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***"Aspects of cellular iron homeostasis: NRAMP
transporter function and eryptosis"***

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FOREWORD

The present study focus attention on “Aspects of cellular iron homeostasis: NRAMP transporter function and eryptosis” combining aspects of both basic (first chapter: “Iron transporters NRAMP1 and NRAMP2 from *Dictyostelium discoideum* as a model of cellular iron homeostasis”) and applied physiology (second chapter: “Effects of xenobiotics on the suicidal death of erythrocytes”). Iron plays a central role in a large number of essential cellular functions but it is also potentially toxic being able to generate reactive oxygen species (ROS), that can damage DNA, phospholipids and proteins. Thus, it is of utmost importance for both the cells and the organism to maintain iron homeostasis ensuring iron supply but preventing accumulation of excess iron. SLC11 and SLC40 families are involved in iron transport and play an important role in the maintenance of iron homeostasis. The SLC11 family is comprised of two members, SLC11A1 and SLC11A2. SLC11A1 is expressed in the phagolysosome of macrophages and in the tertiary granules of neutrophils. It plays an important role in innate resistance against bacterial infection. SLC11A2 (also known as DMT1) is a key player in iron metabolism and is expressed in the proximal duodenum, immature erythroid cells, brain, placenta and kidney. Intestinal iron absorption is mediated by SLC11A2 at the apical membrane of enterocytes and is followed by basolateral exit via SLC40A1. *D. discoideum* represents a model for the study of cellular iron homeostasis, showing subcellular localization of iron transporters resembling that of macrophages. Moreover, *Dictyostelium* cells resemble macrophages for their ability to engulf bacteria and death cell, to discriminate between self and non-self and to fight potential pathogens. The *Dictyostelium* genome shares with mammals many genes regulating iron homeostasis; in particular, *D. discoideum* expresses the ortholog of SLC11A1 transporter in phago-lysosomes and that of SLC11A2 in the contractile vacuole. Mutations that reduce DMT1 activity in human patients are associated with a severe defect in erythroid iron utilization and are correlated with several diseases. DMT1 deficiency leads to an impaired erythroid differentiation hallmarked by accumulation of immature forms of erythroblast, accelerated death of erythroid precursors and a decrease survival in the erythroid progenitors. Thus, iron deficiency is associated with shortened life span of erythrocytes. The accelerated clearance of erythrocytes can be attributed to excessive hemolysis or induction of programmed cell death of erythrocytes, called eryptosis. Eryptosis is fostered by an increase in cytosolic calcium; iron deficient erythrocytes when exposed to stress conditions has been demonstrated to activate Ca^{2+} -permeable cation channel allowing Ca^{2+} entry. Ca^{2+} entry through this channels leads to activation of a scramblase with subsequent phosphatidylserine exposure, and to activation of the Gardos channels leading to KCl loss and cell shrinkage.

PLACES

The work presented in this thesis was mainly performed at the:

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Eryptosis mechanism was investigated in the laboratory headed by Prof. Dr. med. Florian Lang at
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FIRST CHAPTER:

“IRON TRANSPORTERS NRAMP1 AND NRAMP2

FROM Dictyostelium discoideum

AS A MODEL OF CELLULAR IRON HOMEOSTASIS”

1. ABBREVIATIONS

ABCB transporter: subfamily of ABC transporters (ATP-binding cassette transporters)

Bcg: bacille Calmette-Guerin

cDNA: complementary deoxyribonucleic acid

cRNA: complementary ribonucleic acid (*in vitro* synthesis)

CV: contractile vacuole

DCT1: divalent cation transporter 1

Dcytb: duodenal cytochrome b

DMT1: Divalent Metal Transporter-1

DNA: deoxyribonucleic acid

dNTPs: deoxynucleotides

EDTA: ethylenediaminetetraacetic acid

EGTA: glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid

Hepes: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HH: hereditary hemochromatosis

Mes: 2-(N-morpholino)ethanesulfonic acid

MnTH: divalent metal cation transporter

NRAMP: natural resistance-associated macrophage protein

NRAMP1: natural resistance-associated macrophage protein 1

NRAMP2: natural resistance-associated macrophage protein 2

PCR: polymerase chain reaction

rDMT1: rat DMT1

RBCs: red blood cells

ROS: reactive oxygen species

SCL11A1: solute carrier 11A

SD: standard deviation

SEM: standard error of the mean

SLC11: solute carrier family 11

SLC11A1: solute carrier family 11, Member 1

SLC11A2: solute carrier family 11, Member 2

SLC40: solute carrier 40

SLC40A1: solute carrier 40, Member 1

Smf: family of yeast metal ion transporters

Smf1 : family of yeast metal ion transporters 1

Smf2: family of yeast metal ion transporters 2

Smf3: family of yeast metal ion transporters 3

SOFA: single oocyte fluorescence assay

TfR: transferrin receptor

TMA: tetramethylammonium

TMDs: transmembrane domains

V-ATPase: vacuolar-type H⁺-ATPase

2. ABSTRACT

Iron plays a central role in a large number of essential cellular functions but it is also potentially toxic being able to generate reactive oxygen species (ROS). *Dictyostelium discoideum* harbours several iron genes with the exception of transferrin, ferritin and TfR [1, 2] and represents a model for the study of cellular iron homeostasis showing subcellular localization of iron transporters (NRAMP) resembling that of macrophages. The ortholog of NRAMP1 transporter is expressed in phago-lysosomes and is involved in resistance to bacterial infection; that of NRAMP2 is located in the contractile vacuole and contributes synergistically with NRAMP1 to regulate iron homeostasis. To better understand the function of *Dictyostelium* NRAMP proteins, they were expressed in *Xenopus laevis* oocytes by cRNA injection and functionally tested by radiochemical techniques and by two novel assays based on metal-induced changes in calcein fluorescence. To increase surface localization at the plasma membrane, the N- and C- terminus of both proteins were replaced with the corresponding regions of murine transporter DMT1, that is highly expressed at the plasma membrane of *X. laevis* oocytes [3]. Radiochemical assays showed that NRAMP1 induced iron transport is proton-dependent and it is inhibited by Mn^{2+} , Cd^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} and to a lesser extent by Zn^{2+} . In calcein-injected oocytes expressing NRAMP1 and analyzed using confocal microscopy, Fe^{2+} , Mn^{2+} and but not Fe^{3+} or Cu^{2+} led to fluorescence quenching due to their transport and accumulation into the cytoplasm of the oocytes. The novel assay SOFA showed that also Co^{2+} induced calcein quenching due to its transport into the oocytes. To conclude, *Dictyostelium* NRAMP1 is an electrogenic proton-dependent divalent metal ion transporter with a cation selectivity comparable to that of rat DMT1 [4]. NRAMP1 colocalizes with V-ATPase in the membrane of phago-lysosomes. Therefore, it exploits the proton gradient maintained by the V-ATPase to mediate the efflux of iron and manganese from the phago-lysosomes to the cytosol after bacterial engulfment. Preliminary studies showed that *D. discoideum* NRAMP2 can transport ferrous iron at neutral pH and it appears independent from proton gradient, nevertheless its transport activity is strongly reduced compared with that observed for NRAMP1. The transport activity of NRAMP2 is Na^{+} -dependent.

Keywords: NRAMP1, NRAMP2, iron homeostasis, *Xenopus laevis* oocytes

3. INTRODUCTION

3.1 NRAMP family

Metal ions are involved in many metabolic processes in every living cell. On the other hand, these essential nutrients are toxic at elevated levels. For this reason, shortage or excess in metal ions, as a result of genetic disorders as well as malnutrition, may lead to death or severe diseases [5]. Iron plays a central role in a large number of essential cellular functions but it is also potentially toxic being able to generate reactive oxygen species (ROS), that can damage DNA, phospholipids and proteins; therefore, it is of utmost importance, for both the cells and the organism, to maintain iron homeostasis ensuring iron supply and preventing accumulation of excess iron. Abnormal iron uptake has been implicated in hemochromatosis, anemia, atherosclerosis, and in neurological diseases such as Parkinson's, Alzheimer's, Huntington's, Friedreich's ataxia and Pica [5-14]. To prevent such disorders, cells must maintain metal ion homeostasis through highly regulated processes of uptake, storage and secretion [5]. The NRAMP family of metal ion transporters is involved in the maintenance of ion homeostasis and is conserved in different organisms, from bacteria to human [2]. In prokaryotes, MntH family (manganese transporters) is related to the NRAMP proteins [15]. Smf1 and Smf2 are two NRAMP homologs in yeast and they are manganese transporters, while Smf3 probably transports iron from the vacuole to the cytosol [16, 17]. In plants were found NRAMP homologs which play an important role in seed germination [2, 18, 19]. In *Drosophila* the Malvolio protein regulates manganese and ferrous iron homeostasis [20-22]. In mammals, the SLC11A family includes two genes, NRAMP1 (SLC11A1) and DMT1 (SLC11A2, formerly NRAMP2). NRAMP1 is responsible of the metal transport across the phagosomal membrane of macrophages, and defective NRAMP1 causes sensitivity to several intracellular pathogens. DCT1 (or DMT1 or NRAMP2) can transport metal ions at the plasma membrane of cells of both the duodenum and in peripheral tissues, and defective DCT1 may lead to anemia [5, 23]. Functional characterization of Smf1 and mammalian DMT1 in *Xenopus laevis* oocytes have shown that they mediate proton-dependent metal ion transport [24, 25]. DMT1 is an electrogenic symporter of several metals and protons and exploits the proton gradient which gives the energy required for its activity [4, 5, 24]. Mammalian NRAMP1 transport iron, manganese and copper together with protons, but the directionality of transport has long been a matter of debate [5, 26, 27].

3.2 Mammalian iron transporters: families SLC11 and SLC40

The SLC11 family is comprised of two members, SLC11A1 and SLC11A2. SLC11A1 is expressed in the phagolysosome of macrophages and in the tertiary granules of neutrophils. It plays an important role in innate resistance against bacterial infection. SLC11A2 is a key player in iron metabolism and is expressed in the proximal duodenum, immature erythroid cells, brain, placenta and kidney [23]. SLC11A2 also mediates iron transport into the cytosol across the membrane of endocytotic vesicles of the TfR-cycle. Intestinal iron absorption is mediated by SLC11A2 at the apical membrane of enterocytes and is followed by basolateral exit via SLC40A1 [23]. Approximately 80% of the iron comes from the breakdown of hemoglobin following macrophage phagocytosis of senescent erythrocytes. Both SLC11A1 and SLC11A2 play an important role in macrophage iron recycling. SLC40A1 is the unique member of the SLC40 family and is involved in cellular iron efflux. SLC11A1 and SLC11A2 share 66% identity and 82% similarity at the amino acid sequence level [23]. SLC11 transporters are transmembrane proteins that exploit the H^+ -electrochemical gradient as the driving force necessary for their transport activity. They usually transport divalent metal ions, such as Mn^{2+} , Fe^{2+} , Cd^{2+} , Co^{2+} , Zn^{2+} , Ni^{2+} , and Pb^{2+} [3, 5]. It has been shown that mammalian members of the SLC11 family have 12 transmembrane domains (TMDs) [28] with a conserved hydrophobic core of 10 TMDs [29, 30] that plays an important role in H^+ -dependent metal transport [31, 32]. A conserved metal transport signature was found within the cytoplasmic loop between TMD 8 and 9. Furthermore, a DPGN motif between TMD 1 and 2 is conserved across species and is essential for the transport function of these proteins. The loop between TMD 7 and 8 has been shown to be extracellular [33, 34] and the presence of a glycosylated loop in this region is conserved in almost all sequences [23].

3.2.1 SLC11A1: Natural resistance-associated macrophage protein-1 (NRAMP1)

SLC11A1, also known as NRAMP1 (Natural resistance-associated macrophage protein-1), was identified in 1993 by positional cloning of the mouse chromosome 1 locus *Bcg/Lsh/Ity* [23, 35]. NRAMP1 is a 90-100 kDa integral transmembrane protein which is expressed in the phagosome of macrophages and in tertiary granules of neutrophils. It plays an important role in mouse innate resistance to bacterial infection. Naturally occurring or experimentally induced mutations at NRAMP1 cause susceptibility to infection by several intracellular parasites, including *Salmonella*, *Mycobacterium*, and *Leishmania* [23, 36-38]. Furthermore, polymorphic variants of NRAMP1 are associated with human susceptibility to tuberculosis and leprosy [33, 39, 40]. Mouse susceptibility to bacterial infections is associated with a single loss of function mutation at position G169D in the

predicted TMD 4 of the NRAMP1 protein [41]. Schematic secondary structure representation of NRAMP1 is shown in Fig. 1 [26]. The main function of this protein seems to be the regulation of intraphagosomal metal concentration, in particularly of iron and manganese [38]. During the maturation process of the phagosome, NRAMP1 is recruited to the phagosomal membrane after bacterial engulfment and colocalizes with the lysosomal-associated membrane protein 1 (LAMP1) [23]. Moreover, it is present in gelatinase-positive tertiary granules of neutrophils [42]. Mammalian NRAMP1 transports iron, manganese and copper together with protons, but the directionality of transport has been a matter of debate [5, 26, 27].

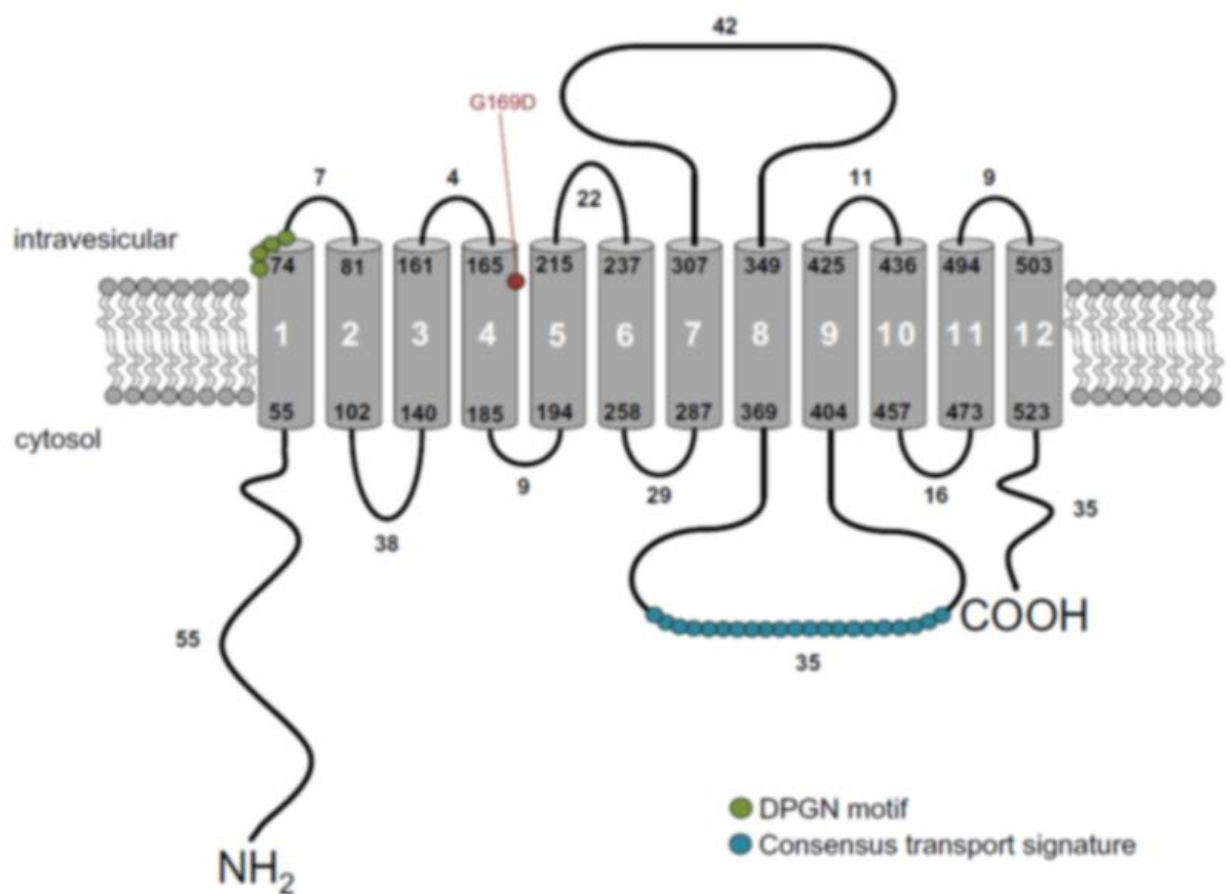


Fig. 1: Schematic secondary structure representation of SLC11A1 [23]. SLC11A1 representation shows the consensus transport signature (blue) and the DPGN motif (green). The amino acid mutated that leads to susceptibility to infection in mice is indicated in red. The N- and C-termini are denoted by NH₂ and COOH. The grey cylinders indicate the proposed transmembrane regions.

3.2.2 SLC11A2: Divalent metal transporter-1

SLC11A2 is also known as DMT1, DCT1 and NRAMP2. This protein was cloned from a rat duodenal cDNA library prepared from rat mRNA fed with a low-iron diet using the *Xenopus* oocytes expression system [23]. NRAMP2 transports divalent metal ions and is involved in iron absorption. DMT1 is the major iron transporter at the apical membrane of intestinal cells, enabling dietary iron uptake in the duodenum [2]. Another isoform is expressed at the cell surface and in endosomes and facilitates transferrin-independent iron uptake in most peripheral tissues [2, 3, 23, 27, 42, 43]. Mutations in this gene are associated with hypochromic microcytic anemia with iron overload [44-46]. Altered DMT1 expression may lead to iron deposition in neuronal cells and neurodegenerative disorders [47, 48]. Multiple transcript variants encoding different isoforms have been found for this gene. It has been shown that the NRAMP2 gene is mutated (G185R) in mk mice and Belgrade (b) rats, both of which display a severe iron deficiency associated with reduced intestinal iron uptake, impaired iron acquisition by peripheral tissues and microcytic anemia [23, 46]. Moreover, NRAMP2 represents the major, transferrin-independent, intestinal iron uptake system of mammals [38]. NRAMP2 is an electrogenic symporter of a variety of metals and protons [4, 5, 24]. Schematic secondary structure representation of NRAMP2 is shown in Fig. 2 [23].

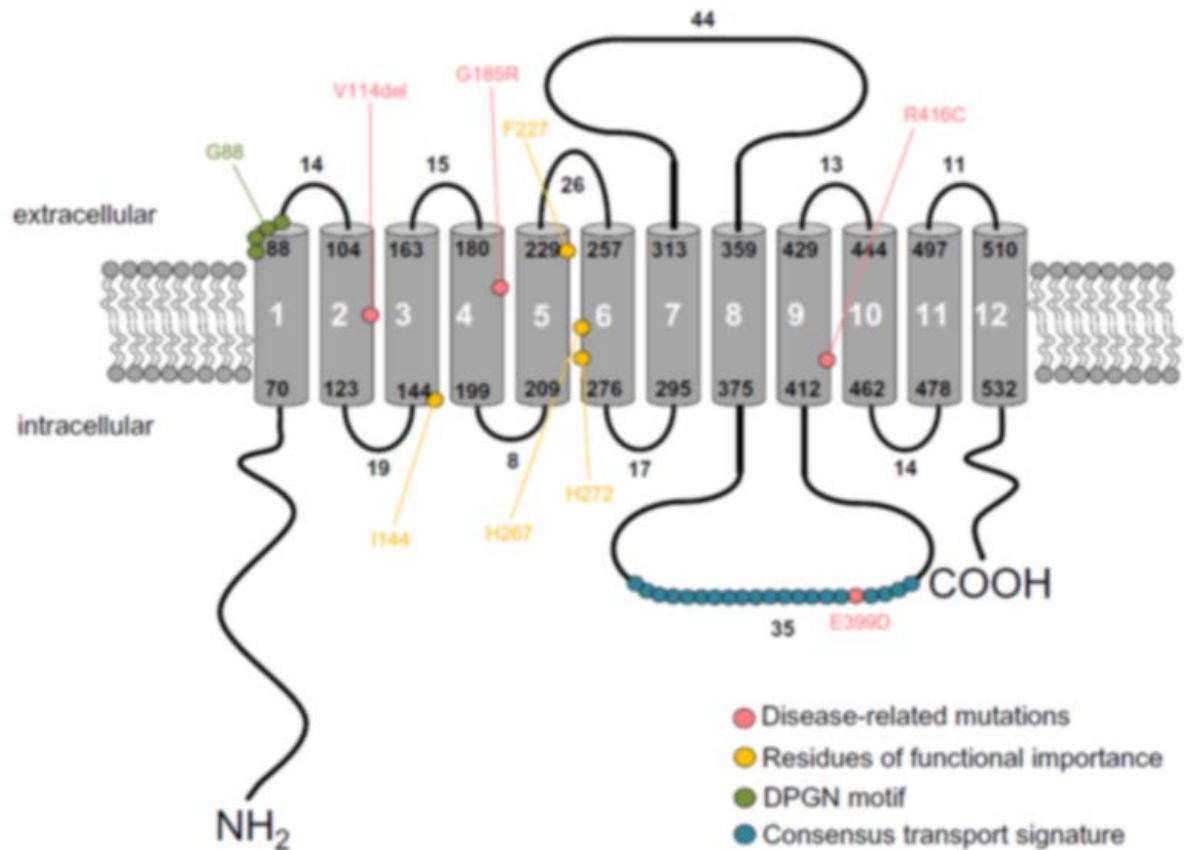


Fig. 2: Schematic secondary structure representation of NRAMP2 [23]. NRAMP2 representation shows the locations of known human mutations resulting in microcytic anemia (pink), the DPGN motif (green), consensus transport signature (blue), and residues directly involved in the transport mechanism of SLC11A2 (yellow). The N- and C-termini are denoted by NH₂ and COOH. The grey cylinders indicate the proposed transmembrane regions.

3.2.3 SLC40A1: Ferroportin 1

SLC40A1 is also known as Ferroportin1, Ireg1 and MTP1. It was described as an iron efflux protein in enterocytes in 2000 [49-51]. SLC40A1 is the unique member of the SLC40 transporter family. The human SLC40A1 gene encodes a protein of about 62 kDa. SLC40A1 is a highly conserved protein with 90-95% homology among human, mouse and rat orthologs [51, 52]. Fig. 3 shows the proposed membrane topology of ferroportin based on the model of Liu *et al.* (2005) [53, 54]. Iron appears to exit cells in a unique common pathway that generally involves SLC40A1. SLC40A1 is expressed in several tissues, in particular duodenum (basolateral portion), macrophages, liver Kupffer cells, placenta and kidney. It plays a critical role in body iron homeostasis [23, 51, 55]. Type IV hereditary hemochromatosis (HH) is also called “ferroportin disease” and is the result of mutations in SLC40A1. Furthermore, it has been demonstrated that SLC40A1 levels may decrease in malignant tissue from breast cancer patients and in malignant breast cancer cell lines [56]. The complete loss

of SLC40A1 expression was found to be lethal in mammals [50]. Recently, it has been shown that SLC40A1 is also a manganese transporter and not only an iron transporter [57-59].

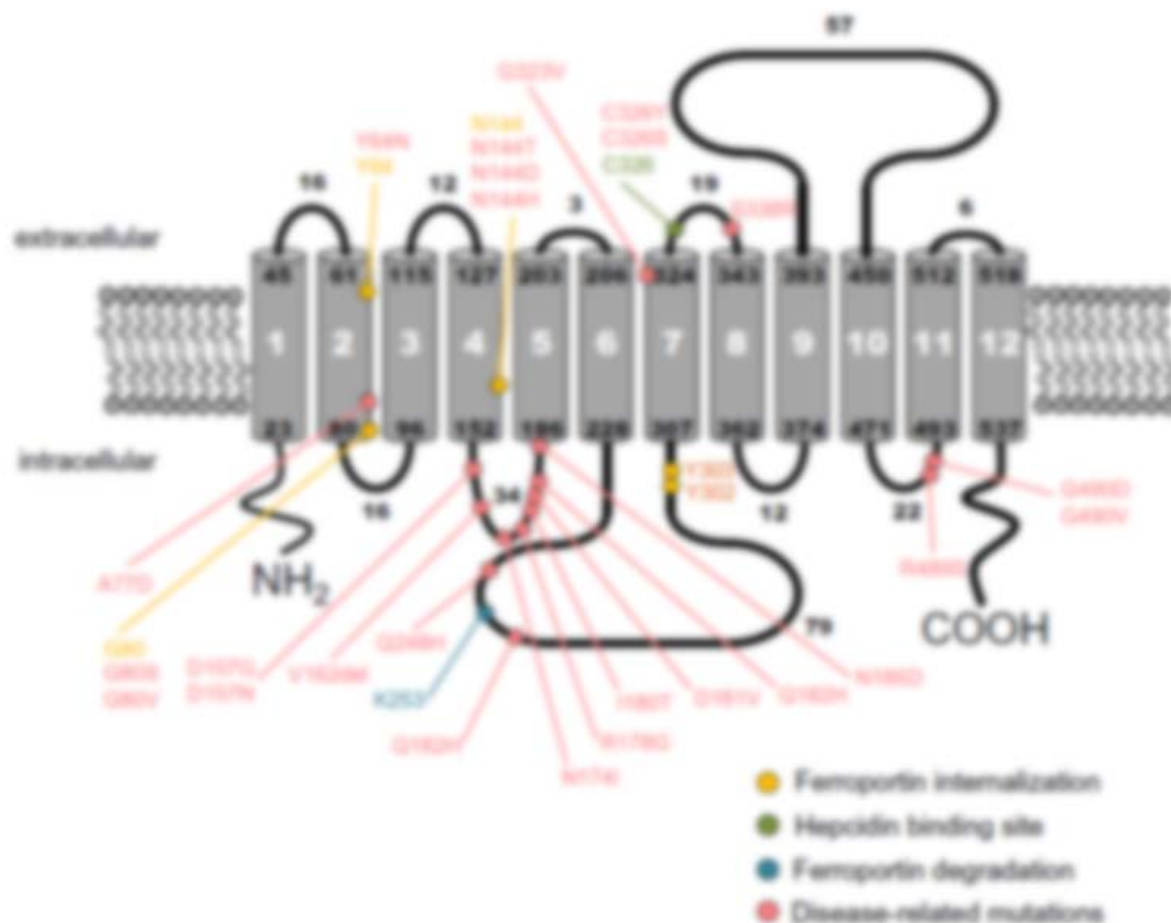


Fig. 3: Schematic secondary structure representation of ferroportin [23]. Highlighted circles indicate the locations of human mutations that result in iron overload disease (red), amino acid substitutions that interfere with hepcidin binding (green), and hepcidin-induced ferroportin internalization (orange) and degradation (blue). The N- and C-termini are denoted by NH₂ and COOH. The grey cylinders indicate the proposed transmembrane regions.

3.3 Systemic iron homeostasis

Iron is an essential element for almost all living organisms and it participates in a wide variety of metabolic processes, including oxygen transport, DNA synthesis and electron transport [60]. Because it may accept or donate electrons, free iron is highly reactive and toxic [61]. Iron is potentially toxic because it may foster ROS production and the generation of highly reactive radicals through Fenton reaction [62, 63]. Disorders of iron metabolism are among the most

common diseases of humans, ranging from anemia to iron overload and possibly to neurodegenerative diseases [60]. Thus, iron concentration in body tissues must be finely regulated in order to avoid several diseases. Proteins may contain iron in the prosthetic form of iron-sulfur clusters or heme, or it can be directly coordinated by amino acid side chains, for example histidine, glutamate, aspartate and tyrosine. Iron-containing proteins can carry or store oxygen (for example hemoglobin or myoglobin). They may catalyze metabolic and antimicrobial redox reactions (cytochromes, ribonucleotide reductase, nitric oxide synthase, NADPH oxidase, myeloperoxidase) and play an important role in iron transport or storage (transferrin, lactoferrin and ferritin) [61]. Iron-containing proteins are essential for energy metabolism and play a role in signaling pathways as well as host defense [61]. The average adult human contains 3-4 g iron, most of which (2-3 g iron) is distributed in the haemoglobin of RBCs and developing erythroid cells and serves in oxygen transport [61]. Iron is also present in macrophages (up to 600 mg), whereas excess body iron is stored in the liver [64, 65]. The liver and the spleen are iron-rich tissues where iron is stored in macrophages and hepatocytes in a specialized cytoplasmic iron storage protein, called ferritin [61]. Iron balance is maintained by the control of dietary iron absorption in the duodenum (Fig. 4). Dietary iron is predominately present in the duodenum as heme bound iron or non-heme ferric iron [23]. Iron uptake involves the reduction of Fe^{3+} in the intestinal lumen by duodenal cytochrome b (Dcytb) before being transported across the intestinal epithelium by DMT1 [61]. Transepithelial iron transport is completed when Fe^{2+} is exported across the basolateral membrane into bloodstream via ferroportin [23]. Fe^{2+} is oxidized by hephaestin and Fe^{3+} is then bound to the serum iron-transport protein transferrin [61]. It is an iron-binding blood plasma glycoproteins that control the level of free iron in biological fluids and regulates also tissue iron transport for utilization and storage. Iron is deposited in liver and cells of the reticuloendothelial system. Transferrin receptor-1 (TfR1) is involved in cellular iron uptake. Iron released from the transferrin-receptor complex into the endosomes is transported out of the phagosome to the cytosol by SLC11A2 [42, 43, 46]. Ferritin may bind most intracellular iron for storage.

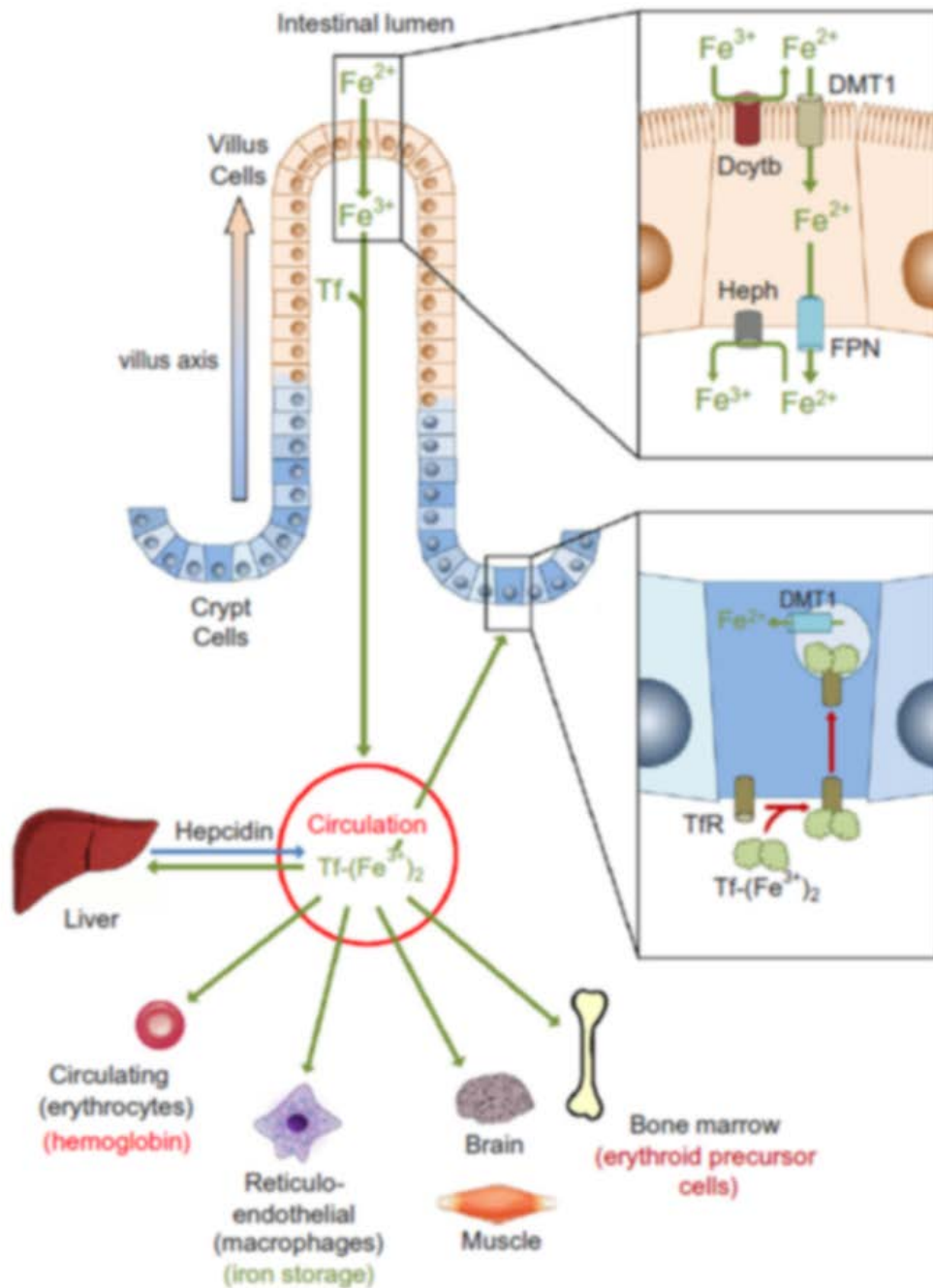


Fig. 4: Intestinal iron absorption [23].

The total iron content of transferrin may undergo daily turnover to sustain erythropoiesis and the transferrin iron pool is replenished by iron recycled from senescent RBCs and by dietary iron. Macrophages play an important role in body iron homeostasis as the main iron supply for erythropoiesis derives from the iron recycled by these cells after phagocytosis of senescent

erythrocytes [38, 66]. This process is achieved by macrophages of the spleen, bone marrow and also in the Kupffer cells. Thus, senescent RBCs are cleared by macrophages, which can metabolize haemoglobin and haem, and release iron into the blood flow [61]. It has been shown that NRAMP2 is associated with erythrocyte-containing phagosomes [38]. Iron released from erythrocytes degradation is transported out of the phagosome by NRAMP2 [23] (Fig. 5). Moreover, NRAMP1 has been suggested to be involved in metal export from phagosomes to the cytosol. Iron transported to the cytosol could be used for metabolic purposes, stored in ferritin or transported out of the cell by ferroportin [23]. Macrophages lacking both NRAMP1 and DMT1 show reduction in iron recycling efficiency [67]. NRAMP1 is involved in iron recycling during conditions of increased erythrophagocytosis [23, 38].

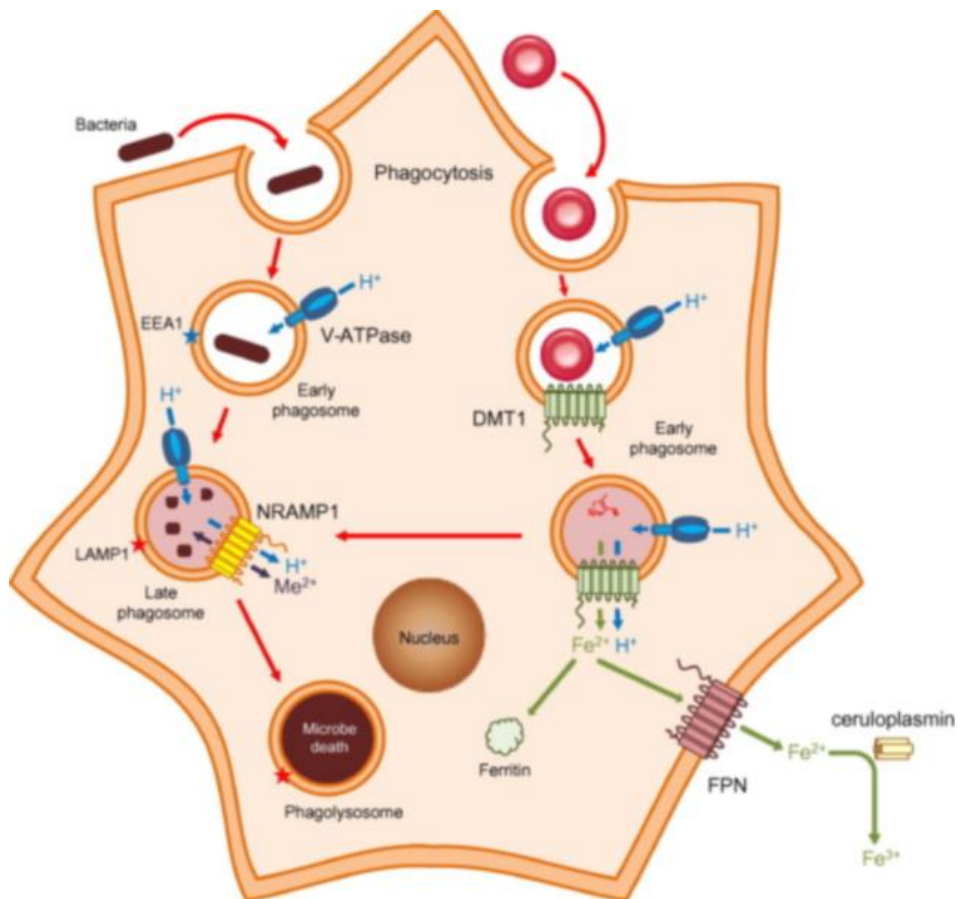


Fig. 5: Macrophages iron transport (NRAMP1 and NRAMP2) [23].

3.4 *Dictyostelium* NRAMP proteins

Dictyostelium discoideum is a forest soil-living amoeba belonging to the phylum *Amoebozoa* and it is exceptional in its ability to alternate between unicellular and multicellular forms (Fig. 6) [1, 68]. In particular, *Dictyostelium* cells proliferate as solitary cell until bacteria are consumed. Many prokaryotic species are present in the forest soil and they can be used as food for this social amoeba [1]. Starvation triggers a change in life cycle, forcing *Dictyostelium* cells to gather into aggregates to produce a multicellular organism, called “slug” [1]. The slug gives rise to the fruiting body composed of a slender stalk bearing on the top a ball of fully differentiated spores [69].

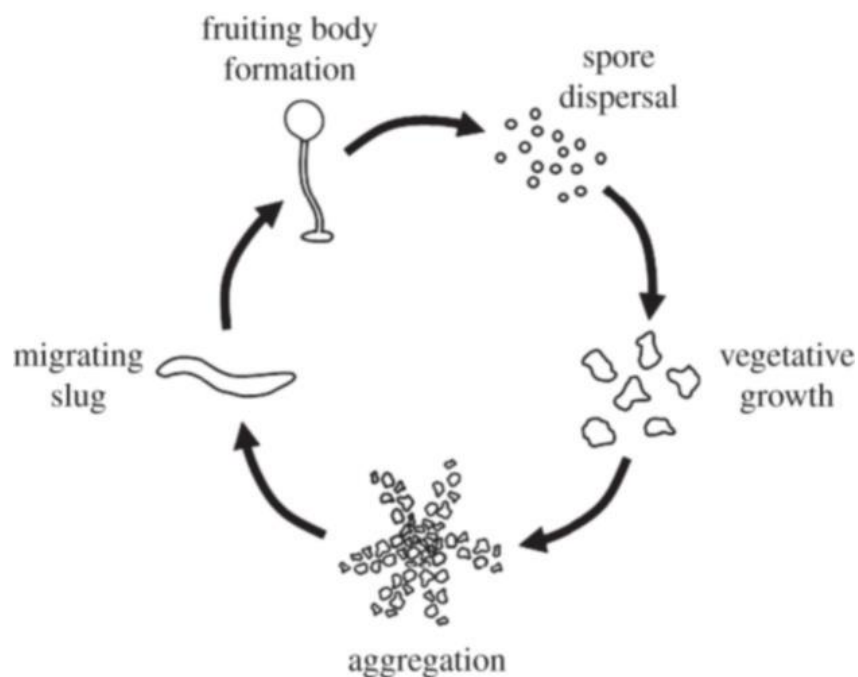


Fig. 6: *Dictyostelium discoideum* (life cycle) [68].

The social amoeba *Dictyostelium discoideum* has been particularly useful for the study of cell motility, chemotaxis, phagocytosis, endocytic vesicle traffic, cell adhesion, pattern formation, caspase-independent cell death, and, more recently, autophagy and social evolution [1, 70]. *D. discoideum* represents a model for the study of cellular iron homeostasis showing subcellular localization of iron transporters resembling that of macrophages. Moreover, *Dictyostelium* cells resemble macrophages for their ability to engulf bacteria and death cell, to discriminate between self and non-self and to fight potential pathogens. The *Dictyostelium* genome shares with mammals many genes regulating iron homeostasis; in particular, *D. discoideum* expresses the ortholog of

NRAMP1 transporter in phago-lysosomes and that of NRAMP2 in the contractile vacuole, a structure involved in osmoregulation. The distinct localization of the two proteins in different compartments suggests that both contribute synergistically to regulate iron homeostasis. Iron is gained by degradation of ingested bacteria and efflux via NRAMP1 from phagosomes to the cytosol after bacterial engulfment (Fig. 7) [1]. Iron transport to the cytosol is important in order to deplete the bacteria from this essential metal and represents the major source of iron for *Dictyostelium* cells. In addition to the NRAMP proteins, the *Dictyostelium* genome encodes other proteins which are known to be involved in cellular iron homeostasis (Fig. 7). Homologs of mammalian mitoferrin, Fe-S and heme ABCB transporters, frataxin and also a cytosolic and a mitochondrial aconitases are found [71, 72]. Two distantly-related ferroportin-like proteins exist, but no homologs for transferrin or transferrin receptors [1].

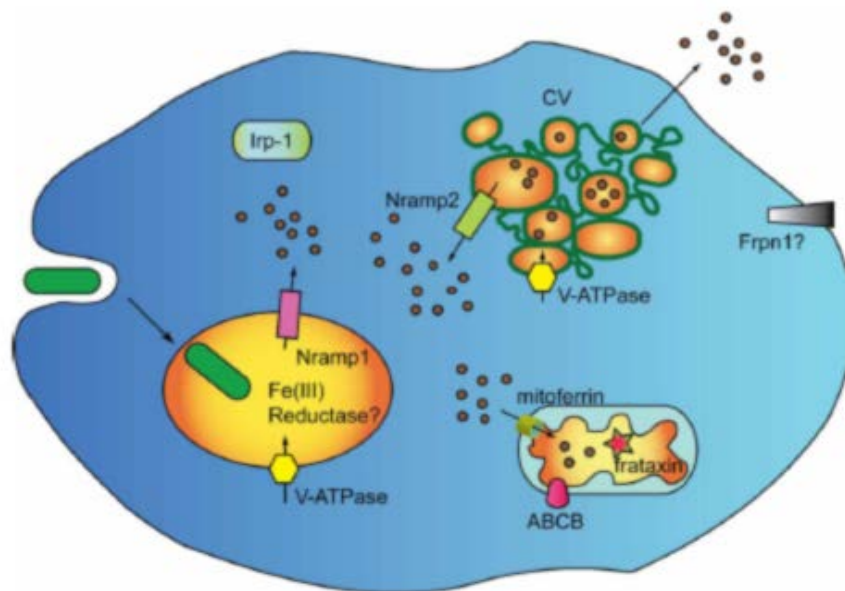


Fig. 7: Genes regulating iron homeostasis in *Dictyostelium*. The major source of iron for *Dictyostelium* cells are engulfed bacteria which are degraded in phago-lysosomes. The NRAMP1 transporter is recruited to phagosome shortly after uptake and is retrieved during post-lysosomal maturation. NRAMP2 is localized in the membrane of the contractile vacuole (CV) [1].

It has been shown that NRAMP gene disruption increases *Dictyostelium* sensitivity to infection, enhancing intracellular growth of *Legionella* or *Mycobacteria* [1]. Iron is an essential element for pathogens. In particular, *Legionella*, *Mycobacteria* or *Salmonella* can assimilate significant amounts of iron for their metabolism and virulence [73-76]. Thus, depleting iron from the phagosome via

NRAMP1 could be an host defense strategy to starve the pathogen for iron. The contractile vacuole membrane is studded with the V-ATPase, which can pump H^+ inside the lumen [77, 78]. Moreover, the V-ATPase is also recruited to phagosomes or macropinosomes shortly after their engulfment [79]. NRAMP1 and NRAMP2 colocalize with the vacuolar ATPase that can provide the electrogenic potential regulating their transport activity. On the other hand, hindering co-recruitment of the V-ATPase by the pathogen, in particular *Legionella*, could avoid acidification of the vacuole, neutralizing NRAMP1-dependent iron transport to the cytosol (Fig. 8) [80].

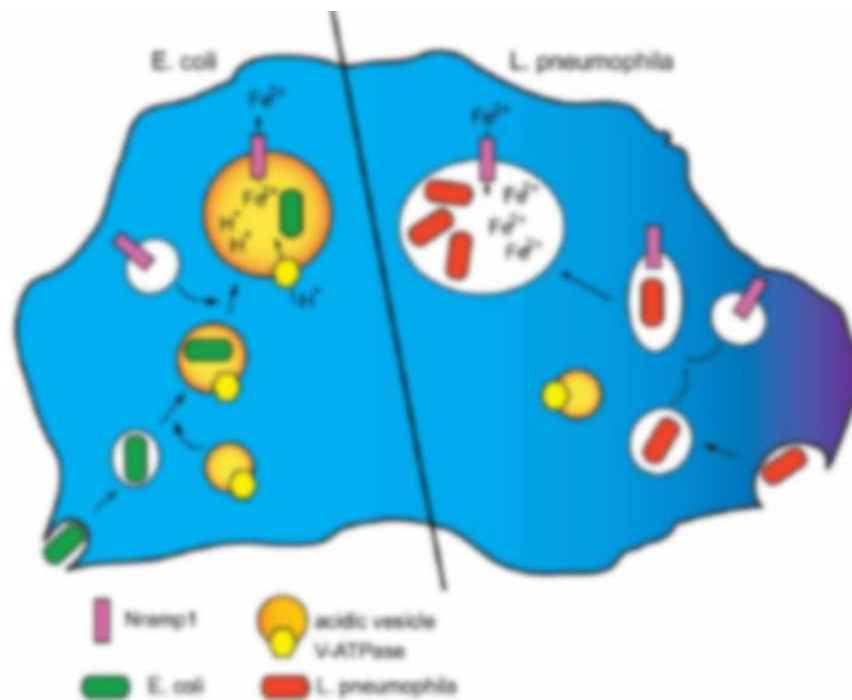


Fig. 8: NRAMP1 activity and its manipulation by Legionella [1]. (Left) NRAMP1 colocalizes with the vacuolar ATPase. The activity of the V-ATPase can provide the electrogenic potential necessary for NRAMP1 transport activity, in order to deplete the bacteria from an essential nutrient element. (Right) *L. pneumophila* is taken up in *Dictyostelium* cells by macropinocytosis. The pathogen may inhibit fusion of its vacuole with acidic vesicles bearing the V-ATPase, thus neutralizing the electrogenic potential.

To better understand the function of *Dictyostelium* NRAMP proteins, they were expressed in *Xenopus laevis* oocytes by cRNA injection and functionally tested by radiochemical techniques and by two novel assays based on metal-induced changes in calcein fluorescence. Injecting cRNA encoding the wild-type proteins resulted in a very low level of functional expression, which was not surprising as both NRAMP1 and NRAMP2 are not plasma membrane proteins. To increase expression at the plasma membrane, both N- and C- terminus of the two proteins were replaced

with the corresponding regions of murine DMT1 [2]. DMT1, which has been successfully expressed in *Xenopus* oocytes [3, 16, 24, 81], is used as internal control for the characterization of iron transport as it shows an high level expression at the plasma membrane of *Xenopus* oocytes. DMT1 is the divalent metal transporter 1, also known as Natural Resistance-Associated Macrophage Protein 2 (NRAMP2) and Divalent Cation Transporter 1 (DCT1) [23].

3.5 *Xenopus* oocytes: a heterologous expression system

X. laevis oocytes were utilized in this study as a system of heterologous expression to characterize NRAMP1 and NRAMP2 iron transporters. *Xenopus laevis* is an African aquatic frog of the *Pipidae* family (Fig. 9) and is a medium-sized aquatic frog native of Africa with a smooth skin and a large clawed rear feet [82]. For many years *X. laevis* was used as a biological assay to establish human pregnancy status but nowadays this frog is utilized in research laboratories for their eggs and oocytes, which are used in developmental biology and for heterologous expression. *Xenopus* is used also as a tool to study vertebrate embryology and development, basic cell and molecular biology, genomics, neurobiology and toxicology.

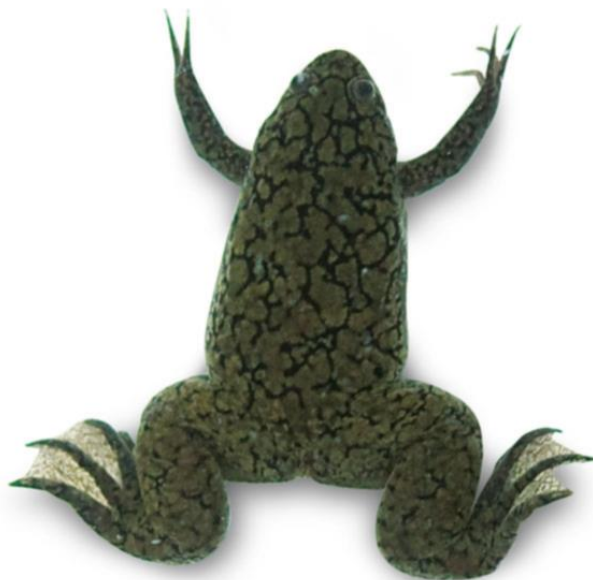


Fig. 9: *X. laevis* frog [82].

Detailed studies by electron and fluorescence microscopy of amphibian oocytes were performed in the last decades [83-85]. The oocytes stage were characterized by Dumont (1972) into six different stages, according to the dimensions and pigmentation of the oocytes [83] (Fig. 10). The first stage consists of small (50 to 100 μm) colorless oocytes and the cytoplasm is transparent. They are also characterized by large nuclei and mitochondrial masses that are clearly visible in the intact oocyte. The second stage oocytes range up to 450 μm in diameter, and appear white and opaque. Pigment synthesis and vitellogenesis begins during Stage III. The yolk accumulation (vitellogenesis) continues through Stage IV (600 to 1000 μm) and the animal and vegetal hemispheres become differentiated. By Stage V (1000 to 1200 μm) the oocytes have nearly reached their maximum size. Stage VI oocytes are characterized by the unpigmented equatorial band. They range in size from 1200 to 1300 μm , are postvitellogenic and ready for ovulation [83].

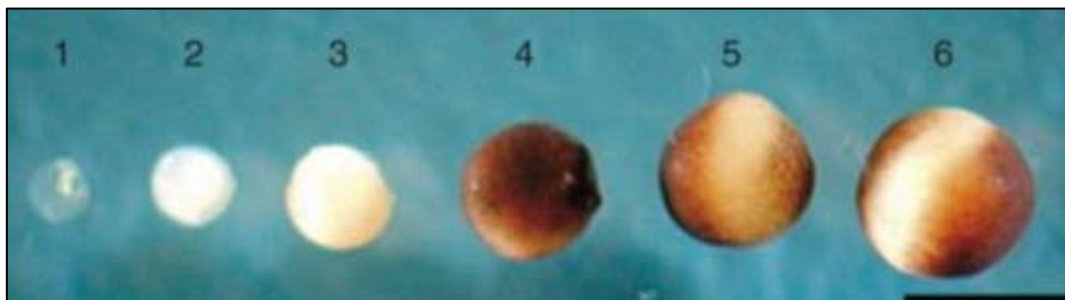


Fig. 10: Maturation stage of *X. laevis* oocytes (Scale bar = 1 mm) [83]. Oogenesis in the anuran *X. laevis* is divided into six stages based on the anatomy of the developing oocyte.

In adult female oogenesis is asynchronous and therefore all the six stages of oocyte development occur at the same time [86]. The oocytes are surrounded by different layers of cellular and non-cellular material. The plasma membrane of the oocytes is surrounded by the vitelline membrane, which is a non-cellular glycoprotein fibrous layer. Moreover, there is a layer of follicle cells electrically connected to the oocyte by gap junctions, a connective tissue layer, and an epithelial cells layer relying the ovary wall. This complete structure is called "follicle" [87-90]. *Xenopus* oocytes provide an important expression system for molecular biology. By injecting DNA or cRNA into the oocyte or developing embryo, scientists can study specific proteins in a controlled system. This allows rapid functional expression of manipulated DNAs (or cRNA); this is particularly useful to characterize membrane transporters or channels (Fig. 11) [91]. To conduct this study, *X. laevis*

oocytes were used as a system of heterologous expression to characterize iron transporters from *Dictyostelium discoideum*.

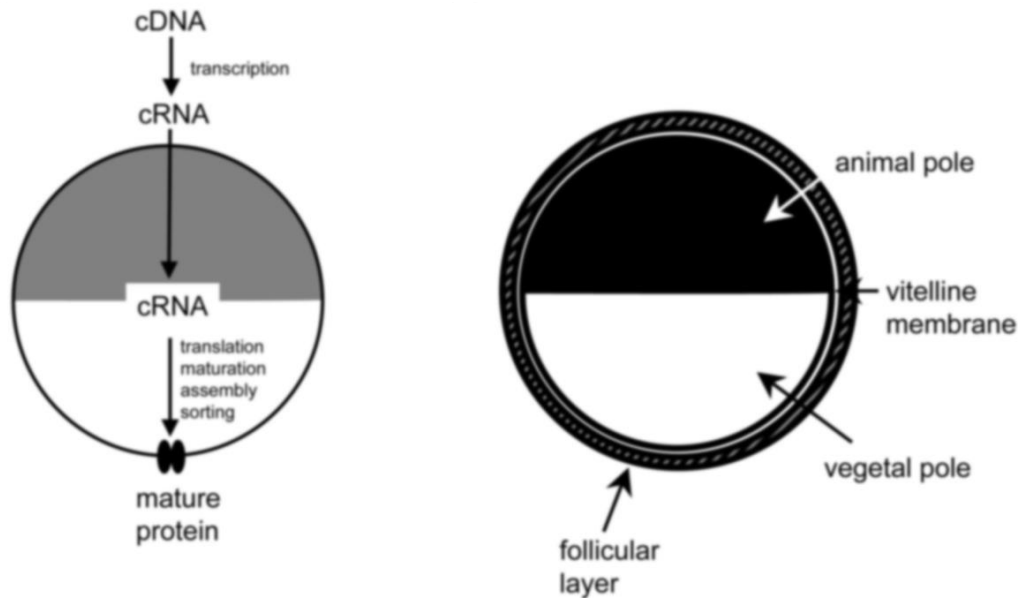


Fig. 11: *Xenopus* oocytes as important heterologous expression system [91]. Heterologous cRNA can be easily injected into *Xenopus* oocytes and mature expressed proteins can be characterized using several techniques, such as radiochemistry, electrophysiology or immunocytochemistry.

4. AIM OF STUDY

The aim of this project is the functional characterization of iron transporters NRAMP1 and NRAMP2 from *Dictyostelium discoideum* as a model of cellular iron homeostasis. *Dictyostelium* NRAMP proteins belong to NRAMP family, which is conserved from bacteria (MntH protein) to humans (SLC11 proteins). Moreover, *Dictyostelium* cells show subcellular localization of iron transporters resembling that of macrophages. The ortholog of NRAMP1 transporter is expressed in phagolysosomes and plays an important role in resistance to bacterial infection; that of NRAMP2 is located in the contractile vacuole, a structure involved in osmoregulation. *D. discoideum* resembles macrophages for its ability to engulf bacteria and dead cell, to discriminate between self and non-self and to fight potential pathogens. It could also be used as a basic model to study iron transport and to investigate the subcellular localization of iron transporters. The functional characterization of *Dictyostelium* NRAMP proteins could be important in order to define their role in physiological and pathological iron-dependent processes, i.e. resistance to bacterial infection, effects on growth, differentiation and development.

5. MATERIALS AND METHODS

5.1 Oocytes harvesting and selection

Oocytes were obtained from adult female of *Xenopus laevis* by means of surgery operation and manually defolliculated [92] after a treatment of 40 minutes using 1 mg/ml Collagenase NB 4 Standard Grade (SERVA) at room temperature in Ca²⁺-free ORII medium (in mM: NaCl, 82.5; KCl, 2; MgCl₂, 1; HEPES/Tris, 5; pH 7.5). Healthy V-VI stadium oocytes [83] were then selected for injection and maintained at 16°C in Barth's solution (in mM: NaCl, 88; KCl, 1; MgSO₄, 0.82; CaCl₂, 0.41; Ca(NO₃)₂, 0.33; NaHCO₃, 2.4; HEPES/Tris, 10; pH 7.5) supplemented with 50 mg/l gentamicin sulfate and 2.5 mM sodium pyruvate. The experiments were carried out according to the institutional and national ethical guidelines (Legislative Decree: 26/2014, permit No. 1/2013 to M. Castagna).

5.2 Oocytes expression of DMT1 and NRAMP proteins

The chimeric constructs NRAMP1/DMT1 and NRAMP2/DMT1 were produced using the scheme shown in Fig. 12 [2]. Chimeric cDNAs and RNAs were prepared as described previously [93]. The restriction sites HpaI at the N-terminus and NsiI at the C-terminus were inserted by PCR primer amplification of the central portion of cDNA coding for the proteins in pGEMT vector. Rat DMT1 in pSPORT1 was instead mutagenized by site directed mutagenesis with overlapping primer to insert the Eco47III site and then the NsiI site. The chimeric constructs were amplified in *Escherichia coli* cells (JM109 strain) and plasmidic DNAs were extracted using affinity column chromatography (KIT "WIZARD clean up system", Promega). The plasmidic DNAs were linearized by *Not1* digestion and corresponding cRNAs were *in vitro* transcribed and capped (Ribo m7G Cap Analog, Promega) using T7 RNA polymerase (Promega). The cRNAs obtained were used to inject *X. laevis* oocytes and functionally tested by radiochemical techniques and by two different novel assays based on metal-induced changes in calcein fluorescence after four days post-injection.



Fig. 12: Scheme of the chimeric proteins [2]. In grey are shown the 12 TMDs of *Dictyostelium* protein NRAMP1 or NRAMP2, while in black are shown the C- and N- terminus of rat DMT1 (NP037305.2). Chimeric cDNAs were prepared by linking together the two portion of cDNA coding for N- (60 amino acids) and C-terminus (19 amino acids) of murine DMT1 in pSPORT1 and the coding sequence of *Dictyostelium* NRAMP1 or NRAMP2. To generate chimeric cDNAs, the restriction sites HpaI at the N-terminus (position +96 and +291 for *Dictyostelium* NRAMP1 and NRAMP2, respectively) and NsiI at the C-terminus (position +1533 and +1749 for NRAMP1 and NRAMP2, respectively) were inserted by PCR primer amplification of the central portion of cDNA coding for the proteins NRAMP in pGEMT vector. Rat DMT1 in pSPORT1 was instead mutagenized by site directed mutagenesis with overlapping primer to insert the Eco47III site (at position +171) and then the NsiI site (at position +1713). The final NRAMP1 in pSPORT1 vector is coding for a protein with the first 59 amino acids of rat DMT1 replacing the 32 residues at the N-terminus of *Dictyostelium* NRAMP1 and with the 21 C-terminal amino acids replacing the last 19 amino acid of the rat protein. In the NRAMP2 chimera, the same N-terminus and C-terminus of rat DMT1 replace the first 97 and the last 46 residues of *Dictyostelium* NRAMP2.

5.3 Radiotracer uptake

Uptake experiments in *Xenopus* oocytes were performed 4 days post cRNA injection [2]. The uptake solution contained $^{55}\text{FeCl}_2$ and in mM: NaCl, 100; KCl, 1.8; CaCl_2 , 0.6; MgCl_2 , 0.6; Mes or Hepes 10 Mm at pH 5.5, 6.5, 7.5 or 8.0. 1 mM ascorbic acid (freshly prepared) was added to maintain iron in the reduced form unless otherwise indicated. Groups of 8-10 oocytes were incubated for 5 or 60 min (as indicated) in uptake solution, washed in ice-cold uptake solution devoid of FeCl_2 and Ca^{2+} (Wash solution), dissolved in 10% SDS solution and then counted in a liquid scintillation counter [94]. The external uptake solution contained in mM: NaCl or ChCl, 98; MgCl_2 , 1; CaCl_2 , 1.8, Hepes or Mes. The final pH values of 5.5, 6.5, 7.5 or 8.0 were adjusted with HCl and NaOH.

5.4 Calcein transport assay and confocal fluorescence imaging

For metal transport in *Xenopus* oocytes, control oocytes and oocytes transfected with cRNA encoding for NRAMP1, NRAMP2 or rDMT1 were injected with a 50 nl drop of a 0.025 mM calcein dissolved in intracellular solution (in mM: KCl, 130; NaCl, 4; MgCl₂, 1.6; EGTA, 5; HEPES, 10; Glucose, 5; pH 7.6). The nominal volume of a 1.2 mm diameter oocyte is 1 µL; therefore, a 50 nL injected drop will be diluted 20 times. Following calcein injection, the oocytes were placed in TMA (tetramethylammonium) solution at pH 5.5 containing or not divalent metals at a final concentration of 0.1 mM and observed at the confocal microscope (Zeiss LSM 5 EXCITER confocal laser-scanning microscope) equipped with a 5x Plan Neofluar 0.15 objective. Images of single oocytes were taken every 10 sec for a total of 10 min, by using excitation at 488 and emission at 505-550 nm. For F/F₀ quantification, the fluorescence intensity at time 0 (F₀) and at subsequent times (F) was calculated in the entire area of the oocytes using ImageJ. Changes in fluorescence intensity in the entire oocyte or in selected spots were proportionally linear with time.

5.5 Single Oocyte Fluorescence Assay (SOFA)

For metal transport in *Xenopus* oocytes, control oocytes and oocytes expressing chimeric NRAMP1 and NRAMP2 or rDMT1 were injected with a 50 nl drop of a 0.025 mM calcein in intracellular solution (in mM: KCl, 130; NaCl, 4; MgCl₂, 1.6; EGTA, 5; HEPES, 10; Glucose, 5; pH 7.6). Following calcein injection, the oocytes were placed for 5 or 60 min in uptake solution. The solutions contained the indicated concentration of FeCl₂ and in mM: NaCl, 100; KCl, 1.8; CaCl₂, 0.6; MgCl₂, 0.6; Mes or Hepes 10 mM at pH 5.5 and 7.5 (the final pH values of 5.5 and 7.5 were adjusted with HCl and NaOH). 1 mM ascorbic acid (freshly prepared) was added to maintain ions in the reduced form. The oocytes were subsequently washed in ice-cold uptake solution devoid of FeCl₂ and Ca²⁺ (Wash solution). SDS 10% and ORII solution were added to each oocyte and subsequently homogenized using CAT Scientific Homogenizer (GLAS-COL, Model 099C K4424, Terre Huate, USA). The oocytes were centrifuged (15000 rpm per 10 min a 4°C) after homogenization. The supernatants were harvested and put in 96-wells black plate (96-well microplates BRANDplates® with F-bottom). Fluorescence was measured from each well utilizing Tecan fluorescence reader (Infinite® F500) by using excitation at 488 and emission at 505-550 nm.

5.6 Statistics

For uptake experiments, the results are expressed as arithmetic means \pm SEM, whereas for calcein assays the data are calculated as mean \pm S.D. As indicated in the figure legends, statistical analysis is performed using Student's t-Test.

6. RESULTS

6.1 NRAMP1 transport activity

6.1.1 Uptake induced by NRAMP1

The present study investigated the transport activity of *Dictyostelium* NRAMP1, the protein recruited to the phagosomal membrane and involved in iron recycling. The bar charts in Fig. 13 show that both NRAMP1 and DMT1 mediate iron uptake when expressed in *X. laevis* oocytes. Moreover, Mn^{2+} competition of 100 μM iron uptake induced by NRAMP1 and 10 μM iron uptake induced by murine DMT1 were analyzed using radioactive uptake techniques. As a result, Mn^{2+} partially inhibits iron uptake induced by NRAMP1 and DMT1. Therefore, these two transporters could be permeable to Mn^{2+} .

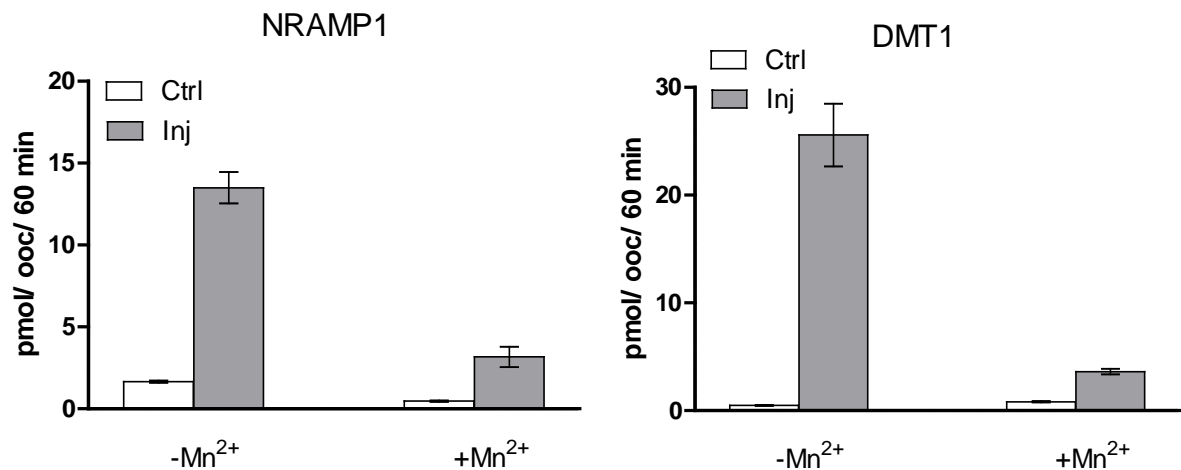


Fig. 13: ⁵⁵FeCl₂ uptake in *Xenopus laevis* oocytes expressing NRAMP1 or DMT1 (internal control) and Mn²⁺ inhibition of ⁵⁵FeCl₂ uptake induced by NRAMP1 or DMT1. 1 mM Mn²⁺ inhibition of 100 μM FeCl₂ uptake induced by NRAMP1 at pH 6.5 (on the left) and 1 mM Mn²⁺ inhibition of 10 μM FeCl₂ uptake induced by DMT1 at pH 6.5 (on the right). The white columns represent the uptake in control oocytes and the grey columns indicate the uptake induced by cRNA injected oocytes. Bars represent the mean \pm S.E.M. of 8-10 oocytes in a representative experiment of three independent experiments. Both NRAMP1 and rDMT1 mediate iron uptake, which is inhibited by Mn²⁺. P<0.001 for NRAMP1 control versus non inj. control; P<0.003 for NRAMP1 Mn²⁺ versus NRAMP1 control (Student's t-test).

Experiments were performed to investigate the H⁺-dependence of NRAMP1 (Fig. 14). NRAMP1 transport activity increased decreasing the pH of the external medium. The highest value of uptake can be reach at pH 5.5, nevertheless its activity is strongly reduced at pH 8. Therefore, NRAMP1 is H⁺-dependent.

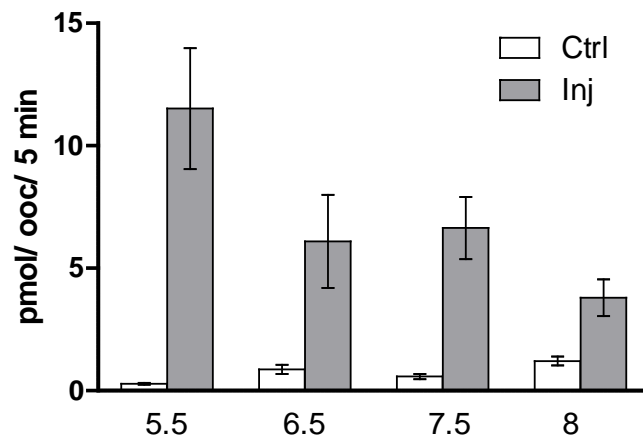


Fig. 14: H^+ -dependence of NRAMP1 activity. $100 \mu\text{M } ^{55}\text{FeCl}_2$ uptake induced by NRAMP1 at different pH (5.5, 6.5, 7.5, 8). The white columns represent the uptake in control oocytes and the grey columns indicate the uptake induced by cRNA injected oocytes. Bars represent the mean \pm S.E.M. of 8-10 oocytes in a representative experiment of three independent experiments. $P < 0.05$ for cRNA inj. pH 5.5 versus cRNA inj. pH 6,5; $P < 0.004$ for cRNA inj. pH 5.5 versus cRNA inj. pH 8 (Student's t-test).

Further experiments were performed in order to investigate the Na^+ effect on the activity of NRAMP1 or DMT1 (Fig. 15). Na^+ does not affect the activity of these transporters. NRAMP1 and DMT1 induce a comparable level of iron uptake in both presence and absence of Na^+ .

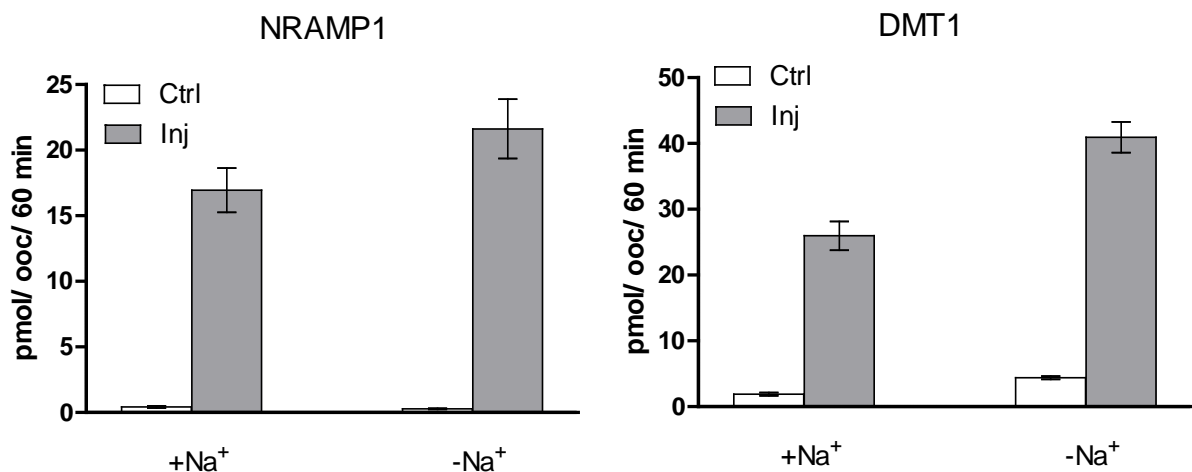


Fig. 15: Na^+ -dependence of $^{55}\text{FeCl}_2$ uptake induced by NRAMP1 or DMT1 (internal control). $100 \mu\text{M } ^{55}\text{FeCl}_2$ uptake induced by NRAMP1 (on the left) or $10 \mu\text{M } ^{55}\text{FeCl}_2$ uptake induced by DMT1 (on the right) in the presence or absence of Na^+ at pH 6.5. The white columns represent the uptake in control oocytes and the grey columns indicate the uptake induced by cRNA injected oocytes. Bars represent the mean \pm S.E.M. of 8-10 oocytes in a representative experiment of three independent experiments. $P < 0.001$ for NRAMP1 or DMT1 versus non inj. control. No statistical significance in the absence or presence of Na^+ .

In order to explore the cation selectivity of NRAMP1, further experiments were performed in the absence or presence of 1 mM of Mn^{2+} , Zn^{2+} , Cd^{2+} , Ni^{2+} , Co^{2+} or Cu^{2+} . As shown in Fig. 16, all these divalent cations partially inhibit radioactive iron uptake and to a lesser extent by Zn^{2+} . Therefore, NRAMP1 seems to be permeable to all these divalent cations.

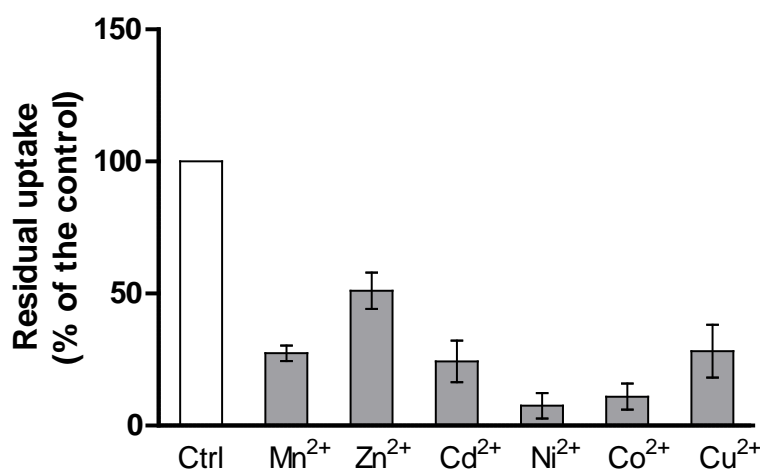


Fig. 16: Divalent ions competition of $^{55}\text{FeCl}_2$ uptake in *Xenopus laevis* oocytes expressing NRAMP1: divalent ions competition of 100 μM FeCl_2 uptake induced by NRAMP1 at a concentration of 1 mM for all cations at pH 6.5. Data shown represent the residual uptake expressed as percent of the control condition in the absence of divalent cations. Bars represent the mean \pm S.E.M. of data obtained from 8-10 of three independent experiments. $P < 0.04$ for each metal versus control (Student's t-test).

6.1.2 Calcein assays in oocytes expressing NRAMP1 or DMT1 using confocal microscopy

Using a novel assay with calcein, this study demonstrates that NRAMP1, similarly to rat DMT1, transports ferrous iron and Mn^{2+} , but not ferric iron or copper (Fig. 17). Uptake experiments allow to study the inhibition of iron uptake induced by divalent metal ions but calcein experiments allow to measure calcein quenching due to the flux of ions through the plasma membrane of *Xenopus* oocytes.

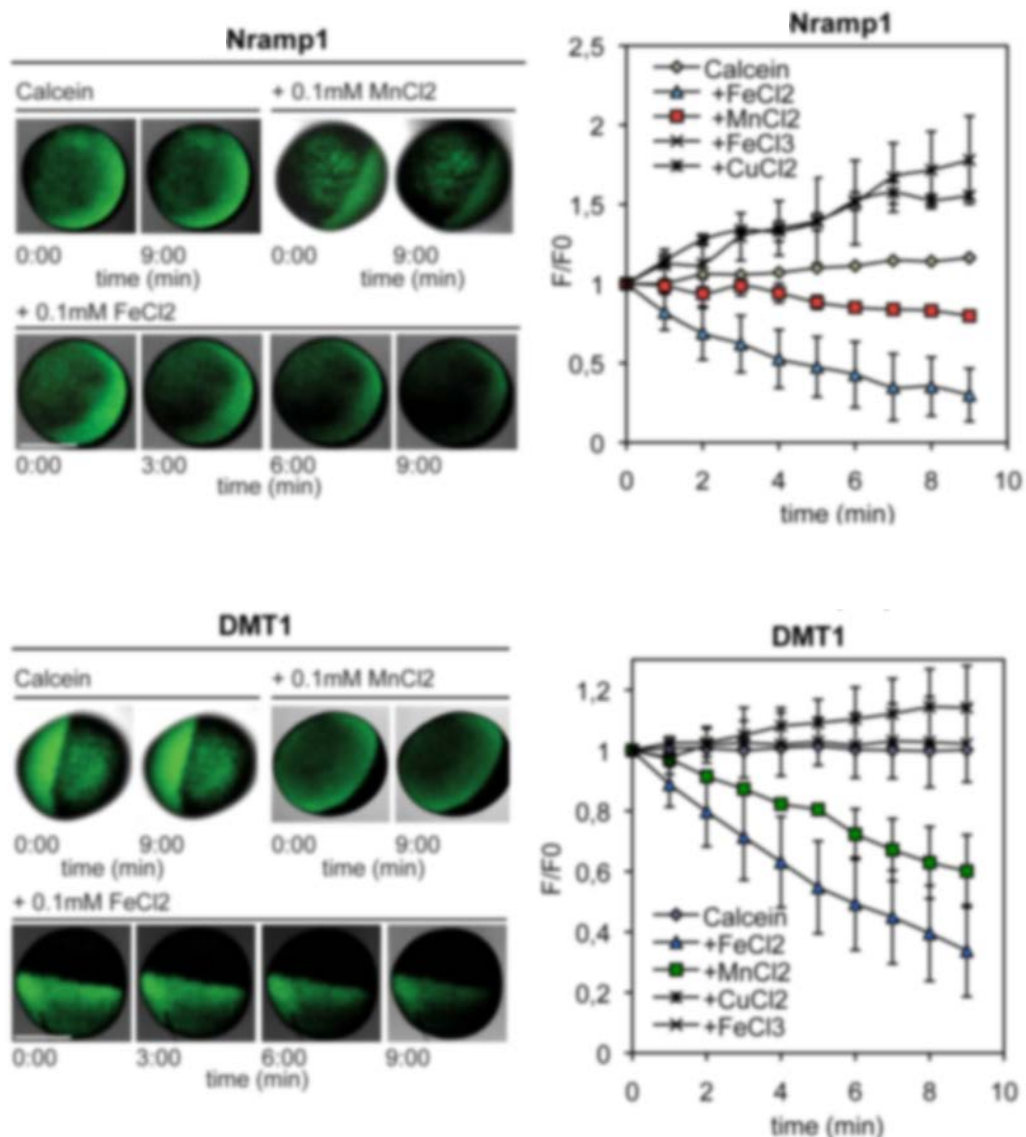


Fig. 17: Live imaging of divalent metals transport induced by NRAMP1 or DMT1 in *Xenopus* oocytes using calcein fluorescence and confocal microscopy. In the presence of Mn^{2+} or Fe^{2+} fluorescence decreased within minutes. The fluorescence changes over time were quantified by measuring the ratio of fluorescence intensity at the time indicated in the abscissa (F) vs. fluorescence at time 0 (F_0). Mean \pm S.D. are shown for each group of oocytes [NRAMP1 n values are: Mn, 3; Fe(II), 8; Fe(III), 4; Cu, 5; DMT1 n values are: Mn, 2; Fe(II), 8; Fe(III), 8; Cu, 7]. Statistical analysis was undertaken at the 9-min time point using a two-tailed Student's t-test, assuming unequal variance: Fe(II) versus control was $P < 0.05$ in all cases; Cu^{2+} versus control was $P < 0.05$ for NRAMP1. Scale bars: 0.5 mm.

6.1.3 Single Oocyte Fluorescence Assay (SOFA) compared to uptake techniques

SOFA (Single Oocyte Fluorescence Assay) is a novel technique based on metal-induced changes in calcein fluorescence. This assay allow to measure calcein quenching due to the flux of ions through the plasma membrane of *Xenopus* oocytes. Preliminary experiments using SOFA confirmed the data

obtained with uptake assay (Fig. 18), showing that both NRAMP1 and DMT1 can transport ferrous iron. As a result, their activity is enhanced at pH 5.5.

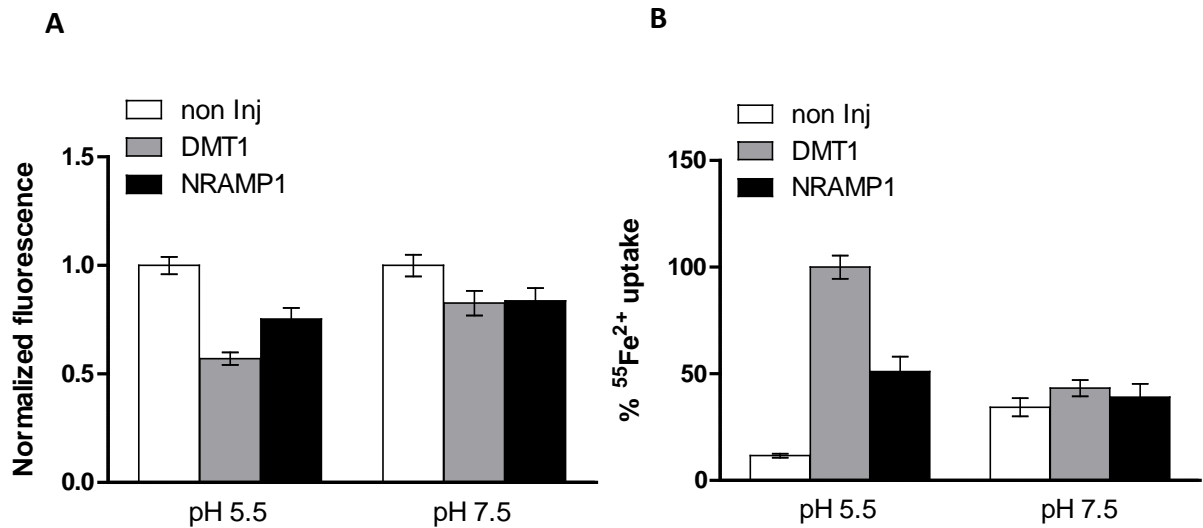


Fig. 18: H^+ -dependence of NRAMP1 and DMT1 activity analyzed using SOFA and uptake techniques. 100 μ M $FeCl_2$ uptake induced by NRAMP1 and DMT1 at pH 5.5 and 7.5 analyzed by SOFA (A.) or uptake techniques (B.). The white columns represent the uptake induced by control oocytes, the grey and the black columns indicate the uptake induced by DMT1 or NRAMP1 expressing oocytes respectively. Bars represent the mean \pm S.E.M. of 8-10 oocytes in a representative experiment of three independent experiments. $P < 0.05$ for all the results shown (Student's t-test).

To explore the divalent metal ions selectivity of NRAMP1 or DMT1, experiments were performed in the absence or presence of Fe^{2+} and Co^{2+} . As shown in Fig. 19, the data obtained with the novel technique SOFA demonstrate that $FeCl_2$ and $CoCl_2$ could induce calcein quenching due to the flux of ions through the plasma membrane of *Xenopus* oocytes. Therefore, NRAMP1 and DMT1 could transport these divalent metals and Co^{2+} is a competitive inhibitor of iron uptake.

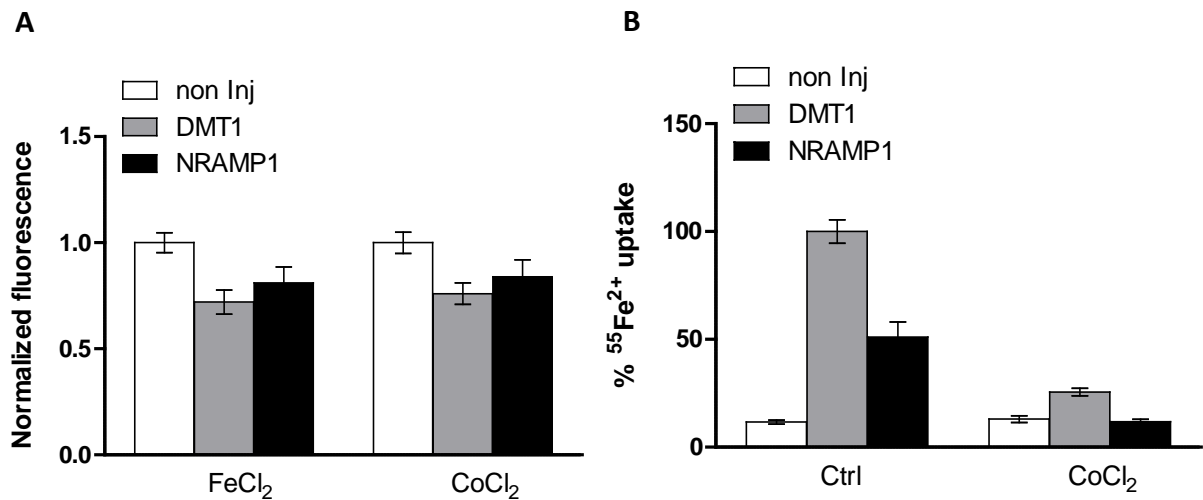


Fig. 19: CoCl₂ uptake induced by NRAMP1 or DMT1 analyzed using SOFA and uptake techniques. Uptake induced by NRAMP1 and DMT1 in the presence of 100 μM FeCl₂ or CoCl₂ at pH 5.5 analyzed with SOFA assay (A.) and 100 μM FeCl₂ uptake induced by NRAMP1 and DMT1 in the presence or absence of 1 mM CoCl₂ at pH 5.5 analyzed using uptake techniques (B.). The white columns represent the uptake induced by control oocytes, the grey and the black columns indicate the uptake induced by DMT1 or NRAMP1 expressing oocytes, respectively. Bars represent the mean ± S.E.M. of 8-10 oocytes in a representative experiment of three independent experiments. P<0.05 for all the results shown (Student's t-test).

6.2 NRAMP2 transport activity

6.2.1 Radioactive iron uptake induced by NRAMP2

The present study investigates the transport activity of NRAMP2, the protein localized in the contractile vacuole of *Dictyostelium discoideum*. NRAMP2 can transport ferrous iron. As a result, its transport activity was highest at pH 7.5 and was strongly reduced at pH 5.5. Therefore, this transporter is not proton activated (Fig. 20).

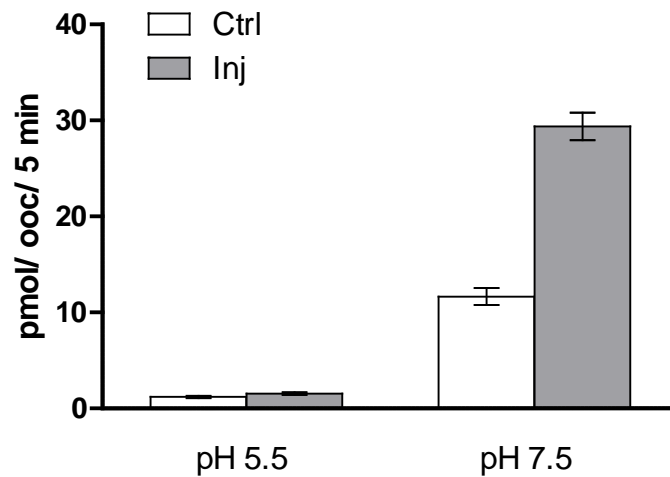


Fig. 20: Uptake of $^{55}\text{FeCl}_2$ in *Xenopus laevis* oocytes expressing NRAMP2: 100 μM FeCl_2 uptake induced by NRAMP2 at pH 5.5 and 7.5. The white columns represent the uptake in control oocytes and the grey columns indicate the uptake induced by cRNA injected oocytes. Bars represent the mean \pm S.E.M. of 8-10 oocytes in a representative experiment of three independent experiments. $P < 0.001$ for cRNA inj. pH 5.5 versus cRNA inj. pH 7.5; NRAMP2 induces significant iron accumulation only at pH 7.5 (Student's t-test).

In order to check the Na^+ -dependence of NRAMP2, experiments were performed in the absence and presence of Na^+ (Fig. 21). The transport activity of NRAMP2 is strongly reduced in the absence of Na^+ in the external medium. Therefore, its transport activity is Na^+ -dependent.

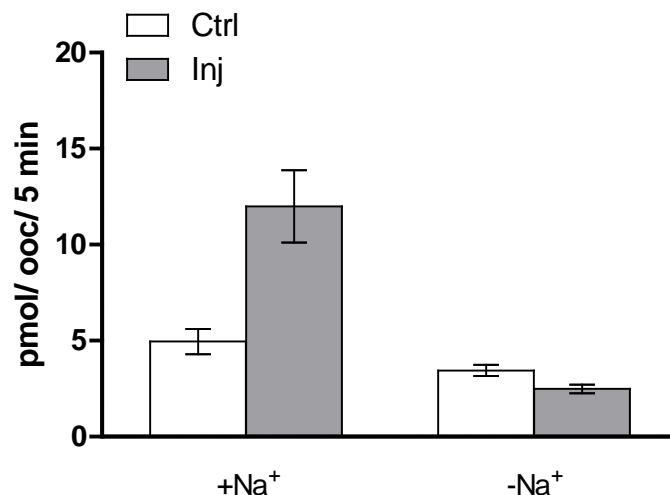


Fig. 21: Na^+ -dependence of $^{55}\text{FeCl}_2$ uptake induced by NRAMP2. 100 μM FeCl_2 uptake induced by NRAMP2 in the presence or absence of Na^+ at pH 7.5. The white columns represent the uptake in control oocytes and the grey columns indicate the uptake induced by cRNA injected oocytes. Bars represent the mean \pm S.E.M. of 8-

10 oocytes in a representative experiment of three independent experiments. $P < 0.05$ for cRNA inj. oocytes in presence versus absence of Na^+ (Student's t-test).

6.2.2 Calcein assays in oocytes expressing NRAMP2 using confocal microscopy

Live imaging of metal transport using confocal microscopy (Fig. 22) confirmed the results obtained with uptake experiments. NRAMP2 transport activity is limited to ferrous iron and no calcein quenching was detected in the presence of $100 \mu\text{M Mn}^{2+}$ in the external solution.

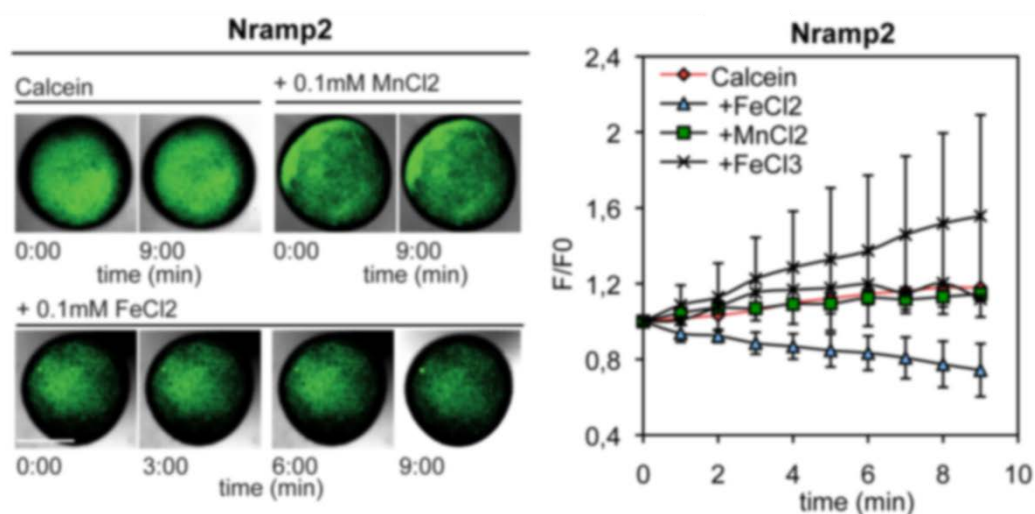


Fig. 22: Live imaging of divalent metal transport induced by NRAMP2 in *Xenopus* oocytes using calcein fluorescence. In the presence of Fe^{2+} calcein fluorescence decreased for NRAMP2 expressing oocytes. The fluorescence changes over time were quantified by measuring the ratio of fluorescence intensity at the time indicated in the abscissa (F) vs. fluorescence at time 0 (F_0). Mean \pm S.D. are shown for each group of oocytes [NRAMP2 n values are: Mn, 2; Fe(II), 7; Fe(III), 11; Cu, 5]. Statistical analysis was undertaken at the 9-min time point using a two-tailed Student's t-test, assuming unequal variance: Fe(II) versus control was $P < 0.05$. Scale bars: 0.5 mm.

7. DISCUSSION

Dictyostelium discoideum harbours several iron genes with the exception of transferrin, ferritin and TfR [1, 2] and represents a model for the study of cellular iron homeostasis showing subcellular localization of NRAMP transporters resembling that of macrophages. *Dictyostelium discoideum* expresses the ortholog of NRAMP1 transporter in phago-lysosomes and that of NRAMP2 in the contractile vacuole. Radiochemical assays showed that NRAMP1 induced ferrous iron transport is proton dependent and it is inhibited by Mn^{2+} , Cd^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} and to a lesser extent by Zn^{2+} . In calcein injected oocytes expressing NRAMP1 and analyzed by confocal microscopy, Fe^{2+} , Mn^{2+} but not Fe^{3+} or Cu^{2+} led to fluorescence quenching due to their transport and accumulation into the cytoplasm of the oocytes [2]. Therefore copper, that in uptake experiment inhibits iron uptake, actually interacts with the transporter, probably blocking its transport activity, and it is a non-competitive inhibitor of iron uptake (no quenching is detectable). Interestingly, metal ions binding studies in crystals of the homolog *ScaDMT* have recently shown that copper binds to the same location as Mn^{2+} and Fe^{2+} , but at a slightly shifted position [95]. This could explain why copper is not transported. Compared to competition experiments with radiolabeled tracer, calcein assays have thus the advantage to discriminate between Fe^{2+} competitors that are transported, such as Mn^{2+} , or that just hinder transport, such as Fe^{3+} or Cu^{2+} [2]. Therefore we can also affirm that Mn^{2+} may compete with iron to be transported by NRAMP1. To better characterize the cation selectivity of NRAMP1, its transport activity was analyzed by a novel approach based on metal-induced changes in calcein fluorescence, called SOFA. This technique showed that also Co^{2+} is transported by NRAMP1 and confirms the data obtained with uptake experiments. SOFA (Single Oocyte Fluorescence Assay) and transport assays analyzed by confocal microscopy are two novel techniques that exploit calcein properties, allowing to measure calcein quenching due to the flux of ions through the plasma membrane of *Xenopus* oocytes induced by a specific transporter. These techniques constitute a valuable alternative to radioactive uptake assays, avoiding the use of radioactive elements, reducing the costs and the risks of the experiment. The functional characterization of *Dictyostelium* NRAMP1 reveals that it is a ferrous iron and manganese transporter and it shows an activity resembling that of murine DMT1 for H^+ -dependence and cationic selectivity. In *Dictyostelium* cells, NRAMP1 is associated with V-ATPase in phago-lysosomes, that maintains the proton gradient between phagosomal membrane and cytosol. Therefore, the functional characterization of NRAMP1 confirms its implication in the efflux of iron and manganese to the cytosol; indeed, iron is likely gained by degradation of ingested bacteria and efflux via NRAMP1 from phagosomes to the cytosol after bacterial engulfment. The transport of iron and manganese to the cytosol is important in order to deplete the engulfed bacteria from these

essential elements, blocking the growth of the bacteria and it represents a source of manganese or iron for *Dictyostelium*. The data about NRAMP2 are preliminary but it can transport ferrous iron at 7.5 and it appears not proton activated. Further experiments reveal that its activity is enhanced by Na^+ . For *D. discoideum*, this could be important in order to allow iron flux between CV and cytosol. This data is in agreement with the fact that the vacuole internal pH is neutral, nevertheless the transport activity of this protein is strongly reduced compared with that observed for NRAMP1. This could be due to a defective localization or to a defective recycling of the transporter at the plasma membrane of *Xenopus* oocytes.

8. BIBLIOGRAPHY

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