysis of iron-dependent growth reveals a novel yeast gene required for vacuolar acidification, J. Biol. Chem. 279 (2004) 4322–4329.
[15] D. Eide, Molecular biology of iron and zinc uptake in eukaryotes, Curr. Opin. Cell Biol. 9 (4) (1997) 573–577.
[16] D. Blaudez, A. Kohler, F. Martin, D. Sanders, M. Chalot, Poplar metal tolerance protein 1 confers zinc tolerance and is an oligomeric vacuolar zinc transporter with an essential leucine zipper motif, Plant Cell 15 (2004) 2911–2928.
[17] S.E. Abdel-Ghany, P. Muller-Moule, K.K. Niyogi, M. Pilon, T. Shikanai, Two p-type ATPases are required for copper delivery in *Arabidopsis thaliana* chloroplasts, Plant Cell 17 (2005) 1233–1251.
[18] J.R. Forbes, P. Gros, Divalent-metal transport by NRAMP proteins at the interface of host–pathogen interactions, Trends Microbiol. 9 (8) (2001) 397–403.
[19] A. Van Ho, D.M. Ward, J. Kaplan, Transition metal transport in yeast, Annu. Rev. Microbiol. 56 (2002) 237–261.
[20] T. Goswami, A. Rolfs, M.A. Hediger, Iron transport: emerging roles in health and disease, Biochem. Cell. Biol. 80 (2002) 679–689.
[21] D. Agranoff, L. Collins, D. Kehres, T. Harrison, M. Maguire, S. Krishna, The Nramp orthologue of *Cryptococcus neoformans* is a pH-dependent transporter of manganese, iron, cobalt and nickel, Biochem. J. 385 (2005) 225–232.
[22] F. Supek, L. Supekova, H. Nelson, N. Nelson, A yeast manganese transporter related to the macrophage protein involved in conferring resistance to mycobacteria, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 5105–5110.
[23] F. Supek, L. Supekova, H. Nelson, N. Nelson, Function of metal-ion homeostasis in the cell division cycle, mitochondrial protein processing, sensitivity to mycobacterial infection and brain function, J. Exp. Biol. 200 (1997) 321–330.
[24] X.F. Liu, F. Supek, N. Nelson, V.C. Culotta, Negative control of heavy metal uptake by the *Saccharomyces cerevisiae* BSD2 gene, J. Biol. Chem. 272 (1997) 11763–11769.
[25] X.Z. Chen, J.B. Peng, A. Cohen, H. Nelson, N. Nelson, M.A. Hediger, Yeast SMF1 mediates H(+)-coupled iron uptake with concomitant uncoupled cation currents, J. Biol. Chem. 274 (1999) 35089–35094.
[26] Y. Nevo, N. Nelson, The mutation F227I increases the coupling of metal ion transport in DCT1, J. Biol. Chem. 27951 (2004) 53056–53061.
[27] M. Cellier, G. Prive, A. Belouchi, T. Kwan, V. Rodrigues, W. Chia, P. Gros, Nramp defines a family of membrane proteins, Proc. Natl. Acad. Sci. U. S. A. 92 (22) (1995) 10089–10093.
[28] H. Gunshin, B. Mackenzie, U.V. Berger, Y. Gunshin, M.F. Romero, W.F. Boron, S. Nussberger, J.L. Gollan, M.A. Hediger, Cloning and characterization of a proton-coupled mammalian metal-ion transporter, Nature 388 (1997) 482–488.
[29] A.H. West, D.J. Clark, J. Martin, Two related genes encoding extremely hydrophobic proteins suppress a lethal mutation in the yeast mitochondrial processing enhancing protein, J. Biol. Chem. 267 (34) (1992) 24625–24633.
[30] R.A. Pollock, F.U. Hartl, M.Y.J. Ostermann, A. Horwich, W. Neupert Cheng, The processing peptidase of yeast mitochondria: the two cooperating components MPP and PEP are structurally related, EMBO J. 7 (11) (1988) 3493–3500.
[31] M. Yang, R.E. Jensen, M.P. Yaffe, W. Oppliger, G. Schatz, Import of proteins into yeast mitochondria: the purified matrix processing protease contains two subunits which are encoded by the nuclear MAS1 and MAS2 genes, EMBO J. 7 (12) (1988) 3857–3862.
[32] C. Witte, R.E. Jensen, M.P. Yaffe, G. Schatz, MAS1, a gene essential for yeast mitochondrial assembly, encodes a subunit of the mitochondrial processing protease, EMBO J. 7 (5) (1988) 1439–1447.
[33] G. Hawlitschek, H. Schneider, B. Schmidt, M. Tropschug, F.U. Hartl, W. Neupert, Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein, Cell 53 (5) (1988) 795–806.
[34] S.M. Vidal, D. Malo, K. Vogan, E. Skamene, P. Gros, Natural resistance to infection with intracellular parasites: isolation of a candidate for *Bcg*, Cell 73 (1993) 469–485.
[35] J.E. Plant, J.M. Blackwell, A.D. O'Brien, D.J. Bradley, A.A. Glynn, Are the *Lsh* and *Ity* disease resistance genes at one locus on mouse chromosome 1? Nature 297 (5866) (1982) 510–511.
[36] M. Arredondo, P. Muñoz, C.V. Mura, M.T. Núñez, DMT1, a physiologically relevant apical Cu^{1+} transporter of intestinal cells, Am. J. Physiol. 284 (2003) C1525–C1530.
[37] Z. Bereczky, H.Y. Wang, V. Schubert, M. Ganal, P. Bauer, Differential regulation of nramp and irt metal transporter genes in wild type and iron uptake mutants of tomato, J. Biol. Chem. 278 (2003) 24697–24704.
[38] P. Marciani, D. Trotti, M.A. Hediger, G. Monticelli, Modulation of DMT1 activity by redox compounds, J. Membr. Biol. 197 (2) (2004) 91–99.
[39] R. Chaloupka, P. Courville, F. Veyrier, B. Knudsen, T.A. Tompkins, M.F. Cellier, Identification of functional amino acids in the Nramp family by a combination of evolutionary analysis and biophysical studies of metal and proton cotransport in vivo, Biochemistry 44 (2005) 726–733.
[40] V.C. Culotta, M. Yang, M.D. Hall, Manganese transport and trafficking: lessons learned from *Saccharomyces cerevisiae*, Eukar. Cell 4 (2005) 1159–1165.
[41] M. Hanikenne, U. Kramer, V. Demoulin, D. Baurain, A comparative inventory of metal transporters in the green alga *Chlamydomonas reinhardtii* and the red alga *Cyanidioschizon merolae*, Plant Ohysiol. 137 (2005) 428–446.
[42] J.K. Pittman, Managing the manganese: molecular mechanisms of manganese transport and homeostasis, New Phytol. 167 (2005) 733–742.
[43] A. Rosakis, W. Koster, Divalent metal transport in the green microalga *Chlamydomonas reinhardtii* is mediated by a protein similar to prokaryotic Nramp homologues, Biometals 18 (2005) 107–120.
[44] T. Takahashi, T. Furuchi, A. Naganuma, A novel role for Bsd2 in the resistance of yeast to adriamycin, J. Cell. Physiol. 202 (2005) 100–104.
[45] W. Zhang, L. Shao, X. Weng, Z. Hu, A. Jin, S. Chen, M. Pang, Z.W. Chen, Variants of the natural resistance-associated macrophage protein 1 gene (NRAMP1) are associated with severe forms of pulmonary tuberculosis, Clin. Infect. Dis. 40 (9) (2005) 1232–1236.
[46] V. Rodrigues, P.Y. Cheah, K. Ray, W. Chia, malvolio, the *Drosophila* homologue of mouse NRAMP-1 (*Bcg*), is expressed in macrophages and in the nervous system and is required for normal taste behaviour, EMBO J. 14 (13) (1995) 3007–3020.
[47] M. Cellier, G. Govoni, S. Vidal, T. Kwan, N. Groulx, J. Liu, F. Sanchez, E. Skamene, E. Schurr, P. Gros, Human natural resistance-associated macrophage protein: cDNA cloning, chromosomal mapping, genomic organization, and tissue-specific expression, J. Exp. Med. 180 (1994) 1741–1752.
[48] S. Gruenheid, M. Cellier, S. Vidal, P. Gros, Identification and characterization of a second mouse *Nramp* gene, Genomics 25 (1995) 514–525.
[49] F. Kishi, Isolation and characterization of human Nramp cDNA, Biochem. Biophys. Res. Commun. 204 (1994) 1074–1080.
[50] F. Kishi, M. Tabuchi, Human natural resistance-associated macrophage protein 2: gene cloning and protein identification, Biochem. Biophys. Res. Commun. 251 (1998) 775–783.
[51] S. Vidal, A.M. Belouchi, M. Cellier, B. Beatty, P. Gros, Cloning and characterization of a second human NRAMP gene on chromosome 12q13, Mammal. Genome 6 (1995) 224–230.
[52] M. Paidhungat, S. Garrett, Cdc1 is required for growth and Mn^{2+} regulation in *Saccharomyces cerevisiae*, Genetics 1484 (1998) 1777–1786.
[53] M. Paidhungat, S. Garrett, Cdc1 and the vacuole coordinately regulate Mn^{2+} homeostasis in the yeast *Saccharomyces cerevisiae*, Genetics 1484 (1998) 1787–1798.

[54] A. cohen, H. Nelson, N. Nelson, Metal-ion transporters from yeast to human diseases, in: M. Cellier, P. Gros (Eds.), The NRAMP family, Eurekah Bioscience Collection, 2003.
[55] S. Orgad, H. Nelson, D. Segal, N. Nelson, Metal ions suppress the abnormal taste behavior of the *Drosophila* mutant *malvoliom*, J. Exp. Biol. 201 (1998) 115–120.
[56] Y. Ben-Shahar, N.L. Dudek, G.E. Robinson, Phenotypic deconstruction reveals involvement of manganese transporter malvolio in honey bee division of labor, J. Exp. Biol. 207 (2004) 3281–3288.
[57] M.D. Fleming, C.C. Trenor, M.A. Su, D. Foernzler, D.R. Beier, W.F. Dietrich, N.C. Andrews, Microcytic anaemia mice have a mutation in *Nramp2*, a candidate iron transporter gene, Nat. Genet. 16 (1997) 383–386.
[58] L.J. DeFelice, Transporter structure and mechanism, Trends Neurosci. 27 (2004) 352–359.
[59] J. Abramson, I. Smirnova, V. Kasho, G. Verner, H.R. Kaback, S. Iwata, Structure and mechanism of the lactose permease of *Escherichia coli*, Science 301 (2003) 610–615.
[60] A. Sacher, A. Cohen, N. Nelson, Properties of the mammalian and yeast metal-ion transporters DCT1 and Smf1p expressed in *Xenopus laevis* oocytes, J. Exp. Biol. 2046 (2001) 1053–1061.
[61] A. Cohen, Y. Nevo, N. Nelson, The first external loop of the metal-ion transporter DCT1 is involved in metal-ion binding and specificity, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 10694–10699.
[62] F. Canonne-Hergaux, M.D. Fleming, J.E. Levy, S. Gauthier, T. Ralph, V. Picard, N.C. Andrews, P. Gros, 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 (2000) 3964–3970.
[63] F. Canonne-Hergaux, A.S. Zhang, P. Ponka, P. Gros, Characterization of the iron transporter DMT1 (NRAMP2/DCT1) in red blood cells of normal and anemic *mk/mk* mice, Blood 98 (2001) 3823–3830.
[64] D.I. Bannon, R. Abounader, P.S. Lees, J.P. Bressler, Effect of DMT1 knockdown on iron, cadmium, and lead uptake in Caco-2 cells, Am. J. Physiol. 284 (2003) C44–C50.
[65] S. Tandy, M. Williams, A. Leggett, M. Lopez-Jimenez, M. Dedes, B. Ramesh, S.K. Srai, P. Sharp, Nramp2 expression is associated with pH-dependent iron uptake across the apical membrane of human intestinal Caco-2 cells, J. Biol. Chem. 275 (2000) 1023–1029.
[66] P. Marciani, D. Trotti, M.A Hediger, G. Monticelli, Modulation of DMT1 activity by redox compounds, J. Membr. Biol. 197 (2) (2004) 91–99.
[67] M. Okubo, K. Yamada, M. Hosoyamada, T. Shibasaki, H. Endou, Cadmium transport by human Nramp 2 expressed in *Xenopus laevis* oocytes, Toxicol. Appl. Pharmacol. 187 (3) (2003) 162–167.
[68] J.R. Forbes, P. Gros, Iron, manganese, and cobalt transport by Nramp1 (Slc11a1) and Nramp2 (Slc11a2) expressed at the plasma membrane, Blood 102 (2003) 1884–1892.
[69] N. Touret, W. Furuya, J. Forbes, P. Gros, S. Grinstein, Dynamic traffic through the recycling compartment couples the metal transporter Nramp2 (DMT1) with the transferrin receptor, J. Biol. Chem. 278 (2003) 25548–25557.
[70] D. Bannon, M.E. Portnoy, L. Olivi, P.S. Lees, V.C. Culotta, J.P. Bressler, Uptake of lead and iron by divalent metal transporter 1 in yeast and mammalian cells, Biochem. Biophys. Res. Commun. 295 (2002) 978–984.
[71] J.P. Bressler, L. Olivi, J.H. Cheong, Y. Kim, D. Bannona, Divalent metal transporter 1 in lead and cadmium transport, Ann. N. Y. Acad. Sci. 1012 (2004) 142–152 (Review).
[72] N.C. Andrews, Iron homeostasis: insights from genetics and animal models, Nat. Rev., Genet. 1 (2000) 208–217.
[73] M. Wessling-Resnick, Iron transport, Annu. Rev. Nutr. 20 (2000) 129–151.
[74] E. Huang, W.Y. Ong, J.R. Connor, Distribution of divalent metal transporter-1 in the monkey basal ganglia, Neuroscience 1283 (2004) 487–496.
[75] Y.Z. Chang, Z.M. Qian, K. Wang, L. Zhu, X.D. Yang, J.R. Du, L. Jiang, K.P. Ho, Q. Wang, Y. Ke, Effects of development and iron status on ceruloplasmin expression in rat brain, J. Cell. Physiol. 204 (2005) 623–631.
[76] F. Canonne-Hergaux, S. Gruenheid, P. Ponka, P. Gros, Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron, Blood 93 (1999) 4406–4417.
[77] L.M. Garrick, K.G. Dolan, M.A. Romano, M.D. Garrick, Non-transferrin-bound iron uptake in Belgrade and normal rat erythroid cells, J. Cell. Physiol. 178 (1999) 349–358.
[78] S. Gruenheid, F. Canonne-Hergaux, S. Gauthier, D.J. Hackam, S. Grinstein, P. Gros, The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes, J. Exp. Med. 189 (1999) 831–841.
[79] M.A. Su, C.C. Trenor, J.C. Fleming, M.D. Fleming, N.C. Andrews, The G185R mutation disrupts function of the iron transporter Nramp2, Blood 92 (1998) 2157–2163.
[80] M. Tabuchi, T. Yoshimori, K. Yamaguchi, T. Yoshida, F. Kishi, Human NRAMP2/DMT1, which mediates iron transport across endosomal membranes, is localized to late endosomes and lysosomes in HEp-2 cells, J. Biol. Chem. 275 (2000) 22220–22228.
[81] Y. Gruper, J. Bar, E. Bacharach, R. Ehrlich, Transferrin receptor co-localizes and interacts with the hemochromatosis factor (HFE) and the divalent metal transporter-1 (DMT1) in trophoblast cells, J. Cell. Physiol. 204 (3) (2005) 901–912.
[82] M.D. Garrick, K. Gniecko, Y. Liu, D.S. Cohan, L.M. Garrick, Transferrin and the transferrin cycle in Belgrade rat reticulocytes, J. Biol. Chem. 68 (1993) 14867–14874.
[83] B.J. Bowen, E.H. Morgan, Anemia of the Belgrade rat: evidence for defective membrane transport of iron, Blood 70 (1987) 38–44.
[84] N. Jabado, F. Canonne-Hergaux, S. Gruenheid, V. Picard, P. Gros, Iron transporter Nramp2/DMT-1 is associated with the membrane of phagosomes in macrophages and Sertoli cells, Blood 100 (2002) 2617–2622.
[85] H. Gunshin, Y. Fujiwara, A.O. Custodio, C. DiRenzo, S. Robine, N.C. Andrews, Slc11a2 is required for intestinal iron absorption and erythropoiesis but dispensable in placenta and liver, J. Clin. Invest. 115 (2005) 1258–1266.
[86] S. Lam-Yuk-Tseung, G. Govoni, P. Gros, Iron transport by NRAMP2/DMT1: pH regulation of transport by two histidines in transmembrane domain 6, Blood 101 (2003) 3699–3707.
[87] M.P. Mims, Y. Guan, D. Pospisilova, M. Priwitzerova, K. Indrak, P. Ponka, V. Divoky, J.T. Prchal, Identification of a human mutation of DMT1 in a patient with microcytic anemia and iron overload, Blood 105 (3) (2005) 1337–1342.
[88] S. Lam-Yuk-Tseung, M. Mathieu, P. Gros, Functional characterization of the E399D DMT1/NRAMP2/SLC11A2 protein produced by an exon 12 mutation in a patient with microcytic anemia and iron overload, Blood Cells Mol. Diseases 352 (2005) 212–216.
[89] A.T. McKie, D. Barrow, G.O. Latunde-Dada, A. Rolfs, G. Sager, E. Mudaly, M. Mudaly, C. Richardson, D. Barlow, A. Bomford, T.J. Peters, K.B. Raja, S. Shirali, M.A. Hediger, F. Farzaneh, R.J. Simpson, An iron-regulated ferric reductase associated with the absorption of dietary iron, Science 2, 291 (5509) (2001) 1755–1759.
[90] H. Gunshin, C.N. Starr, C. Direnzo, M.D. Fleming, J. Jin, E.L. Greer, V.M. Sellers, S.M. Galica, N.C. Andrews, Cybrd1 (duodenal cytochrome b) is not necessary for dietary iron absorption in mice, Blood 1068 (2005) 2879–2883.
[91] C.D. Vulpe, Y. Kuo, T.L. Murphy, L. Cowley, C. Askwith, N. Libina, J. Gitschier, G.J. Anderson, Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the *sla* mouse, Nat. Genet. 21 (1999) 195–199.
[92] A. Donovan, A. Brownlie, Y. Zhou, J. Shepard, S.J. Pratt, J. Moynihan, B.H. Paw, A. Drejer, B. Barut, A. Zapata, T.C. Law, C. Brugnara, S.E. Lux, G.S. Pinkus, P.D. Kingsley, J. Palis, M.D. Fleming, N.C. Andrews, L.I. Zon, Positional cloning of zebrafish *ferroportin1* identifies a conserved vertebrate iron exporter, Nature 403 (2000) 776–781.
[93] A.T. McKie, P. Marciani, A. Rolfs, K. Brennan, K. Wehr, D. Barrow, S. Miret, A. Bomford, T.J. Peters, F. Farzaneh, M.A. Hediger, M.W. Hentze, R.J. Simpson, A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation, Mol. Cell 5 (2000) 299–309.
[94] S. Abboud, D.J. Haile, A novel mammalian iron-regulated protein involved in intracellular iron metabolism, J. Biol. Chem. 275 (2000) 19906–19912.
[95] C. Delaby, N. Pilard, A.S. Goncalves, C. Beaumont, F. Canonne-Hergaux, Presence of the iron exporter ferroportin at the plasma membrane of macrophages is enhanced by iron loading and down-regulated by hepcidin, Blood 106 (12) (2005) 3979–3984.

[96] D.M. Frazer, G.J. Anderson, The orchestration of body iron intake: how and where do enterocytes receive their cues? Blood Cells Mol. Diseases 30 (2003) 288–297.
[97] J. Petrak, D. Vyoral, Hephaestin — a ferroxidase of cellular iron export, Int. J. Biochem. Cell Biol. 37 (6) (2005) 1173–1178 (Review).
[98] N. Nelson, A. Sacher, H. Nelson, The significance of molecular slips in transport systems, Nat. Rev. Mol. Cell Biol. 3 (11) (2002) 876–881.
[99] A. Cohen, H. Nelson, N. Nelson, The family of SMF metal ion transporters in yeast cells, J. Biol. Chem. 275 (43) (2000) 33388–33394.
[100] E. Pinner, S. Gruenheid, M. Raymond, P. Gros, Functional complementation of the yeast divalent cation transporter family SMF by NRAMP2, a member of the mammalian natural resistance-associated macrophage protein family, J. Biol. Chem. 272 (46) (1997) 28933–28938.
[101] J.J. Bullen, The significance of iron in infection, Rev. Infect. Dis. 3 (6) (1981) 1127–1138.
[102] S.M. Payne, Iron acquisition in microbial pathogenesis, Trends Microbiol. 1 (2) (1993) 66–69.
[103] D.D. Agranoff, S. Krishna, Metal ion homeostasis and intracellular parasitism, Mol. Microbiol. 28 (3) (1998) 403–412.
[104] A.W. Segal, A. Abo, The biochemical basis of the NADPH oxidase of phagocytes, Trends Biochem. Sci. 18 (2) (1993) 43–47.
[105] Y. Chan, R.S. Xing, Magliozzo, B.R. Bloom, Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages, J. Exp. Med. 175 (4) (1992) 1111–1122.
[106] B.S. Zwilling, D.E. Kuhn, L. Wikoff, Role of iron in Nramp1-mediated inhibition of mycobacterial growth, Infect. Immun. 67 (3) (1999) 1386–1392.
[107] D.E. Kuhn, B.D. Baker, W.P. Lafuse, B.S. Zwilling, Differential iron transport into phagosomes isolated from the RAW264.7 macrophage cell lines transfected with Nramp1^{Gly169} or Nramp1^{Asp169}, J. Leukoc. Biol. 66 (1999) 113–119.
[108] D.E. Kuhn, W.P. Lafuse, B.S. Zwilling, Iron transport into *Mycobacterium avium*-containing phagosomes from an *Nramp1* ^{Gly169}-transfected RAW264.7 macrophage cell line, J. Leukoc. Biol. 69 (2001) 43–49.
[109] T. Goswami, A. Bhattacharjee, P. Babal, S. Searle, E. Moore, M. Li, J.M. Blackwell, Natural-resistance-associated macrophage protein 1 is an H^+/bivalent cation antiporter, Biochem. J. 354 (2001) 511–519.
[110] V. Mulero, S. Searle, J.M. Blackwell, J.H. Brock, Solute carrier 11a1 (Slc11a1; formerly Nramp1) regulates metabolism and release of iron acquired by phagocytic, but not transferrin-receptor-mediated, iron uptake, Biochem. J. 363 (2002) 89–94.
[111] P.G. Atkinson, C.H. Barton, High level expression of Nramp1G169 in RAW264.7 cell transfectants: analysis of intracellular iron transport, Immunology 96 (4) (1999) 656–662.
[112] N. Jabado, A. Jankowski, S. Dougaparsad, Natural resistance to intracellular infections: natural resistance-associated macrophage protein 1 (Nramp1) functions as a pH-dependent manganese transporter at the phagosomal membrane, J. Exp. Med. 192 (9) (2000) 1237–1248.
[113] V. Picard, G. Govoni, N. Jabado, P. Gros, Nramp 2 (DCT1/DMT1) expressed at the plasma membrane transports iron and other divalent cations into a calcein-accessible cytoplasmic pool, J. Biol. Chem. 275 (2000) 35738–35745.
[114] P. Lee, T. Gelbart, C. West, C. Halloran, E. Beutler, The human Nramp2 gene: characterization of the gene structure, alternative splicing, promoter region and polymorphisms, Blood Cells Mol. Diseases 24 (1998) 199–215.
[115] P. Courville, R. Chaloupka, F. Veyrier, M.F. Cellier, Determination of transmembrane topology of the *Escherichia coli* natural resistance-associated macrophage protein (Nram p) ortholog, J. Biol. Chem. 279 (2004) 3318–3326.
[116] N. Hubert, M.W. Hentze, Previously uncharacterized isoforms of divalent metal transporter (DMT)-1: implications for regulation and cellular function, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 12345–12350.
[117] D. Tchernitchko, M. Bourgeois, M. Martin, C. Beaumont, Expression of the two mRNAC isoforms of the iron transporter Nramp2/DMT1 in mice and function of the iron responsive element, Biochem. J. 363 (2002) 449–455.
[118] E.M. Wright, D.D. Loo, M. Panayotova-Heiermann, M.P. Lostao, B.H. Hirayama, B. Mackenzie, K. Boorer, G. Zampighi, 'Active' sugar transport in eukaryotes, J. Exp. Biol. 196 (1994) 197–212.
[119] B. Mackenzie, M.L. Ujwal, M.H. Chang, M.F. Romero, M.A. Hediger, Divalent metal-ion transporter DMT1 mediates both H(+)-coupled Fe(2+) transport and uncoupled fluxes, Pflugers Arch. 451 (2006) 544–558.