Doctoral Thesis

Molecular insight into transition metal transport in the green microalga Chlamydomonas reinhardtii

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MOLECULAR INSIGHT INTO TRANSITION METAL TRANSPORT IN THE GREEN MICROALGA CHLAMYDOMONAS REINHARDTII

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

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presented by

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**Chapters submitted for publication:**


**Abbreviations:**

Cd: cadmium  
CDF: cation diffusion facilitator  
Cu: copper  
DMT1: divalent metal transporter  
EDTA: ethylene diamine tetra acetic acid  
EGTA: ethylene glycol-bis(2-aminoethyl)-tetra acetic acid  
Fe: iron  
Mn: manganese  
Nramp: natural resistance associated macrophage protein  
SOD: superoxide dismutase  
TM: transmembrane domain  
ZIP: Zrt, Irt-like protein  
Zn: zinc
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References
Trace amounts of certain transition metals often referred to as “heavy metals” act as cofactors for proteins involved in vital cellular processes. The very same metals as well as others with no essential function can be harmful to the cell if they exceed physiological concentrations. Consequently, mechanisms preserving a stable metal environment within the cell are of outstanding importance. In this context, a decisive role is played by systems which enable the passage of these micronutrients across biological membranes. Supply of the cell with the necessary amounts of metals is guaranteed by uptake systems or systems which release metal stores from intracellular compartments, and metal resistance can be achieved by systems serving the reverse function. The molecular background of these transport processes in algae is not as extensively studied as in other organisms. We chose the green microalga *Chlamydomonas reinhardtii* as a model organism in order to identify systems which are used by algae to facilitate metal transport. *C. reinhardtii* is suitable for such a study since there are some sequence data available and it is established as a laboratory organism. In the *C. reinhardtii* EST (expressed sequence tags) database I was able to identify a sequence which on the amino acid level showed high similarity to members of the Nramp family. Nramps (natural resistance associated macrophage proteins) constitute a group of proteins acting in the transport of divalent metal cations. They occur in a great number of organisms sharing remarkable sequence similarity and displaying broad substrate specificity. We reasoned that the wide distribution and the high degree of conservation of these proteins underscore their importance to cell survival. Therefore, the main part of this work concentrates on the cloning and functional characterization of the *C. reinhardtii* Nramp homologue, which was named DMT1 in the present study.

To study DMT1 function, I performed yeast complementation assays with three different yeast mutant strains deficient in manganese (*smf1*), zinc (*zrt1/zrt2*) or iron uptake (*fet3/fet4*). After transforming the strains with a plasmid moderately expressing DMT1, I monitored the effect of expression of the foreign gene in growth experiments. For this, maximal growth rates of each yeast mutant strain in minimal medium (control) and in minimal medium with the metal chelators EGTA or EDTA (metal deficient conditions) were compared. The DMT1 expressing mutant strains were compared to the control strain (mutant transformed with the empty vector) in terms of change in growth rate under the different
conditions. In the case of the manganese and iron uptake deficient strains, stable expression of DMT1 (verified by western blotting) improved growth under metal limitation conditions compared to the control cells. These results clearly demonstrated that DMT1 is capable of metal transport.

To further investigate the specificity of the transport protein, I performed growth experiments – in a reverse fashion than above – and checked whether expression of DMT1 limits growth by conferring increased sensitivity to elevated metal concentrations. I tested growth of the smfl yeast strain, which is an Nramp knockout, under different culture conditions and showed that DMT1 expression rendered the smfl mutant sensitive to manganese, copper and the non-essential metal cadmium, suggesting that these metals are transported by DMT1. Thus, the broad specificity described for Nramps was also confirmed for the C. reinhardtii homologue. In the case of zinc, the growth rate under metal limiting conditions did not change when DMT1 was expressed. However, addition of zinc reduced sensitivity of the smfl strain to increased manganese concentrations. This might indicate that zinc can compete with manganese for binding sites on the DMT1 protein even if it is not transported.

In further experiments, I tested mutants of the DMT1 protein on expression and function, gaining information on the essentiality of individual amino acid residues. Only one of the three mutated protein versions was functional in a yeast complementation assay although an absolutely conserved amino acid was substituted in this protein.

Recently, a first draft of the C. reinhardtii genome was made available (Doe Joint Genome Institute, California, http://genome.jgi-psf.org/chlre1/chlre1.home.html [52]). I conducted a thorough search in the genomic sequence which allowed a more general insight into the metal transport systems the alga possesses. By this search I identified several putative transport systems belonging to different protein families and a total of five Nramp homologues in the genome.
Zusammenfassung


Um die Funktion von DMT1 zu untersuchen, benutze ich drei verschiedene Hefe-Mutantenstämme, bei denen die Transportaktivität für Mangan (*smf1*), Zink (*zrt1/zrt3*) und Eisen (*fet3/fet4*) stark beeinträchtigt ist. Nachdem ich die Stämme mit einem Plasmid transformiert hatte, das eine moderate Expression von DMT1 erlaubte, verfolgte ich die Auswirkung der Expression des Fremdgens mittels Wachstumstests. Dafür wurden die
maximalen Wachstumsraten jedes Hefestammes in Minimalmedium (Kontrolle) und in Minimalmedium mit den Metalchelatoren EGTA oder EDTA (Metallmangel) verglichen. Die Mutanten, die DMT1 exprimierten, wurden mit dem Kontrollstamm (Mutante transformiert mit leerem Vektor) hinsichtlich einer Veränderung der Wachstumsrate unter verschiedenen Kultivierungsbedingungen verglichen. Stabile Expression von DMT1 (bestätigt mittels western blots) verbesserte das Wachstum der smfl und fet3/fet4 Mutanten unter Metallmangel-Bedingungen, im Vergleich zur Kontrollkultur. Diese Ergebnisse zeigten deutlich, dass DMT1 Metallionen transportierte.

Um die Frage der Spezifität des Transporters weiter zu untersuchen, führte ich Wachstumstests durch, und untersuchte ob die Expression von DMT1 eine erhöhte Sensitivität gegenüber hohen Metallkonzentrationen bewirken und somit das Wachstum der Zellen beeinträchtigen kann. Ich testete das Wachstum der smfl Mutante, welche ein Nramp Knockout ist, unter verschiedenen Metallbedingungen und zeigte, dass DMT1 Mangan-, Kupfer- und Cadmium-Ionen transportieren kann. Somit wurde die breite Spezifität, die für andere Nramps beschrieben wurde, auch für das C. reinhardtii Homolog bestätigt. Was die zrt1/zrt3 Mutante betrifft, bewirkte die DMT1 Expression keine Veränderung im Wachstum unter Metallmangelbedingungen. Jedoch verminderte die Zugabe von Zink die Sensitivität eines smfl Stammes gegenüber erhöhten Mangankonzentrationen. Das könnte ein Hinweis darauf sein, dass Zink mit Mangan um die Bindestellen auf dem DMT1 Protein konkurrieren kann, auch wenn Zink nicht transportiert wird.

Ferner untersuchte ich Mutationen im DMT1 Protein, um Informationen bezüglich der Bedeutung einzelner Aminosäuren für die Expression und die Funktion des Proteins zu bekommen. Nur eines der drei mutierten Proteinderivate war funktionell in einem Hefe-Komplementierungssystem, obwohl in diesem Protein eine absolut konservierte Aminosäure ersetzt war.

Unlängst wurde im Internet eine erste Version der genomischen Sequenz von C. reinhardtii aufgeschaltet (Doe Joint Genome Institute, California, http://genome.jgi-psf.org/chlrel/chlrel.home.html, [52]). Eine gründliche Recherche in der genomischen Sequenz erlaubte Einsicht in verschiedene Metallsysteme, die die Alge besitzt. Ich identifizierte mehrere mutmassliche Transportsysteme, die zu verschiedenen Proteinfamilien gehören, sowie fünf Nramp-Homologe im Genom der Alge.
Chapter 1

General Introduction

Transition metals are involved in a broad range of cellular functions acting especially as structural or functional cofactors for proteins. Several mechanisms have evolved in order to guarantee the supply and also the homeostasis of these micronutrients in the cell. In this regard great importance is attached to systems which transport metals into and out of the cytoplasm or the cellular organelles. This introduction gives an overview on metal function and general transport principles, followed by a description of selected metal transport systems. At the end of the chapter the organism studied in this project will be shortly introduced.

1.1 What are “heavy metals”? A short insight into a confusing terminology

Metals are defined as “elements which conduct electricity, have a metallic luster, are malleable and ductile, form cations, and have basic oxides” [6]. Following this definition over ¾ of the elements in the periodic table are metals (Fig. 1.1). Usually, the terms “metal” and “heavy metal” are used referring not only to the pure element but also to all possible chemical species in which the element may exist (reviewed in [35]). This implies that the pure metal and all its compounds have the same physicochemical, biological and toxicological properties, which does not correspond to reality. In addition to this discrepancy, the term “heavy metal” is very loosely defined (based on density, atomic weight, atomic number or other chemical properties, depending on the definition of the respective author) and is often misused as a synonym to toxic or harmful metal species, assuming that “heaviness” and “toxicity” are identical. In order to guard against confusion it was suggested that a chemical classification of metallic elements following the periodic table may be more appropriate [35]. In the periodic table the elements are grouped according to valence electron configuration and thus by chemical reactivity. In this classification the metallic elements are divided into s-block, p-block, d-block-transition and f-block elements (Fig. 1.1). Elements referred to as “heavy
metals” are found in the class of d-block elements, also called transition metals. Transition metals are characterized by incompletely filled 3d orbitals, while the 4s orbital is already filled. They do not always use the same number of electrons in chemical reactions since the 4s and 3d electrons are energetically close (e.g. iron gives sometimes its two 4s electrons (Fe²⁺), and sometimes adds a 3d electron (Fe³⁺)). Thus, transition metals can take up different oxidation states and are very versatile for biochemical reactions.

In my PhD thesis, the term “transition metals” will be preferred over the term “heavy metals”, referring to the biologically relevant elements. For practical reasons, the word “metal” or the name of an element will be used in the context of “metal transport”, “metal tolerance” etc, and where appropriate the ionic form will be specified (e.g. ferrous ion or Fe²⁺).

![Figure 1.1 Periodic table of the elements (adapted from [44]). f-block elements (lanthanides and actinides) are not shown. The group of the d-block/transition elements is highlighted by a square. Most “trace elements” are found within this group (boxed). Bulk essential elements are circled. The black line is the border between metallic elements (on the left) and non-metals (on the right). This border is not strict, since the elements near it do not possess features strictly metallic or non-metallic, respectively. It is noteworthy that the elements essential for life are the lighter elements, which are also most abundant on earth [44].](image-url)
1.2 Functions of metals

A large number of analyses of several plant and animals’ species, as well as organs and tissues made clear that of all the naturally occurring elements 11 are absolutely essential for life (bulk biological elements, Fig. 1.1). In the example of the human body 99% of the atoms present are constituted by hydrogen and oxygen (88.2%, due to the high water content of all living organisms), and carbon and nitrogen (10.8%). These are the basic elements of the organic structures. Seven additional essential elements are sodium, potassium, calcium, magnesium, phosphorus, sulfur and chlorine, comprising 0.9%. The remaining 0.1% are elements required by most but not all biological systems, and in varying amounts: vanadium, chromium, manganese, iron, cobalt, nickel, copper, zinc, molybdenum, boron, silicon, selenium, fluorine, iodine, arsenic, bromine, and tin, where for the last three elements it is not entirely clear whether they are indeed essential. Iron and zinc occur in all species except for a few bacteria [44].

Essential elements which belong to the group of transition metals are only needed in trace amounts. However, this does not derogate their importance. On the contrary, the cellular reactions these ions are involved in comprise a major part of the cells metabolism. A deficiency in these metals can have severe consequences at cellular and organism level. Manganese is mainly known for its involvement in the oxygen production in the oxygen evolving complex in photosystem II and for its action in the dismutation of the superoxide anion as a cofactor in MnSOD (manganese superoxide dismutase). Both are highly important cellular reactions which establish manganese as an essential element in the cell. Iron and copper have the facile ability to donate and accept electrons and can easily shift between two oxidation states. They are cofactors of a large number of metalloproteins which catalyze redox reactions. Zinc is essential for the formation of the “zinc-finger”, a structural element important for the functionality of transcription factors in transcriptional regulation. It is also a cofactor of enzymes involved in the synthesis of nucleic and amino acids. A short overview of some specific functions of selected metal cations is presented in table 1.1.
Table 1.1 Selected enzymes utilizing transition metals as cofactors [44].

<table>
<thead>
<tr>
<th>Metal</th>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td>Cytochrome oxidase</td>
<td>respiratory electron transfer chain in mitochondria (reduction of $O_2$ to $H_2O$ linked to proton gradient formation)</td>
</tr>
<tr>
<td></td>
<td>Cu, Zn-superoxide dismutase (see also zinc)</td>
<td>conversion of superoxide radical to hydrogen peroxide (cytosol, mitochondria) $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$</td>
</tr>
<tr>
<td></td>
<td>Blue copper proteins</td>
<td>electron transfer (membranes, thylakoid vesicles)</td>
</tr>
<tr>
<td></td>
<td>Caeruloplasmin (multicopper oxidase)</td>
<td>converts Fe$^{2+}$ to Fe$^{3+}$ in the presence of molecular oxygen (which is reduced to $H_2O$)</td>
</tr>
<tr>
<td></td>
<td>Plastocyanin</td>
<td>photosynthetic electron transfer chain in chloroplasts</td>
</tr>
<tr>
<td>Manganese</td>
<td>Mn-superoxide dismutase</td>
<td>conversion of superoxide radical to hydrogen peroxide (mitochondria, chloroplasts, prokaryotes)</td>
</tr>
<tr>
<td></td>
<td>Oxygen Evolving Complex, Photosystem II</td>
<td>dioxygen release (chloroplast)</td>
</tr>
<tr>
<td></td>
<td>Isocitrate dehydrogenase</td>
<td>citric acid cycle (mitochondria)</td>
</tr>
<tr>
<td></td>
<td>Mn-catalase</td>
<td>oxidative stress defense: $2H_2O_2 \rightarrow 2H_2O + O_2$</td>
</tr>
<tr>
<td></td>
<td>Imidazole glycerol phosphate dehydratase</td>
<td>histidine synthesis</td>
</tr>
<tr>
<td></td>
<td>Galactosyl transferase</td>
<td>formation of oligosaccharides of glycoproteins (Golgi)</td>
</tr>
<tr>
<td></td>
<td>Glutamate synthetase</td>
<td>glutamine synthesis</td>
</tr>
<tr>
<td>Zinc</td>
<td>Cu, Zn-superoxide dismutase (see also copper)</td>
<td>copper is the functional cofactor, zinc is an extra stabilizing factor</td>
</tr>
<tr>
<td></td>
<td>Zinc finger proteins</td>
<td>transcription factors (binding to promoter sequences)</td>
</tr>
<tr>
<td></td>
<td>Alcohol dehydrogenase</td>
<td>redox reaction, $NAD^+ + C_2H_5OH \rightarrow NADH + CH_3CHO + H^+$</td>
</tr>
<tr>
<td></td>
<td>Aspartate transcarbamylase</td>
<td>nucleic acid synthesis</td>
</tr>
<tr>
<td></td>
<td>Dehydroquinate synthase</td>
<td>synthesis of aromatic amino acids phenylalanine, tyrosine, tryptophan</td>
</tr>
<tr>
<td></td>
<td>RNA polymerase</td>
<td>RNA synthesis</td>
</tr>
<tr>
<td></td>
<td>Reverse transcriptase</td>
<td>DNA synthesis</td>
</tr>
<tr>
<td></td>
<td>tRNA synthase</td>
<td>transfer RNA synthesis</td>
</tr>
<tr>
<td>Iron</td>
<td>Haemoglobin</td>
<td>transport of $O_2$</td>
</tr>
<tr>
<td></td>
<td>Transferrin</td>
<td>glycoprotein, transport vehicle for Fe$^{3+}$</td>
</tr>
<tr>
<td></td>
<td>Ferritin</td>
<td>storage of Fe$^{3+}$ (cytoplasm)</td>
</tr>
<tr>
<td></td>
<td>Fe-superoxide dismutase</td>
<td>conversion of superoxide radical to hydrogen peroxide (chloroplasts, prokaryotes)</td>
</tr>
<tr>
<td></td>
<td>Cytochromes</td>
<td>electron transfer chain (respiration, photosynthesis)</td>
</tr>
</tbody>
</table>
1.3 Transition metal toxicity and availability

Despite their enormous physiological significance, essential transition metals are needed only in trace concentrations and can become toxic for the cell when they exceed physiological levels. Similarly, metal ions for which no physiological function has been shown can be detrimental when they enter the cell in concentrations exceeding the tolerance limit. Redox activity, a capacity of transition metals like iron and copper, which is routinely exploited by the cell to generate reactive chemical centers for physiologically essential purposes, may lead to substantial damages. Reactive oxygen species (ROS) can be produced by the Fenton and Haber-Weiss reaction in the presence of Cu, Fe, Cr or V [7] leading eventually to damage of DNA, proteins or lipids.

$$\begin{align*}
Fe^{3+} + O_2^- & \rightarrow Fe^{2+} + O_2 \\
Fe^{2+} + H_2O_2 & \rightarrow Fe^{3+} + OH^- + OH' \text{ (Fenton reaction)}
\end{align*}$$

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH' \text{ (Haber-Weiss reaction)}$$

Redox-inactive metals like Cd, Hg, Ni, and Pb may induce oxidative stress indirectly by displacing native metals from cellular binding sites (toxicity of the superoxide anion $O_2^-$ is believed to be mediated indirectly via release of Fenton catalysts from iron-sulfur enzymes, [7]). Other toxicity mechanisms include binding to macromolecules altering their conformation, inactivation of enzymes by displacement of the native metal ion, and interference with membrane permeability.

It is interesting to note that the elements essential for life are the lighter elements which are also the most abundant on earth (Fig. 1.1, [44]). Some elements have analogous properties and would play a similar biological role, but they differ markedly in their abundance. Presumably one reason why the bulk biological metals sodium, potassium, magnesium and calcium were selected in preference to lithium, rubidium, cesium, beryllium, strontium, and barium is that they are more abundant and can perform the tasks that other elements of the same subgroups of the periodic table can perform [44]. But besides the general abundance of the elements their biological availability is of particular importance for
living organisms. These two attributes do not necessarily go together. Most strikingly iron, one of the major players in cellular metabolism, stands at the bottom of the ladder when it comes to its accessibility by biological systems (Fig. 1.2), although it is one of the most abundant transition metals in earth crust [44]. The reason is that iron spontaneously oxidizes in an aerobic environment and at neutral pH and occurs in the form of ferric complexes which are insoluble. However, living organisms are extremely efficient in overcoming limited availability of metals and are able to maintain metal concentrations which exceed the environmental concentrations by several orders of magnitude (Tab. 1.2 [44]). Obviously, a sophisticated system of uptake, storage and detoxification is at play which not only supplies the cells with essential metals, but also maintains the desired concentration balance of the various metal elements.

---

**Figure 1.2** Ease of extraction of elements from the earth to the sea as a guide to the ease with which biological systems will have access to elements, no matter what the absolute abundance [44]. The top of the figure indicates the speciation.
### Table 1.2 Accumulation of metallic elements in algae [134].

<table>
<thead>
<tr>
<th>Concentration factor*</th>
<th>Metal</th>
<th>Type of algae</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,700</td>
<td>Zn</td>
<td>Green algae</td>
</tr>
<tr>
<td>21,600</td>
<td>Zn</td>
<td>diatoms</td>
</tr>
<tr>
<td>ca. 4,000</td>
<td>Zn, Cu, Cd</td>
<td>Chlorococcus parisi</td>
</tr>
<tr>
<td>2,000,000</td>
<td>Cd</td>
<td>Chlorella pyrenoidosa</td>
</tr>
<tr>
<td>&lt;4,000</td>
<td>Cd</td>
<td>Chlorella pyrenoidosa</td>
</tr>
<tr>
<td>1,000-100,000</td>
<td>Cd, Pb, Hg</td>
<td>various spp.</td>
</tr>
<tr>
<td>4,000</td>
<td>U</td>
<td>Chlorella</td>
</tr>
<tr>
<td>&gt;100,000</td>
<td>Pu, Am, Np</td>
<td>various spp.</td>
</tr>
<tr>
<td>500-30,000</td>
<td>various</td>
<td>various spp.</td>
</tr>
<tr>
<td>3056</td>
<td>Cu</td>
<td>Chlorella</td>
</tr>
<tr>
<td>849</td>
<td>Ni</td>
<td>Chlorella</td>
</tr>
<tr>
<td>2327</td>
<td>Cd</td>
<td>Chlorella</td>
</tr>
<tr>
<td>570-31,000</td>
<td>Pb</td>
<td>various spp.</td>
</tr>
<tr>
<td>2,000-25,000</td>
<td>Pb</td>
<td>Cladophora glomerata</td>
</tr>
<tr>
<td>16,000-20,000</td>
<td>Pb</td>
<td>Cladophora glomerata</td>
</tr>
<tr>
<td>620-7,700</td>
<td>Pb, Cd, Zn, Cr</td>
<td>Green algae</td>
</tr>
</tbody>
</table>

*metal in biomass/ metal in solution

### 1.4 Transition metal transport

As mentioned above, different elements do not occur at the same concentration in the environment, are not equally bioavailable, and must be maintained at different concentrations in the cell. A stunning number of uptake mechanisms for micronutrients such as metal ions have evolved and many of them have been characterized in pro- and eukaryotes. The general principles are valid in all organisms. Metal depletion of the growth medium induces the activity of high affinity uptake systems, while under more favorable conditions low affinity systems are active. Compartmentalization into different cellular organelles requires intracellular transport systems, and detoxification mechanisms may also be based on transporter systems which extrude unwanted metals or direct them into intracellular compartments.

#### 1.4.1 Transport systems

The passage of solutes across membranes can occur via protein-independent or protein-dependent transport processes. The latter can be grouped into classes according to energetic criteria [1].
1) Protein-independent diffusion
Diffusion is a transport process which does not require a protein to facilitate passage of the solute through the membrane. Some small molecules like water, ethanol and gases (O₂, CO₂, NH₃) cross the membrane more or less freely following their concentration gradient and with no consumption of metabolic energy. Diffusion is not an adequate process under conditions which demand fast and efficient solute movement (e.g. nutrient limitation, fast growth, weak solute gradient) and cannot guarantee specificity.

2) Protein-mediated transport
In this group one or more proteins act as transporters, expanding the possibilities of the transport process regarding direction, substrates and velocity.

a) Channels
Although a channel facilitates permeation of the membrane, the solutes still follow their concentration gradient without requirement of energy, similarly to diffusion. Aquaporins are such proteins which form channels for the entrance of water into the cell (a molecule which is also able to enter by diffusion).

b) Carriers
Carriers are at play when transport has to occur against a concentration gradient and consequently requires energy. In order to take place this uptake reaction must be coupled to another energy releasing reaction.

i) Primary active
In this class, transport can be driven by ATP hydrolysis or synthesis. ATP synthases in bacteria, mitochondria and chloroplasts convert primary energy, such as respiration- and photosynthesis-coupled movement of H⁺ in the direction of the membrane potential, into ATP. ATPases in the plasma membrane (eukaryotic Na⁺/K⁺ ATPase, bacterial K⁺ ATPase) pump ions at the expense of ATP. ABC (ATP binding cassette) transporters are widely spread and also couple transport of a variety of substrates to ATP hydrolysis. No ABC-type uptake systems have been identified in eukaryotes, but ABC-type
efflux systems abound in both prokaryotes and eukaryotes. Another example for primary active transporters is the CPx-ATPase family described in 1.4.2.

ii) Secondary active

Here, the chemical coupling makes use of the free energy produced by the electrochemical potential of another solute. Pre-existing gradients which are maintained by the action of primary systems are used as driving forces. The process can take place as a uniport, a symport or an antiport. The uniport is insofar a special case as the transported solute uses its own electrochemical gradient. Examples described in more detail in 1.4.2 include the ZIP, CDF and Nramp family.

iii) Group translocators

This group has only been described in bacteria and involves phosphorylation of the substrate during the transport process.

1.4.2 Transport protein families

Transport proteins are grouped into families according to sequence and functional similarity. Several classes of proteins have been proposed or demonstrated to play a role in metal transport. On the following I will concentrate on four major families mediating transition metal transport in eukaryotic organisms (but are also found in prokaryotes).

I) The CPx-type ATPases

CPx-type ATPases are primary active transporters expressed in a wide range of organisms and transporting essential and potentially toxic metal cations across cell membranes (e.g. Cu$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Pb$^{2+}$). They compose a subgroup of the large superfamily of P-type ATPases, which pump a variety of charged substrates across biological membranes at the expense of ATP, and are distinguished by the formation of a phosphorylated intermediate during their catalytic reaction cycle [135]. The divalent metal cation P-type ATPases were defined as CPx-ATPases due to a conserved intramembranous cysteine-
proline-cysteine/histidine-serine (CPx) motif which is thought to function in metal cation transduction [119] (Fig. 5.1).

In cyanobacteria, the CPx-ATPases PacS and CtaA were suggested to supply Cu for photosynthesis [128]. PacS, localized in thylakoid membranes, may also play a role in detoxification by pumping excess Cu into the thylakoid lumen. CtaA is localized in the cytoplasmic membrane. In S. cerevisiae, Cu bound to the copper chaperone Atx1p is transferred to the CPx-ATPase Ccc2p and transported into post-Golgi vesicles. The Cu is then loaded on the multicopper ferroxidase Fet3p [137].

The first CPx-ATPase reported in higher plants was PAA1 (P-type ATPase of Arabidopsis) from Arabidopsis thaliana, identified by sequence homology to CtaA [122]. PAA1 was shown to localize in the chloroplast inner envelope membrane and was suggested to transport Cu for the Cu/Zn superoxide dismutase across the plastid envelope membrane [118]. Another homologue, PAA2, might transport Cu across the thylakoid membrane since it possesses a plastid targeting signal. HMA5, a third homologue discussed by the same authors, may supply mitochondrial proteins such as cytochrome c oxidase with Cu, since it possesses a putative mitochondrial targeting signal sequence [118]. RAN1 (responsive to antagonist 1) was shown to rescue a yeast ccc2 mutant [65]. In Arabidopsis RAN1 is required for loading copper into the active site of the ethylene receptor [64].

CPx-ATPases might be important not only in obtaining sufficient amounts of transition metal ions for essential cell functions but also in preventing accumulation of these ions to toxic levels [135]. It was shown for prokaryotic CPx-ATPases that they mediate efflux (CadA in S. aureus exports cadmium [95], and ZntA in E. coli catalyzes efflux of Zn, Cd, Pb, and Hg [106]).

II) The CDF family

CDF (cation diffusion facilitator) proteins are a family of metal cation transporters identified in bacteria, archaea and eukaryotes (reviewed in [46]). Transport is coupled to the electrochemical H⁺ potential. Many members of this family have been implicated specifically in the transport of zinc in a direction away from the cytoplasm, i.e. out of the cell or into organelar compartments. CDFs are divided based on sequence similarities into subfamilies I, II and III. Subfamily I includes mostly eubacterial and archaeal proteins, while in II and III pro- and eukaryotic proteins are distributed equally. Both the C- and the N-terminus of a
typical CDF protein are predicted to be cytoplasmic with 6 transmembrane domains (TM) between them (Fig. 5.1). A long loop region is found between TM IV and V, frequently containing a histidine-rich motif (HX)n (where n=3-6 and X often S or G). This loop was shown to act in metal binding in *A. thaliana* ZAT1 [11]. Proteins lacking the loop were stably expressed but no longer bound zinc.

In the yeast *S. cerevisiae* the CDF homologue Zrc1p is involved in zinc and cadmium resistance [69]. Another homologue, Cot1p was shown to act in cobalt transport [25] and in zinc detoxification by zinc compartmentalization [24, 25]. Both proteins are expressed on the vacuolar membrane and are thought to sequestrate zinc into the vacuole, driven by the proton potential which is maintained by the V-ATPase (vacuolar ATPase) [80]. In mammals, two CDF homologues seem to play a role in zinc detoxification. Znt1 is expressed on the plasma membrane and exports zinc [99], while Znt2 transports zinc into endosomal/lysosomal vesicles, thereby protecting the cell against toxic zinc concentrations [98].

The *A. thaliana* CDF homologue ZAT1 is expressed in all organs and is involved in zinc resistance. Overexpression in plants leads to enhanced zinc resistance and zinc accumulation in roots, showing that zinc must be transported into a compartment (vacuole) in the root cells [129]. When ZAT1 was purified and reconstituted in proteoliposomes, it did not transport cobalt, and transported cadmium only at 1% of the zinc transport rate, showing that ZAT1 is specific for zinc [11].

### III) The ZIP family

The abbreviation ZIP stands for Zrt, Irt-like protein. Zrt1 (zinc regulated transporter 1) is an *S. cerevisiae* zinc transporter [138], Irt1 (iron regulated transporter 1) functions as an iron transporter in *Arabidopsis thaliana* [37]. The ZIP family includes members from plants, protozoa, insects, mammals and fungi, as well as from eubacteria and archaea [56]. Guerinot et al [56] divided the ZIP proteins into subfamily I (fungi and plants) and II (mammals and nematodes), with many members involved in zinc uptake. Gaither and Eide add in their review two more groups to the ZIP family [46]. The gufA subfamily includes prokaryotic proteins of unknown function, like the GufA of *Myxococcus xanthus* [90], and also eukaryotic proteins like the Zrt3p of *S. cerevisiae*. For ZupT from *E. coli* a function in Zn transport was demonstrated by uptake measurements [51]. The Liv-1 subfamily includes only eukaryotic proteins. Liv-1 is expressed highly in certain metastatic breast cancer tissues [89] but its
function is unknown. This group is only based on a high sequence similarity to the other ZIP proteins but none of the Liv-1 subgroup members was functionally tested to date.

Most ZIP proteins were predicted to possess eight transmembrane domains with the amino- and carboxyterminal ends located on the outside of the plasma membrane [56] (Fig. 5.1). A remarkable feature of many ZIPS is a long cytoplasmic loop between transmembrane domains III and IV (called the “variable region”, because its sequence is not notably conserved [56]), which accommodates many histidine residues. Since histidines are often involved in metal binding, this loop is a potential metal binding site of the proteins. Residues important for determining substrate specificity of IRT1 were mapped to the loop region between TM II and III, which is extracytoplasmic and may be the initial site of substrate binding [110]. Residues important for function of IRT1 were mapped in TMs IV and V, and include three histidines [110]. TMs IV and V are highly conserved and contain histidine residues often with adjacent polar or charged amino acids. Thus, they are predicted to line an aqueous cavity in the transporter through which the cationic substrate passes [38]. The energy source for transport has not been characterized, but these systems probably function as secondary active carriers. A Zn^{2+}/HCO_3^- symport mechanism was proposed for the human hZip2 [45].

In yeast, Zrt1p and Zrt2p are responsible for the high and low affinity zinc uptake, while Zrt3p probably mobilizes the vacuolar zinc store (which is maintained by the action of the CDF members Zrc1p and Cot1p) [87, 138, 139].

In the A. thaliana genome 18 ZIP members were identified, spread among all ZIP subfamilies except subfamily II. IRT1 (iron regulated transporter 1) from A. thaliana was the first ZIP ever identified [37]. The protein complemented a yeast iron transport mutant increasing the iron content of the yeast cells [37], but was also shown by metal uptake assays to transport Zn^{2+}, Mn^{2+} and Cd^{2+} [74]. Since IRT1 is expressed only in the roots of iron limited plants, its function seems to lie mainly in the accumulation of iron, not excluding that other metal cations may also be transported under these conditions. In fact, it was shown that iron-limited plants accumulated higher levels of Zn^{2+}, Mn^{2+} and Cd^{2+} [23, 74, 133]. IRT1 is considered as the major iron uptake system from the soil. Four other ZIP proteins in A. thaliana, ZIP1-4, may play a role in zinc transport [53]. Overexpression of ZIP 1-3 in S. cerevisiae increased zinc uptake by the yeast. ZIP 4 failed to complement perhaps due to poor expression or mislocalization.
IV) The Nramp family

Nramps (natural resistance associated macrophage proteins) form a group of divalent metal cation transporters which contribute to cell metal homeostasis and have been highly conserved throughout evolution. They are expressed in many different organisms belonging to the pro- and eukaryotic domains (Tab. 1.3), and share high similarity at the amino acid level. In the yeast *Saccharomyces cerevisiae* three Nramp homologues have been identified and characterized, *SMF1*, *SMF2* and *SMF3* [103, 121]. Smf1p is a high affinity Mn$^{2+}$ transporter expressed at the cell surface, but it was also shown to mediate Fe$^{2+}$ uptake [21] and contribute to copper and cadmium accumulation [84]. Smf2p was suggested to mobilize Mn$^{2+}$ stores from intracellular vesicles and to supply the manganese superoxide dismutase (MnSOD) in the mitochondria with these essential ions [86]. It was also shown to transport cobalt [84]. Smf3p, expressed at the vacuolar membrane, is believed to export iron from the vacuole and thus mobilize iron stores [103]. Although two other proteins (Fthlp and Fet5p, homologous to high affinity iron uptake proteins Ftl1p and Fet3p) are suggested to mobilize vacuolar iron stores, there may exist multiple pathways for moving iron out of the vacuole. Smf3p may be analogous to the low affinity Fet4p transporter on the plasma membrane, which takes up a variety of divalent metal cations, including Fe$^{2+}$ [33].

In the thale cress *Arabidopsis thaliana*, AtNramp1, 3 and 4 contribute to manganese, iron and cadmium transport as was shown by yeast complementation assays [30, 127]. AtNramp3 was recently characterized in more detail and the following functional model was suggested [126]. AtNramp3 localizes to the vacuolar membrane as was shown by fluorescent microscopy and GFP fusion proteins, and acts in the mobilization of metal stores, i.e. exports metal ions from the vacuole into the cytoplasm. This would explain the previously observed Cd sensitivity in AtNramp3-overexpressing *A. thaliana*, which was not accompanied by increase in total Cd content [127]. Similarly, expression of the primary iron uptake transporter IRT1 and of the root ferric chelate reductase FRO2, which is regulated by iron [27, 107], was altered when AtNramp3 was overexpressed. Since total Fe content did not change, it seems probable that AtNramp3 moved iron from the vacuole into the cytosol. Furthermore, AtNramp3 might also influence accumulation of Zn and Mn by downregulating IRT1 (IRT1 was shown to transport Mn and Zn under iron deficient conditions, [74]).

In mice with defects in nutritional iron absorption (microcytic anemia in *mk* mice), direct iron injection did not correct the clinical picture [8], suggesting a second block in iron uptake by peripheral tissues [124]. It was demonstrated that in *mk* mice the Nramp2
gene is mutated [42]. In parallel, Nramp2 (DMT1, DCT1) was identified as a gene expressed in the duodenum of rats and capable of transporting divalent metal ions, leading to the suggestion that this protein may be responsible for intestinal iron absorption [57]. Recently, a thorough study on the localization of human Nramp2 showed that the protein colocalizes with markers for late endosomes/lysosomes but not with the transferrin receptor (TfR, which is a marker for sorting and recycling endosomes and the plasma membrane) in non-intestinal cells [124]. In the light of these results it was suggested that Nramp2 is involved in the distribution of iron in non-intestinal cells as well as in absorption of intestinal iron (see below).

Three pathways have been proposed in which Nramp2 may play a role: i) nutritional iron absorption in enterocytes, ii) recycling of iron from phagocytosed erythrocytes, and/or iii) transferrin-dependent iron uptake in erythrocytes and other cells [124]. Figure 1.3 visualizes the ways in which Nramp2 is thought to act in iron distribution from the intestine to peripheral cells [88, 124]: Expressed at the apical side of enterocytes Nramp2 can take up nutritional Fe$^{2+}$ (reduced by DcytB) by utilizing the proton gradient maintained by the action of Na$^+$/K$^+$ ATPase and Na$^+$/H$^+$ exchanger. Iron is distributed to other cells through the blood, bound to the plasma protein transferrin (Tf). The loading of iron to transferrin requires oxidation of Fe$^{2+}$ to Fe$^{3+}$, probably by the multicopper oxidase hephaestin, and efflux out of the enterocytes by Ireg1. When Fe$^{3+}$-loaded transferrin binds to a transferrin receptor (TfR) expressed at the cell surface of erythrocytes or other cells it is internalized by endocytosis. Fe$^{3+}$ is released from transferrin by acidification of the endosome via the action of vacuolar H$^+$-ATPase. The apo-Tf-TfR complex is recycled to the cell surface. The free Fe$^{3+}$ in the endosome is reduced, probably by an oxidoreductase, to Fe$^{2+}$. Maturation of the endosome is still obscure but probably involves recruitment of Nramp2 to the endosomal membrane. The transporter exports Fe$^{2+}$ ions into the cytoplasm by using the proton gradient established by the vacuolar H$^+$-ATPase. In a similar way, Nramp2 can contribute to recycling of iron from phagocytosed erythrocytes, by releasing the metal from the phagosome. A similar model was proposed for murine Nramp2 for which co-localization with transferrin and expression in the phagosome and the endosomes was demonstrated [54].

Mammalian Nramp1, the first identified member of the Nramp family was shown to be expressed in the lysosomal vesicles of macrophages and to be recruited to the phagosomal membrane upon phagocytosis of pathogens [55]. However, it is not believed to play a major role in iron distribution since a knockout mouse does not show defects in iron absorption [124]. Instead, it was reported that mutation of Nramp1 causes susceptibility to infections by intracellular pathogens [132]. According to the direction of transport which is not yet settled
General Introduction

(see Mode of transport), Nramp1 is thought to either extrude metal ions from the phagosome or to concentrate them within it. Thereby, the phagocytosed pathogen would either be deprived from metal ions essential for the production of defense proteins (e.g. Fe-SOD), or the increase of the metal concentration in the phagosome would support the production of oxygen radicals to combat the pathogens.

Some pathogens have also been described to express Nramp homologues – MntH in the case of the Gram-negative bacterium Salmonella typhimurium –, which might compete with Nramp1 for metal ions in the phagosome. The MntH proteins from Salmonella typhimurium and Escherichia coli are specific Mn^{2+} transporters (but Cd may also be a substrate) although only few enzymes are known to need Mn^{2+} in these organisms (e.g. Mn-SOD) [72]. Since a knock-out mutant of mntH resulted in increased H_2O_2 sensitivity, it was suggested that Mn^{2+} may play a protective role independent of any enzyme perhaps by direct dismutation of peroxide [72]. Additionally, Mn^{2+} may scavenge the superoxide radical as shown for some Lactobacillus species which lack a cytoplasmic Mn-SOD [3].
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Figure 1.3 Model of the functions of mammalian Nramp2 (adapted from [88, 124]). Nutritional Fe\(^{3+}\) is reduced at the surface of an enterocyte by DcytB to Fe\(^{2+}\) which is taken up by Nramp2 together with H\(^{+}\) in a 1:1 stoichiometry. The proton gradient required for the Fe\(^{2+}\) uptake is maintained by the Na\(^{+}/K^{+}\)-ATPase and the Na\(^{+}/H^{+}\) exchanger. Fe\(^{2+}\) in the cytoplasm is converted to Fe\(^{3+}\) and stored bound to ferritin. For distribution via the blood, iron may be exported by the transporter Ireg1 in parallel with the oxidation of Fe\(^{2+}\) to Fe\(^{3+}\) by the ferroxidase hephaestin. In the blood, Fe\(^{3+}\) is bound to transferrin (Tf). Iron-loaded transferrin can bind to the transferrin receptor (TfR) and the complex can be internalized by erythrocytes or other cells via endocytosis. In the endosome, Fe\(^{3+}\) is released from Tf (acidification of the endosome via H\(^{+}\)-ATPase favors this process) and is reduced to Fe\(^{2+}\) by an oxidoreductase. The empty Tf-TfR complex is recycled to the cell surface. Maturation of the endosome to a late endosome is not well understood but may involve recruitment of Nramp2 to the endosome membrane. Nramp2 exports Fe\(^{2+}\) to the cytoplasm as an Fe\(^{2+}/H^{+}\) symporter. The H\(^{+}\) gradient is maintained by the H\(^{+}\)-ATPase. DcytB: Ferric reductase; Hephaestin: Multicopper ferroxidase; Ireg1 (iron-regulated transporter 1, ferroportin, metal transporter protein-1 MTP1): Protein for iron efflux.
Mode of transport

It is generally believed that transport via Nramps is driven by the electrochemical potential. Studies with Smf1p expressed in Xenopus oocytes showed that metal transport was dependent on extracellular pH [21], and Mn$^{2+}$ influx in *E. coli* and *S. typhimurium* overexpressing their native Nramp transporters was also sensitive to pH [72]. Transport of rat Nramp2 occurred as a divalent metal cation/proton symporter as was shown by two-microelectrode voltage clamp analysis in *Xenopus* oocytes [57]. However, there is some controversy regarding direction and mode of transport of Nramp1. Studies on the regulation of murine Nramp1 ([13-15], see also Nramp regulation) support the model of metal cations being imported into the endosome. Since the phagosome is an acidic compartment, Nramp1 must in this case act as a metal cation/proton antiporter. This model is also supported by electrophysiological measurements on *Xenopus* oocytes, which showed that the Nramp1 transport process is dependent on the pH in a way which speaks for an antiporter mechanism [50]. This suggests that Nramp2 and Nramp1 may have different modes of transport despite the high degree of sequence identity. In contrast, it was shown that phagosomes isolated from Nramp1-expressing mice extruded Mn$^{2+}$ faster than Nramp1-deficient mice, and dissipation of the pH-gradient inhibited Mn$^{2+}$ extrusion [68]. This would speak for a metal cation/proton symporting mechanism. In addition, HA-tagged Nramp1 inserted into the plasma membrane in the same orientation as Nramp2 and mediated uptake of Fe$^{2+}$, Mn$^{2+}$ and Co$^{2+}$ down the proton gradient, again as seen for Nramp2 [43].

Both transport directions seem possible when the role of Nramp1 is taken into consideration. Metal cation movement into the phagosome would catalyze production of toxic hydroxyl radicals by the Fenton/Haber-Weiss reaction in the microenvironment surrounding the pathogen [140]. On the other hand, extrusion of metal cations from the phagosome would deprive the pathogen from factors essential for defense (e.g. metals as cofactor for SOD, [68]).

Nramp regulation

Regulation of Nramps is discussed at several levels and seems to vary from homologue to homologue. Inactivation by mutation of the gene BSD2 (bypass SOD deficiency) suppresses oxidative damage in yeast lacking the Cu/Zn superoxide dismutase and increases sensitivity toward Cd and Cu toxicity [81]. Western blots in a yeast *bsd2* mutant
showed that Bsd2p controls stability of the yeast Nramp homologue Smflp protein and that the half life of Smflp was increased when BSD2 was deleted [83]. The pathway leading to degradation of Smflp via Bsd2p did not involve endocytosis of the transporter protein or ubiquitination for subsequent degradation in the proteasome. Instead, Bsd2p probably targets Smflp to the vacuole for degradation under conditions of sufficient manganese supply. In fact, the bulk of Smflp is not localized at the plasma membrane under favorable conditions, but is directed to the vacuole. Only when manganese becomes limiting, SMF1 is distributed to the plasma membrane, this time by a Bsd2p-independent pathway. Because Bsd2p localizes at the endoplasmic reticulum, the fate of the Smflp protein is decided in the secretory pathway, depending strictly on manganese concentration, since addition of zinc, copper or cobalt did not change Smflp steady state levels. This post-translational control of Smflp effectively minimizes the hyperaccumulation of toxic metals and also provides a rapid switch for inducing metal uptake under conditions of metal starvation, without the need for new protein synthesis [83]. The same regulatory pathway was shown for Smf2p with the difference that the protein is distributed to intracellular vesicles instead of the plasma membrane when manganese is limiting [103].

Rat Nramp2 was proposed to be post-transcriptionally regulated in a way similar to regulation of the transferrin receptor (TfR). The mRNA of TfR contains an iron responsive element (IRE). An IRE binding protein binds to this sequence and confers stability to the transcript, thus allowing transcription of TfR and accumulation of the protein in the plasma membrane. This type of regulation seems possible for rat Nramp2 since there has been an IRE postulated on the transcript [57].

Regulation on the level of mRNA synthesis is proposed for yeast SMF3. The promoter of the gene contains two binding sites for AFT – an iron dependent regulatory factor – and the transcription is induced when iron levels are low [103]. Transcriptional regulation was also studied in tomato and in mouse. In tomato (Lycopersicon esculentum), LeNramp1 complemented an smfl yeast mutant, suggesting a role in metal transport [9]. LeNramp1 and LeIrt1 (a homologue of A. thaliana Irt1) showed a similar regulatory pattern. Both genes were up-regulated under iron deficient conditions and required a functional FER transcription factor and nicotianamine synthase (NAS) for proper regulation. [NAS is the enzyme which produces the metal chelator nicotianamine (NA) found in all plants tested to date [125]. The role of NA is thought to lie in the internal transport of iron and other metals.] In the fer mutant, LeIrt1 and LeNramp1 expression was undetectable irrespective of iron supply conditions. In the chloronerva mutant (nas mutant) LeIrt1 and LeNramp1 expression was higher in iron
sufficient and lower in iron deficient conditions compared to the wildtype. A *chloronerva/fer*
double mutant gave the same picture as the *fer* single mutant, i.e. expression of LeIrt1 and
LeNramp1 was hardly detectable. Since the *fer* mutant allele had an epistatic effect on the
*chloronerva* mutant allele, the two gene products probably act in the same pathway. The
authors suggest that NA acts in the *fer* gene signaling pathway as the sensor for iron
availability [9].

Another group concentrated on murine Nramp1 regulation going more into the
structure of the involved promoter [13-15]. The core promoter of Nramp1 contains multiple
non-canonical E-boxes (E1-E6) or Myc-Max binding sites, two initiator elements (Inr) and an
Sp1 site, but lacks a TATA box. Co-transfection with Nramp1 promoter-constructs and the
bHLH transcription factor c-Myc showed that c-Myc represses promoter activity. Further
experiments suggested a role for the transcription factor Miz-1 in Nramp1 activation. The
model suggested by the authors is that in proliferating cells c-Myc, possibly as a dimer with
Max, is bound at E6 and loops to interact with Inr-bound Miz-1 for transcriptional repression
of Nramp1. In non-proliferating cells the heterodimer Mad-Max replaces Myc-Max
complexes of proliferating cells thereby allowing Nramp1 transcription. This is consistent
with the finding that Nramp1, which is repressed by c-Myc, is expressed only in mature (but
not immature) macrophages.

c-Myc was shown to positively or negatively regulate genes that increase or decrease,
respectively, the labile iron pool (cytosolic iron pool). The authors argue that since c-Myc
represses Nramp1, the function of the transporter should lie in depletion of iron from the
cytoplasm. Thus, mode of transport would be as a divalent cation / proton antiporter [13-15],
in contrast to Nramp2 which was shown to be a symporter ([57], see also chapter Mode of
transport).

In *S. typhimurium* the Nramp homologue MntH is involved in H$_2$O$_2$ resistance and is
regulated on the transcriptional level by the transcriptional repressors MntR and Fur, which
bind to the promoter in the presence of Mn$^{2+}$ or Fe$^{2+}$, respectively, and by the transcriptional
activator OxyR which activated transcription in the presence of H$_2$O$_2$ [72]. Fe$^{2+}$-Fur and
Mn$^{2+}$-MntR regulation was also shown for *E. coli* MntH [100].
<table>
<thead>
<tr>
<th>Name</th>
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<th>Specificity</th>
<th>Function</th>
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<td>Plasma membrane</td>
<td>Mn$^{2+}$, Cu$^{2+}$, Cd$^{2+}$</td>
<td>Mn$^{2+}$ uptake [23], Fe$^{2+}$ transport in Xenopus oocytes [21]</td>
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<td>Mobilization of Mn stores, which are delivered to Mn-SOD [86], Fe$^{2+}$ transport in Xenopus oocytes [21]</td>
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<td>Vacuolar membrane</td>
<td>Fe$^{2+}$</td>
<td>Mobilization of Fe stores [103]</td>
<td>Transcriptional by iron: two potential Aft binding sites (iron regulatory factors) in the promoter [102]</td>
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<td>Divalent metal cations</td>
<td>Fe$^{2+}$, Mn$^{2+}$, Cd$^{2+}$ transport in yeast complementation assays (<em>S. cerevisiae smf1, fet3/fet4</em>) and in <em>A. thaliana</em> overexpression leads to increased resistance to toxic iron concentrations</td>
<td>Induced by iron starvation</td>
<td>[30, 127]</td>
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<tr>
<td>AtNramp3</td>
<td><em>A. thaliana</em> (thale cress)</td>
<td>Vacuolar membrane</td>
<td>Divalent metal cations</td>
<td>Mobilization of metal stores from vacuole</td>
<td>Induced by iron starvation</td>
<td>[126]</td>
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<td>Fe$^{2+}$, Mn$^{2+}$, Cd$^{2+}$</td>
<td>Transport in yeast complementation assays (<em>S. cerevisiae smf1, fet3/fet4</em>) and in <em>A. thaliana</em></td>
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<td>[127]</td>
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<td>Divalent metal cations</td>
<td>Complementation of <em>S. cerevisiae smf1</em></td>
<td>Via FER (TF) and NAS (nicotianamine synthase), induced by iron deficiency</td>
<td>[9]</td>
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<td>Mn^{2+}, Fe^{2+}</td>
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<td>MvL</td>
<td><em>Drosophila melanogaster</em></td>
<td>Mature neurons, macrophages</td>
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<td></td>
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<td>[97, 109]</td>
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<td>MntH</td>
<td><em>Escherichia coli</em></td>
<td>Cell membrane</td>
<td>Specific for Mn^{2+}</td>
<td>Mn-transporter; involved in response to reactive oxygen [72]</td>
<td>Repressed by Fe-Fur and Mn-MntR</td>
<td>[71, 100]</td>
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<tr>
<td>MntH</td>
<td><em>S. typhimurium</em></td>
<td>Cell membrane</td>
<td>Specific for Mn^{2+}</td>
<td>Mn-transporter; involved in response to reactive oxygen [72]</td>
<td>Transcription activated by hydrogen peroxide via the transcriptional activator OxyR. Repressed by Fe-Fur and Mn-MntR</td>
<td>[71, 100]</td>
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<td>MntH</td>
<td><em>Bacillus subtilis</em></td>
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<td>Transcription repressed under Mn excess conditions via the metalloregulator MntR</td>
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**Functional analysis**

For Smf1p of *S. cerevisiae* a functional analysis was conducted by generation of several mutated versions of the protein [82] (Tab. 1.4). Two point mutations, G190A in transmembrane domain 4 (TM4) and G424A in the consensus transport motif (CTM), both resulted in a loss of function phenotype as was shown by functional complementation assays. In addition, those mutants did not respond to regulation by Bsd2p or depending on metal conditions. Localization experiments by immunofluorescence microscopy showed that these protein variants were not redistributed to the cell membrane under metal deplete conditions. Four other mutations in the CTM were functional. However, two of them (Q419A and E423A) did not allow response to decreased metal levels and the proteins were not redistributed to the plasma membrane as shown by immunofluorescence microscopy. This was not the case for the other two functional mutants (Q408A and G418A). Apparently, a functional Smf1p is essential for targeting to the vacuole by Bsd2p but is not sufficient for redistribution to the cell membrane.

An extensive functional analysis was also conducted for murine Nramp2. Several amino acids were replaced and function of the mutated proteins was checked by complementation assays in yeast and by fluorescent metal transport assays in CHO cells [79, 101]. Expression of the mutated proteins in yeast was checked by western blotting of membrane fractions and in CHO cells expression on the cell surface was tested by biotin labeling. Table 1.4 gives a summary of the mutational phenotypes. Interestingly, two point mutations in the Nramp2 CTM (Q384E, G394V [101]) prevented complementation of the yeast mutant, while the corresponding mutated variants of Smf1p (Q408A, G418A [82]) were functional (see above). Perhaps this finding reflects the need for specific interaction partners for correct localization or function of the mammalian protein, missing in the yeast system.

G185R is a naturally occurring point mutation in TM4 and is associated with severe iron deficiency and microcytic anemia in mice [42]. Nramp2<sup>G185R</sup> was unable to complement the yeast *smf1/smf2* mutant but showed significant transport activity in a metal transport assay [79]. This might indicate that the mutated protein was targeted to a transport-incompetent compartment in the yeast, suggesting that the mutation affected correct localization [79], as already seen above for Nramp2 mutants Q384E and G394V. Interestingly, the naturally occurring G169D mutation in Nramp1 – a residue adjacent to G185 in Nramp2 – totally disrupts function of Nramp1 and leads to susceptibility to pathogen infections in mice. This
mutation probably causes a structural alteration which prevents accumulation of the mature Nramp1 protein in macrophages [132]. Two further mutations were inserted in Nramp2. The two evolutionary absolutely conserved histidine residues H267 and H272 were substituted and found to be essential for function of Nramp2 [79]. Strikingly, some of the histidine mutants were rescued by lowering the pH from 6 to 5. The authors suggested that protonation of H267/H272 is required for the protein to assume a functional conformation. Loss of these residues would shift the pH for maximal transport to a more acidic value, requiring protonation of other groups or side chains to create the same conformational state [79].

Table 1.4 Mutational analysis of Smf1p and Nramp2.

A: The effect of individual mutations is shown. Protein expression was stable for all mutants and all proteins were checked for expression in the yeast membrane fraction. For transport experiments with CHO cells Nramp2 expression/localization on the cell surface was determined by biotin labeling. Smf1p localization of G190A, Q419A E423A and G424A was checked by immunofluorescence microscopy. Corresponding residues of Nramp2 and Smf1p are in the same column. Bold residues are evolutionary absolutely-highly conserved. nd: not determined.

B: Graphical representation of the Smf1p and Nramp2 amino acid chain (not drawn to scale). The black bars symbolize the transmembrane regions of the proteins (numbered 1 through 12) and mutated amino acid residues are indicated above or below the corresponding TM. CTM: consensus transport motif.
Table 1.4 Mutational analysis of Smf1p and Nramp2.

A

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<th>SMF1</th>
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<th>G418A</th>
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1.5 Algae and transition metal transport

Molecular data on transition metal transport in algae is scarce. Only recently an iron assimilation pathway in the green microalga *C. reinhardtii* was identified and characterized [61, 78] (Fig. 1.4). Based on these studies high affinity iron uptake in *C. reinhardtii* is mediated by a similar mechanism as in *Saccharomyces cerevisiae*. In the yeast, Fe$^{3+}$ is firstly reduced to Fe$^{2+}$ by the Fe$^{3+}$-chelate reductases Fre1p and Fre2p. Subsequently, the metalloenzyme multicopper ferroxidase Fet3p oxidizes Fe$^{2+}$ to Fe$^{3+}$, which is then imported by the iron permease Ftr1p into the cell. In *A. thaliana* (Strategy I plant), Fe$^{3+}$ is also reduced to Fe$^{2+}$ by the reductase FRO2 [107] but it is taken up by the Fe$^{2+}$ transporter IRT1 without reoxidation to Fe$^{3+}$. Grasses (Strategy II plants) utilize a different mechanism and take up iron as a Fe$^{3+}$-phytosiderophore complex.

Similar to yeast, a multicopper ferroxidase (FOX1/FLP [61, 78]) and an iron permease (FRT1 [78]) were identified and characterized in the alga *C. reinhardtii*, two proteins which may account for the oxidation of Fe$^{2+}$ to Fe$^{3+}$ and subsequent uptake of the trivalent ion, respectively. The proposed mechanism is supported by the finding that iron uptake in *C. reinhardtii* involves an obligatory reduction of Fe$^{3+}$-chelates [36]. Activity of FOX1/FLP was not directly demonstrated, but ferroxidase activity in cell suspensions increased under iron depleted conditions and was inhibited by a multicopper oxidase inhibitor [62]. The transcripts for the ferroxidase and the iron permease accumulated under iron deficiency, independently of copper concentration. Accumulation of the ferroxidase protein, however, was dependent on cellular copper status. Thus, copper is not required for the iron-responsive regulation of the ferroxidase, but for the accumulation of the protein itself [78]. The identified ATX1 copper chaperone and a putative Cu-transporting ATPase may work together to supply Cu for the biosynthesis of FOX1/FLP [78], in a similar way as was shown for yeast [137].

With the reoxidation of iron to Fe$^{3+}$ for uptake, *C. reinhardtii* possesses an iron assimilation system not found in higher plants so far. However, in addition to the FOX1/FLP-FRT1 system, a further iron assimilation pathway must be present in the alga since copper deficiency did not lead to secondary iron deficiency by influencing iron uptake, although ferroxidase accumulation was reduced. This backup system must be independent of the copper nutritional status in order to guarantee sufficient iron supply under copper deficiency [78] (see also chapter 6).

Another protein with a possible role in iron assimilation in *C. reinhardtii* is H43. The gene for the previously described CO$_2$-inducible polypeptide H43 [73] was recently isolated.
H43 is not related to any other protein in the database except for HCR1 from the marine alga *Chlorococcum littorale*, which is induced by high CO₂ and iron deficiency [117]. Induction of H43 mRNA under conditions of cadmium toxicity and iron deficiency was demonstrated. Based on their results, the authors suggest that cadmium causes an iron deficiency in the cell by saturating or inhibiting the iron transporters, leading to an iron stress response which includes induction of H43 [112]. They propose that H43 and HCR1 may represent novel, algal-specific proteins that may facilitate iron transfer from the ferric reductase to the iron transporters [112] (Fig. 1.4). Induction by CO₂ might be indirect. In *C. littorale* incubation under high CO₂ conditions resulted in increased photosynthesis, possibly needing more iron for the biosynthesis of proteins involved in photosynthesis (cytochromes, ferredoxin) [117].

In contrast to the iron uptake system, a copper assimilation mechanism hasn’t been identified yet in *C. reinhardtii*. Also for plants information regarding copper uptake is scarce. Only recently a family of putative copper transporter proteins was identified (COPT family), with COPT1 and COPT2 shown to mediate high affinity Cu uptake when overexpressed in a yeast mutant deficient in copper uptake [70, 116]. Although a Cu reductase activity has been observed in tomato plasma membrane vesicles [66] and pea roots [133] the enzyme responsible for this activity has not been identified and a possible role for a Cu reductase in plant copper uptake has not been established yet. Only one member of the FRO family of putative ferric chelate reductases has been characterized to date but no relation to copper homeostasis has been demonstrated [26].

In *C. reinhardtii*, induced cupric chelate reductase activity was measured together with ferric chelate reductase activity under iron and/or copper deplete conditions [61]. The question whether the cupric reductase and the ferric reductase activities are displayed by two or by a sole enzyme – as is the case in yeast – is unexplored yet, because the identity of the enzyme(s) is unknown. In yeast, Cu²⁺ reduction to Cu⁺ is performed by the same reductases as Fe³⁺ reduction to Fe²⁺ (Fre1p and Fre2p). Cu⁺ is then taken up by the Cu transporters Ctr1p and Ctr3p [130]. In *C. reinhardtii*, a copper uptake system has not been identified yet, but a radioisotopic copper uptake assay demonstrated the existence of a high affinity copper transport pathway which is more active in copper-deficient than in copper-sufficient cells [63] (Fig. 1.4). The affinity constant was unaffected by copper conditions but the activity of the transport system was 20 times higher in copper-deficient cells, indicating that the activity or expression of the system is induced in copper-deficiency. In the same study cupric reductase
activity and copper uptake were repressed by equal amounts of copper suggesting that they are regulated coordinately [63]. It is therefore possible that copper uptake in C. reinhardtii may resemble that in yeast, which includes reduction and subsequent uptake of Cu.

Figure 1.4 Scheme of copper and iron homeostasis in C. reinhardtii. FOX1: multicopper ferroxidase, FTR1: iron permease, ATX1: copper chaperone, H43: putative iron chaperone, DMT1: divalent metal transporter 1. When copper is limiting (-Cu / +Fe) cupric chelate reductase activity [61] and high affinity copper uptake is induced [63]. Under iron limitation (-Fe / +Cu) ferric chelate reductase activity and iron uptake via the high affinity FOX1/FTR1 system is induced [61, 78]. Fe2+ may be delivered from the reductase to FOX1 by H43 [112]. ATX1 and Cu-transporting ATPase are also induced and may contribute to FOX1 biosynthesis by copper delivery to the secretory system [78]. Under copper and iron limitation (-Cu / -Fe) an alternative uptake pathway for iron must exist since FOX1 cannot be synthesized [78]. Both reductase activities are induced under all three conditions [61]. The role of DMT1 in uptake of iron or copper as indicated in the figure will be discussed in chapter 6.
1.6 *Chlamydomonas reinhardtii* – a short introduction to the “green yeast”

**Taxonomy**

Superkingdom: *Eukaryota*

Kingdom: *Viridiplantae* (green plants)

no rank: *Chlorophyta* (green algae)

Class: *Chlorophyceae*

Order: *Volvocales*

Family: *Chlamydomonadaceae*

Genus: *Chlamydomonas*

Species: *Chlamydomonas reinhardtii*

*Chlamydomonas* is a genus of unicellular green algae, including more than 500 different species, found in soil, fresh and marine water, and in snow [59]. The cells have a glycoprotein wall and two anterior equal flagella. The nucleus is enclosed in a cup-shaped chloroplast, which contains a single large pyrenoid, a proteinaceous structure where starch is formed from photosynthetic products. Two small contractile vacuoles, which have an excretory function, are located near the flagella and a red pigment spot which is light-sensitive, allows the cell to swim towards light (Fig. 1.5). The genome counts 100 Mbp.

![Figure 1.5 The morphology of *Chlamydomonas reinhardtii* [31]. C: chloroplast, ES: eye spot, F: flagellum, M: mitochondrion, N: nucleus, P: pyrenoid, V: vacuole](image)
The most widely used laboratory species is *Chlamydomonas reinhardtii*. Cells of this species are about 10μm long. They can grow photosynthetically on a simple medium of inorganic salts, but they can also grow heterotrophically in total darkness if acetate is provided as an alternative carbon source. *C. reinhardtii* has a fast mitotic life cycle but can also reproduce in a controlled sexual way. Under adverse conditions (e.g. nitrogen deprivation) haploid cells of opposite mating types can fuse to form a diploid zygospore with a hard outer wall. When conditions improve, the diploid zygote undergoes meiosis releasing four haploid cells that resume the vegetative life cycle.

*C. reinhardtii* is a suitable laboratory organism and is especially used as a model system for research on photosynthesis and flagellar motility: It is a eukaryotic organism, but can be handled by standard microbial techniques. The generation time (6 hours) is short, which is a great advantage for research purposes. Cultivation is simple and cheap, and a wide range of mutant stocks is available at the Chlamydomonas Genetics Center, Duke University, Durham. Mutant phenotypes are immediately expressed since the genome during the vegetative life cycle is haploid.

A major drawback of *C. reinhardtii* is that an efficient system for directed disruption of nuclear genes is lacking. Homologous recombination between introduced DNA and the nuclear genome occurs at a low frequency (3 recombinants out of 2000 transformants, [94]) and is often accompanied by deletion events (2 out of 3 recombinants, [94]). At such low recombination frequencies the availability of a good screening system in order to detect replacement mutants of the gene of interest is particularly important. In the case of a gene where the mutant phenotype is unknown this method is hardly applicable. Concerning the DMT1 protein, studied in this project, precise function was unknown and it seemed more fruitful to start characterization by an alternative method. Yeast functional complementation assays allowed a thorough investigation of the transport function of the protein.

*C. reinhardtii* is sometimes referred to as the “green yeast” because it is unicellular, grows quickly, forms colonies on plates, and has two mating types, which allows it to switch between a haploid and a diploid state. On February 2003 a first draft of the complete genome of *C. reinhardtii* was released (version 1.0, http://genome.jgi-psf.org/chlre1/chlre1.home.html, Doe Joint Genome Institute, California, [52]). More information on *C. reinhardtii* is available on http://www.biology.duke.edu/chlamy/ and in [59].
1.7 Motivation and aim of the thesis

Transition metals deserve a lot of attention because of their dual action as essential micronutrients and as potentially toxic substances. A lot of work concentrates on molecular aspects of acquisition or extrusion of metallic ions by cells or cellular organelles, and several protein families involved in transition metal homeostasis have been identified and characterized to date. In this regard, little is known about analogous molecular mechanisms present in algae. Yet, these organisms have a significant status not only in aquatic ecosystems being the basis of the food chain, but also in the overall ecology of the planet, contributing more than 90% of the worlds’ photosynthetic activity, which makes them the most important source of oxygen. Algae, being photosynthetic organisms, are especially dependent on a precise regulation of metal balance. The photosynthetic process requires manganese for water splitting, and oxidative stress resulting from photosynthesis must be antagonized by metalloenzymes.

The aim of this work was to gain insight on the alternatives green algae have when it comes to metal transport, on a molecular level. For this, the chosen approach was to explore the existence of, to identify and to investigate functionally a metal transport system in the green microalga Chlamydomonas reinhardtii. The strategy was first to search in a C. reinhardtii partial sequence database (the genomic sequence was not yet available) for potential transport systems belonging to various protein families and, if such a transporter was identified, to proceed with molecular cloning of the complete coding sequence. A detailed functional characterization of the transporter in vivo by the means of yeast complementation assays in several yeast mutants was to follow, together with studies on the specificity. This approach was successful and the results are presented in chapters 2 and 3.

Further, elucidation of the regulation of the identified transporter was attempted (chapter 4). Finally, the database of the recently released genomic sequence of C. reinhardtii was utilized to perform a thorough investigation on the presence of different metal transport systems in this alga. The insights of this study are presented in chapter 5.
Divalent metal transport in the green microalga *Chlamydomonas reinhardtii* is mediated by a protein similar to prokaryotic Nramp homologues

Information about the molecular mechanisms of metal transport in algae is scarce, despite the significant status these organisms have in aquatic ecosystems. In the present study, we describe the cloning and functional characterization of a divalent metal transporter (named DMT1) in the green microalga *Chlamydomonas reinhardtii*. The longest open reading frame of the cloned DMT1 cDNA encodes a protein of 513 amino acids with 11 putative transmembrane domains. The protein belongs to the Ntamp family of divalent metal transporters and shows surprisingly higher homology to prokaryotic than to eukaryotic polypeptides. Especially the N-terminus, which is longer than of every other homologue considered in this study, displays – uniquely among eukaryotic Ntramps – exclusively prokaryotic characteristics. Functional complementation experiments in yeast strains with impaired metal transport systems, revealed that *C. reinhardtii* DMT1 has a broad specificity, acting in the transport of several divalent metals (manganese, iron, cadmium, and copper), but excluding zinc.

2.1 Introduction

Metal ions are indispensable for cell survival since they fulfil important functions at several sites in the cell, including stabilisation of cellular structures, enabling of redox reactions and activation of enzymes [44]. Different metal elements do not occur at the same concentration in the environment, are not equally bioavailable, and have to be maintained at different concentrations in the cell. Iron for instance, is needed in greater amounts than copper, but occurs mainly in the form of insoluble and thus not readily bioavailable ferric complexes. This reality forces the cell to express a variety of transport systems in order to acquire a broad range of these essential micronutrients in the required concentrations. A number of metal
transport proteins have been characterized in bacteria, yeast, mammals and plants, some of them displaying similar features and belonging to the same family (for an overall classification see [114]). Less is known regarding divalent metal transporters in green algae at a molecular level. Only recently, the molecular identification and characterization of genes involved in high affinity iron transport in the green microalga *Chlamydomonas reinhardtii* [61, 78] was published.

Nramps (natural resistance associated macrophage proteins) form a group of divalent metal cation transporters which contribute to cell metal homeostasis and have been highly conserved throughout evolution. They are expressed in many different organisms belonging to the pro- and eukaryotic domains, and share high similarity at the amino acid level. In the yeast *Saccharomyces cerevisiae* three Nramp homologues have been identified and characterized, SMF1, SMF2 and SMF3 [103, 121]. SMF1 is expressed at the cell surface and is specific for Mn\(^{2+}\) ions, but it was also shown to contribute to copper and cadmium accumulation [84]. SMF2 is expressed in the membrane of intracellular vesicles and was suggested to supply the manganese superoxide dismutase (MnSOD) in the mitochondria with the essential Mn\(^{2+}\) ions [86]. SMF2 was also shown to transport cobalt [84]. SMF1 and SMF2 are regulated at the posttranslational level by the protein Bsd2p [83, 84]. SMF3, expressed in the vacuole, is believed to mobilize vacuolar iron stores and is down-regulated by iron [103]. In the thale cress *Arabidopsis thaliana* several Nramp homologues were found. AtNrampl, 3 and 4 contribute to manganese, iron and cadmium transport as was shown by yeast complementation tests [30, 127]. Mammalian *Nramp2* (DMT1, DCT1) is described as the major transferrin-independent iron transport system in the intestine. It functions as a pH-dependent divalent cation transporter and displays broad specificity [57]. The mammalian *Nramp1* is expressed in the endosomal/lysosomal vesicles of macrophages and is recruited to the phagosomal membrane upon phagocytosis of pathogens [55]. It was shown to transport manganese (Mn\(^{2+}\)) ions out of the phagosome, suggesting that its role lies in mediating metal depletion of divalent metals from the phagosomal space [68]. Since Mn\(^{2+}\) is a cofactor for MnSOD, its lack would deprive the pathogen from an important defence mechanism, resulting to decreased pathogenicity. Some pathogens have also been described to express Nramp homologues – MntH in the case of the Gram-negative bacterium *Salmonella typhimurium* – which might compete with Nramp1 for metal ions in the phagosome. The MntH proteins from *Salmonella typhimurium* and *Escherichia coli* were shown to be Mn\(^{2+}\) transporters involved in the response to reactive oxygen [72]. Several other organisms were described to express Nramp homologues [20, 34, 39, 109] and putative genes encoding
The Nramp homologue of C. reinhardtii members of the Nramp family have been identified in many other organisms based on sequence homology.

The present study describes the identification, cloning and functional characterization of a divalent metal transporter from the green microalga Chlamydomonas reinhardtii. The protein designated DMT1 shows highest amino acid sequence homology to prokaryotic members of the Nramp family. Functional complementation in yeast strains with impaired manganese, zinc and iron transport systems revealed that the DMT1 protein displays broad specificity, transporting manganese, cadmium, copper and iron but no zinc.

2.2 Materials and methods

2.2.1 Strains and plasmids

Saccharomyces cerevisiae strain Y16272 (BY4742; MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; sml1::kanMX4) is a yeast mutant in which the SFL1 gene has been disrupted and was obtained from EUROSCARF, Frankfurt (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). The following two strains were kindly provided by Dr. D. Eide, University of Missouri-Columbia and have disruptions in genes for zinc and iron transport, respectively: S. cerevisiae strain ZHY3 (MATα; ade6; can1-100oc; his3; leu2; trp1; ura3; zrt1::LEU2; zrt2::HIS3) [139] and strain DDY4 (MAT α; ura3; trp1; leu2; his3; can1; fet3::HIS3; fet4::LEU2) [32]. For a listing of yeast strains see also table 2.1. C. reinhardtii strain cw15arg7 (kindly provided by E. H. Harris [59]) was used for RNA isolation for RACE and RT-PCR.

To express the C. reinhardtii DMT1 in yeast the corresponding full-length cDNA was cloned into the yeast expression vector pUG35 (kindly provided by J. H. Hegemann, University of Düsseldorf, Germany; Guldener, U., and Hegemann, J. H. manuscript in preparation). pUG35 has a CEN/ARS origin of replication (1-2 copies per cell) and uses the MET-25 promoter for moderate expression of heterologous genes.
### Table 2.1 List of *S. cerevisiae* strains used in this study

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<td>SMFl MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; smfl::kanMX4</td>
<td>EUROSCARF, Frankfurt <a href="http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html">http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html</a></td>
</tr>
<tr>
<td>ZHY3</td>
<td>ZRT1/2 MATα; ade6; can1-100oc; his3; leu2; trp1; ura3; zrt1::LEU2; zrt2::HIS3</td>
<td>Dr. D. Eide, Department of Nutritional Sciences, University of Missouri Columbia</td>
</tr>
<tr>
<td>DDY4</td>
<td>FET3/4 MATα; ura3; trp1; leu2; his3; can1; fet3::HIS3; fet4::LEU2</td>
<td>Dr. D. Eide</td>
</tr>
</tbody>
</table>

#### 2.2.2 Culture conditions

*S. cerevisiae*: For selection of transformants cells were grown on solid synthetic minimal medium containing 0.17% yeast nitrogen base (without amino acids and ammonium sulphate, DIFCO), 0.5% ammonium sulphate, 2% glucose, a mixture of amino acids and 1.8% agar. For complementation experiments cells were grown in liquid medium with the same composition as above supplemented with various concentrations (see results) of metals or metal chelators EGTA (Ethylene glycol-bis(2-aminoethyl)-tetra acetic acid) and EDTA (Ethylene diamine tetra acetic acid). For growth tests 5 ml medium were inoculated with a single colony and incubated with shaking (200 rpm) at 30°C for a few hours. 45 ml of growth medium in a 250 ml Erlenmeyer flask were inoculated with this yeast preculture and incubated with shaking at 30°C overnight. The overnight culture was diluted to an optical density (OD<sub>600</sub>) of 0.1-0.3 at 600nm and divided into several flasks containing medium with and without stress (metal chelators or metals). These were then incubated with shaking at 30°C for the course of the growth experiment. Growth was monitored by measuring OD<sub>600</sub> at 1-2 hour intervals for 7-10 hours. The maximal growth rate of each strain in the medium without any stress conditions was defined as 100% growth of the strain and growth of the same cells in different concentrations of stress was expressed in relation to it. All growth tests were performed at least in triplicate and either a representative experiment or the mean of several experiments is shown in the corresponding figures.

*C. reinhardtii*: For RNA isolation *C. reinhardtii* strain cw15arg7 was grown at 25°C in liquid Tris-acetate-phosphate medium TAP [59] supplemented with 50mg/l arginine under constant illumination and agitation.
2.2.3 Cloning

A search in the *C. reinhardtii* EST (expressed sequence tag) library of Kazusa DNA Research Institute, Japan identified a partial EST sequence (GenBank accession no. AV389837) with high homology to known Nramps. The 5’ end of the corresponding cDNA was identified by 5’ RACE (rapid amplification of cDNA ends). Full-length cDNA was then amplified by nested PCR following reverse transcription and cloned into the yeast vector pUG35. Total RNA of *C. reinhardtii* was extracted with Trizol Reagent (GibcoBRL) according to supplier’s instructions. RACE-primers were designed specific to the partial EST-sequence and RACE was performed as described elsewhere [58]. Briefly, one reverse transcription step was followed by a tailing reaction and by two PCR steps. For cloning of the full-length cDNA by RT-PCR, two restriction sites were inserted at the 5’ (XbaI) and 3’ (EcoRI) end of the cDNA in the second PCR step by use of appropriate primers. A haemagglutinin tag (HA-tag) was fused to the 5’ end immediately after the start codon and the resulting plasmid was named pAR1. The insert of pAR1 contains the start codon, followed by the HA-tag and ends 129 bp downstream of the native stop codon. Yeast mutants were transformed with pAR1 and pUG35 with the YEASTMAKER yeast transformation system (Clontech).

2.2.4 DMT1 expression

Heterologous expression of *DMT1* in *S. cerevisiae* was tested by western blotting according to standard protocols [115]. Total yeast protein content was extracted by the method described in [96]. SDS gel electrophoresis was performed according to standard protocols [115] and proteins were blotted onto nitrocellulose membrane (Schleicher&Schuell). Anti-HA High Affinity (Roche) was used as the primary antibody and Anti-Rat-IgG (peroxidase conjugate, Sigma) as the secondary. Subsequent detection was performed with the ECL kit (Amersham Biotech).
2.2.5 GenBank accession numbers

Cloned *C. reinhardtii* DMT1: AF515631
EST clone from Kazusa institute: AV389837

Nramp homologues in figure 2.3: *Escherichia coli* MntH P77145; *Salmonella enterica* MntH CAD07646; *Deinococcus radiodurans* Nramph AAF11265; *C. reinhardtii* DMT1 AF515631; *Nostoc* sp. PCC 7120 MntH BAB77244; *Xylella fastidiosa* 9a5c Nramph AAF83825; *Pseudomonas aeruginosa* MntH2 Q9RPF2; *Staphylococcus aureus* Nramph NP_374223; *Oryza sativa* Nramph1 S62667; *Arabidopsis thaliana* Nramph1 AAF36535; *Saccharomyces cerevisiae* Smf1 CAA64547, Smf2 AAB68900, Smf3 NP_013134; *A. thaliana* Nramph2 AF141204, Nramph4 AAF13279, *O. sativa* Nramph2 AAB61961; *A. thaliana* Nramph5 CAC27822; *Homo sapiens* Nramph1 AAG15405, Nramph2 P49281.

Nramp homologues in figure 2.5: *Salmonella enterica* MntH CAD07646; *Escherichia coli* MntH P77145; *Yersinia pestis* MntH CAC92226; *Chlorobium tepidum* Nramph NP_661903; *Wigglesworthia brevipalpis* Nramph NP_714977; *Listeria monocytogenes* Nramph CAC99502; *Nostoc* sp. PCC 7120 MntH BAB77244; *Leuconostoc mesenteroides* Nramph ZP_00063650; *Enterococcus faecalis* Nramph NP_815583; *Streptococcus agalactiae* Nramph NP_689011; *Lactococcus lactis* Nramph NP_267238; *Oenococcus oeni* Nramph ZP_00069508; *Lactobacillus brevis* Nramph BAB47552; *Xylella fastidiosa* 9a5c Nramph AAF83825; *Xanthomonas axonopodis* Nramph NP_642362; *Pseudomonas aeruginosa* MntH2 Q9RPF2; *Mesorhizobium loti* Nramph BAB49617; *Brucella melitensis* MntH NP_539486; *Agrobacterium tumefaciens* Nramph NP_354720; *Ralstonia solanacearum* Nramph NP_522081; *Staphylococcus aureus* Nramph NP_374223; *Deinococcus radiodurans* Nramph AAF11265; *Chlamydomonas reinhardtii* DMT1 AF515631; *Bos taurus* Nramph NP_777077; *Ovis aries* Nramph1 P49280; *Homo sapiens* Nramph1 AAG15405, *Mus musculus* Nramph1 NP_038640; *Homo sapiens* Nramph2 P49281; *Macaca fascicularis* Nramph2 AF153279; *Rattus norvegicus* Nramph2 O54902; *Takifugu rubripes* Nramph CAD43053; *Ictalurus punctatus* Nramph AAM73759; *Gallus gallus* Nramph1 P51027; *Drosophila melanogaster* Mvl S56140; *Caenorhabditis elegans* Nramph NP_509132; *Arabidopsis thaliana* Nramph1 AAF36535, Nramph3 Q9SNV9, Nramph4 AAF13279, Nramph2 AF141204, Nramph5 CAC27822; *Oryza sativa* Nramph1 S62667; *Schizosaccharomyces pombe* Nramph T38466; *S. cerevisiae* Smf1 CAA64547, Smf2 AAB68900, Smf3 NP_013134.
2.3 Results

2.3.1 Identification and cloning of the *Chlamydomonas reinhardtii* DMT1 gene

A search for Nramp homologous sequences in the *C. reinhardtii* EST database of the Kazusa DNA Research Institute led to the identification of an EST sequence displaying high similarity to genes of the *Nramp* family. Sequencing of the clone (CM051d09, GenBank accession no. AV389837) obtained from the Kazusa Institute revealed that the cDNA was complete only at the 3’ end. We identified the 5’ end with the help of 5’RACE and cloned the full-length cDNA via RT-PCR (GenBank accession no. AF515631). The cloned gene is named *DMT1* (divalent metal transporter 1) in this study, in analogy to *DMT1* (*DCT1, Nramp2*) in mammals. The longest cDNA product obtained by RT-PCR reached 1936 nucleotides and the longest open reading frame, which showed homology to Nramps, consisted of 1542 base pairs (bp) with the translation start (ATG) at position 64. The 3’ untranslated region was 330 nucleotides long and contained a putative polyadenylation signal (TGTAA) 13 bp upstream the polyadenylation site, as has already been described for other *C. reinhardtii* genes [108]. The *DMT1* GC content of 62.9% reflects the typical codon usage of *C. reinhardtii*, which favors codons containing a G or C at the third position.

Recently, sequencing of the *C. reinhardtii* genome was accomplished at the Doe Joint Genome Institute, California [52] and the sequence is available in http://genome.jgi-psf.org/chlre1/chlre1.home.html since February 2003. Homology search in this database (version 1.0) revealed that the coding sequence of the cloned *DMT1* corresponds to genomic sequence scaffold_489, contig 3, base pairs 30332:34793. Comparison of the genomic sequence to the cDNA sequence determined by us showed that the *DMT1* gene consists of 13 exons and 12 introns (Fig. 2.1).
The Nramp homologue of C. reinhardtii

Figure 2.1 Organisation of the DMT1 gene. Total length of the genomic DMT1 DNA region is 4859 base pairs, of mRNA without the poly-A tail (cDNA) 1936 nucleotides and of the coding region 1542. Exons and the coding region are represented by black, introns by white and the 3’ and 5’ untranslated regions by grey boxes. Number 1 indicates the first nucleotide of the sequence which was obtained by 5’RACE and the other numbers mark the position of start and end of exons (genomic DNA) or of the coding sequence (mRNA). Boxes are drawn to scale in order to give an impression of the organization of exons and introns in the genomic sequence.

The introns are rather small with the largest one reaching 504 bp and the smallest 110 bp. However, total length of intronic sequence is 2923 bp, while the coding sequence reaches only 1542 bp. This is rather unusual for C. reinhardtii genes where total intronic sequence is described to equal total coding sequence [108]. Analysis of the boundaries between exons and introns (Tab. 2.2) showed that they correspond well to the consensus sequence for C. reinhardtii splice sites [108]. At least three further Nramp homologues were identified by comparative sequence analysis [111] in the recently released genome of C. reinhardtii [52].
Table 2.2 Splice sites in the genomic sequence of DMT1. Shown are the sequences at the splice sites of each intron (1-12) of DMT1. On the left part of the table the 5' splice sites are listed and on the right the 3' splice sites. The positions of the nucleotides relative to the splice site are indicated by numbers on the top. Underneath, the consensus sequence for \textit{C. reinhardtii} splice sites (according to [108]) is typed in bold. Nucleotides identical to the consensus sequence appear in capital letters, variant nucleotides in small.

2.3.2 Deduced amino acid sequence analysis

Translation of the \textit{DMT1} cDNA gives rise to a 513 amino acids protein sequence which contains key structural features of the Nramp family (Fig. 2.2). DMT1 is predicted to comprise 11 transmembrane domains (by the programme TMHMM 2.0, [76]), with the N-terminus in the cytoplasm and the C-terminus extracellularly. Comparison of the amino acid content of the intra- and extracellular loops linking the putative transmembrane domains showed that positive charged amino acids (lysine and arginine) are found more often at the cytoplasmic side than at the extracellular side of the membrane. The CTM (consensus transport motif) sequence motif described for other Nramp homologues [16] is also present in DMT1 (Fig. 2.2). Putative N-glycosylation sites as described for other Nramps [57, 132] were predicted only with low probability by the programme NetNGlyc 1.0 (Gupta, R., Jung, E., and Brunak, S. manuscript in preparation, http://www.cbs.dtu.dk/services/NetNGlyc).
Interestingly, comparison of Nramp homologues from various species to the *C. reinhardtii* Nramp showed that the predicted protein sequence of DMT1 shares more similarity to the prokaryotic than to the eukaryotic Nramps (Fig. 2.3). Pairwise alignment of the hydrophobic core (as defined in [16]) of the proteins in figure 2.3 reveals the best similarity of DMT1 to the Nramp homologue of the cyanobacterium *Nostoc sp. PCC 7120* (58.06% identities) and lowest similarity to *Oryza sativa* Nramp2 (32.51% identities, see Fig. 2.4 for an alignment).
Figure 2.3 Homology tree of selected members of the Nramp family. The homology tree for the hydrophobic core (as defined by [16]) of 19 Nramp homologues was constructed by the program DNAMAN. The first two letters correspond to the initials of the organism (e.g. Ec for E. coli) with the designation of the protein following. Nramp stands for Nramp homologue. Prokaryotic and eukaryotic groups of Nramp homologues are boxed. Accession numbers for the used protein sequences in order of appearance in the tree can be found in the materials and methods section.
Another interesting feature of DMT1 involves the N-terminus of the protein. We compared the N-termini of selected Nramp homologues and found that the length of the \textit{C. reinhardtii} DMT1 N-terminus exceeds that of the other analyzed homologues (Fig. 2.5). Presuming that the N-terminus faces the inside of the cell as predicted, it might form an extra domain with a function distinct from the actual transport function of the protein and for example play a role in the regulation of protein stability. However, analysis of the first 80 amino acids with ScanProsite [48] produced only patterns with a high probability of occurrence (e.g. phosphorylation sites) but no apparent protein motif known to play a role in

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{alignment.png}
\caption{Alignment of the hydrophobic core of \textit{Nostoc} sp. PCC 7120 MntH, \textit{Oryza} sativa Nramp2 and \textit{C. reinhardtii} DMT1. The hydrophobic core was defined after [16]. Identical amino acid residues are marked by a star and similar residues by a dot in the consensus sequence.}
\end{figure}
regulatory posttranslational modification (e.g. ubiquitination, which was shown for yeast
Zrt1p [49], data not shown).

In a further investigation of the DMT1 N-terminus we compared selected Nramp
homologues in respect of the N-terminal sequence, which is not as conserved as the
hydrophobic core. For this alignment we used the N-terminal sequence overlapping the first 9
amino acids of the core up to the first completely conserved residue (glycine). Despite the
overall variability of the sequence there are distinct conserved residues shared only by
eukaryotic or prokaryotic homologues (Fig. 2.5).
The Nramp homologue of C. reinhardtii

Figure 2.5 Alignment of the N-terminal sequence of selected Nramp homologues and of C. reinhardtii DMT1. The alignment was performed with the N-terminal sequence of selected Nramp homologues including the first 9 amino acids of the core region, which display high similarity among the organisms (rightmost in the alignment). The alignment is split in two blocks. The upper block is composed of prokaryotic Nramps and CrDMT1 (bold), the lower block of eukaryotic Nramps. White letters shaded in black or dark grey represent residues conserved in both blocks. Letters shaded in green represent residues conserved only in the prokaryotic block and CrDMT1, and letters shaded in yellow represent residues conserved only in the eukaryotic block. The cross, the circle and the star indicate residues which are conserved in both blocks, but are somewhat shifted (because of additional amino acid stretches in the eukaryotic block). The first two letters correspond to the initials of the organism (e.g. Se for Salmonella enterica) with the designation of the protein following. Nramp stands for Nramp homologue. Accession numbers for the used sequences (in order of appearance in the alignment) can be found in the materials and methods section.

Some prokaryotic Nramps (e.g. Salmonella, E. coli, Yersinia, Chlorobium, Ralstonia) do not include these conserved residues mainly because they are shorter. On the other hand, there are also eukaryotic homologues which break the ranks, namely the plants, yeasts, Drosophila and C. elegans, which do not possess the typical eukaryotic conserved amino acids or possess them to a lesser extend. Nevertheless, the vertebrates display a matching pattern. Seemingly, the two major groups formed regarding the N-terminal sequence correspond to the pro- and eukaryotic domains. Clearly, the C. reinhardtii DMT1 takes its place among the prokaryotic Nramp homologues since it only displays prokaryotic and no eukaryotic features on the N-terminus. This is in agreement with the placement of DMT1 in the prokaryotic cluster when the sequences of the core are compared (Fig. 2.3). Once again, DMT1 takes a unique position among the analyzed Nramps as a eukaryotic homologue displaying prokaryotic characteristics.

2.3.3 Heterologous expression of C. reinhardtii DMT1 in S. cerevisiae

For functional analysis of the C. reinhardtii DMT1 protein an approach utilizing yeast S. cerevisiae transport mutants was chosen, since targeted nuclear gene disruption by homologous recombination in C. reinhardtii is not established (but see [94]). The complete cDNA of DMT1 was cloned into the yeast vector pUG35 resulting in plasmid pAR1, which was transformed into various yeast mutants (Tab. 2.1). Expression of the DMT1 gene in yeast was confirmed by western blot analysis (Fig. 2.6). A specific immunoreactive band was detected in cells transformed with pAR1, but not in cells transformed with the control vector pUG35 or in untransformed cells. As was already observed with highly hydrophobic membrane proteins [10, 75] heating of the cell extract in SDS sample buffer at 95°C before
loading on the gel lead to aggregation of the DMT1 protein which barely entered the separation gel (Fig. 2.6, lane 4). When the cell extract was loaded on the gel without previous heating, the DMT1 protein was identified as a rather diffuse band in the gel corresponding to the predicted 57 kDa (Fig. 2.6, lane 1). Expression of the DMT1 gene was confirmed in all studied mutants (data not shown).

![Figure 2.6](image.png)

**Figure 2.6** Heterologous expression of DMT1 in *S. cerevisiae* strain Y16272 (snf1). Yeast strain Y16272 was transformed with pAR1 or with the control vector pUG35 and whole cell extracts were analyzed for DMT1 expression by western blotting. The molecular weight in kDa is indicated on the right and arrows point to the DMT1 protein band. Samples 1-3 were loaded on an SDS PAGE gel without previous boiling, samples 4-6 were boiled. Lanes 1 and 4: mutant snf1 with DMT1, lanes 2 and 5: mutant snf1 with control plasmid, lanes 3 and 6: mutant snf1.

### 2.3.4 DMT1 restores growth of a *S. cerevisiae* snf1 mutant under manganese limiting conditions

The Snf1p protein of *S. cerevisiae* has been identified as a manganese transporter belonging to the Nramp family [121] and shows 38% similarity to *C. reinhardtii* DMT1. Yeast strains with disrupted SMFL gene fail to grow on synthetic medium containing the divalent cation chelator EGTA. As a first step to elucidate the function of DMT1, its ability to complement the yeast strain Y16272 (snf1) with respect to growth restoration in inhibiting concentrations of EGTA was tested. For this, yeast strain Y16272 (snf1) was transformed with plasmid pAR1 containing *C. reinhardtii* Nramp homologue DMT1, or with the empty yeast overexpression vector pUG35 as a control. Growth tests were performed in liquid minimal medium containing 0–10 mM EGTA and the maximal growth rate of each strain in
medium with no EGTA was defined as 100% growth of the strain. Growth rates of the two strains were different and the strain with the control vector showed higher sensitivity to EGTA than the strain expressing DMT1 (Fig. 2.7). At an EGTA concentration of 5 mM, relative growth of strain smfl/pUG35 was reduced to 78%, whereas relative growth of strain smfl/pAR1 remained at 100%. Higher EGTA concentrations eventually impaired growth of both strains. These results indicate that the strain smfl/pAR1 is able to compete with EGTA for manganese ions, in contrast to strain smfl/pUG35, indicating that DMT1 acted as a metal transporter. At EGTA concentrations below 5 mM, growth of strain smfl/pAR1 exceeded 100%. This might suggest that the overexpressed DMT1 protein imported too many metal ions into the cell in medium without the metal chelator and thus lead to a slight growth decline.

Figure 2.7 Yeast strain smfl/pAR1 was tested for altered EGTA tolerance. The yeast manganese transport mutant strain Y16272 (smfl) was transformed with the DMT1 containing plasmid pAR1 (filled squares) or with the control expression vector pUG35 (open squares) and growth of the two strains was compared in medium containing increasing concentrations of the metal chelator EGTA. The maximal growth rate of each strain in the medium without EGTA was defined as 100% and growth of the same cells in different concentrations of EGTA was expressed in relation to it. The graph is derived from two independent experiments.
2.3.5 Overexpression of DMT1 in *S. cerevisiae* mutant *snf1* increases sensitivity to manganese, cadmium, and copper, but not to zinc

The fact that metals can become toxic when their concentration exceeds physiological levels in the cell was utilised to study the specificity of the DMT1 protein. If uptake of a particular metal ion is mediated by DMT1, the strain expressing the DMT1 protein should be less tolerant to that metal than the strain with the control vector. In the following experiments the two strains used above (*snf1*/pUG35 and *snf1*/pAR1) were tested for differences in growth under increasing metal concentrations.

The first element tested was manganese, in order to confirm the result of the previous experiment, which pointed to a role of DMT1 in manganese (Mn$^{2+}$) uptake. For this, cells were grown in liquid minimal medium containing 0, 10, 100 or 1000 µM MnCl$_2$. Addition of MnCl$_2$ showed the expected effect. That is, the yeast mutant expressing *DMT1* was more sensitive to increasing concentrations of MnCl$_2$ than the control strain with the empty vector (Fig. 2.8A). The maximal growth rate of each strain in medium with no MnCl$_2$ was defined as 100% growth of the strain and growth of the same cells in different concentrations of MnCl$_2$ was expressed in relation to it. Relative growth of strain *snf1*/pUG35 was still 100% at a MnCl$_2$ concentration of 100 µM and 99% at 1000 µM MnCl$_2$. Growth in strain *snf1*/pAR1 was reduced to 89% and 83%, respectively. These results indicate that Mn$^{2+}$ ions must have entered the *snf1*/pAR1 cells via the DMT1 transporter where they reached toxic concentrations leading to growth impairment.

In order to investigate the spectrum of metal cations transported by DMT1 in addition to Mn$^{2+}$, cadmium (Cd$^{2+}$), copper (Cu$^{2+}$) and zinc (Zn$^{2+}$) were tested for potential growth inhibition of the yeast mutant strain *snf1* expressing *DMT1* on the plasmid pAR1. Addition of 1 µM CdCl$_2$ into the medium led to an average growth reduction of 18% in the strain expressing *DMT1* but only 2% in the strain with the control vector (Fig. 2.8B). At 10 µM CdCl$_2$ strain *snf1*/pAR1 showed a growth reduction of 52% whereas relative growth of *snf1*/pUG35 decreased for only 9%. These results led to the conclusion that DMT1 is able to transport cadmium ions into the yeast cells. Addition of CuSO$_4$ to the medium had a similar effect and a clear growth difference between the two strains was observed at a concentration of 1 mM CuSO$_4$ (Fig. 2.8C). Strain *snf1*/pAR1 showed a growth reduction of 73%, while growth of strain *snf1*/pUG35 was reduced by 48%. The fourth metal tested was zinc. In this
case there was no difference in the response of the two strains after addition of up to 10 mM ZnCl₂ into the growth medium. Both strains showed growth inhibition in the same degree at concentrations exceeding 1 mM ZnCl₂ (Fig. 2.8D), showing that the yeast strain expressing DMT1 did not have any growth disadvantage over the strain with the empty vector in toxic zinc concentrations.

Figure 2.8 Altered sensitivity to divalent metal ions in yeast strain snfl/pAR1. The yeast manganese transport mutant strain Y16272 (snfl) was transformed with the DMT1 containing plasmid pAR1 (filled squares) or with the control expression vector pUG35 (open squares) and growth of the two strains was compared in minimal medium containing increasing concentrations of a metal solution. The maximal growth rate of each strain in medium without additional metal was defined as 100% and growth of the same cells in different concentrations of the metal solution was expressed in relation to it. A: MnCl₂, B: CdCl₂, C: CuSO₄, D: ZnCl₂. The graphs for MnCl₂ and CdCl₂ are derived from three independent experiments and for CuSO₄ and ZnCl₂ a representative experiment is shown.
2.3.6 DMT1 does not complement EDTA sensitivity of *S. cerevisiae* zrt1/zrt2 double mutant

ZHY3 is a yeast mutant in which the high and low affinity zinc transporter genes *ZRT1* and *ZRT2* have been disrupted and which, consequently, shows reduced growth in medium with the metal chelator EDTA [139]. The mutant was transformed with the *DMT1* containing plasmid pAR1 or the control vector pUG35 in order to test whether *DMT1* expression would alter the growth behaviour of the strain. Growth tests were performed in liquid minimal medium containing 1 mM EDTA and 0–1 mM ZnCl₂. Maximal growth rate of each strain under zinc replete conditions (EDTA 1 mM, ZnCl₂ 1 mM) was defined as 100% growth of the strain and growth of the same cells in lower concentrations of ZnCl₂ was expressed in relation to it (Fig. 2.9). At lower zinc concentrations in the medium growth of both strains was reduced to the same degree and no clear difference was observed between them. Since the strain expressing the *C. reinhardtii* DMT1 did not show any advantage in growth, it can be assumed that the overexpressed protein was not able to import zinc ions into the cell. This is in agreement with the results obtained for the smf1 mutant grown in medium with ZnCl₂, which showed no enhanced sensitivity to zinc when expressing *DMT1*.

![Figure 2.9](image-url)  

*Figure 2.9* Growth of yeast strain *ZHY3/pAR1* under zinc limiting conditions. The yeast zinc transport mutant strain *ZHY3* [139] was transformed with the *DMT1* containing plasmid pAR1 (filled squares) or with the control expression vector pUG35 (open squares) and growth of the two strains was compared in minimal medium containing 1 mM EDTA and increasing concentrations of ZnCl₂. The maximal growth rate of each strain under zinc replete conditions (EDTA 1 mM, ZnCl₂ 1 mM) was defined as 100% and growth of the same cells in limiting concentrations of ZnCl₂ was expressed in relation to it. Shown is a representative experiment.
2.3.7 DMT1 function in iron transport

Next, we investigated whether DMT1 acts in iron transport. Yeast mutant DDY4 (fet3/fet4), in which high and low affinity iron transport is impaired [32], was tested for growth improvement after transformation with the \textit{DMT1} containing plasmid pAR1, and compared to the same strain transformed with the control vector pUG35. Growth tests were performed in liquid minimal medium containing 1 mM EDTA and 0–1.25 mM FeCl₃. Maximal growth rate of each strain in iron excess conditions (EDTA 1 mM, FeCl₃ 1.25 mM) was defined as 100% growth of the strain and growth of the same cells in lower concentrations of FeCl₃ was expressed in relation to it. At lower FeCl₃ concentrations, growth of all strains was impaired (Fig. 2.11). However, the strain expressing \textit{DMT1} had clearly a growth advantage over the strain with the control vector (growth reduction of approximately 40% as opposed to 70% at a FeCl₃ concentration of 0.5 mM). This result suggests a role of DMT1 in iron transport.

![Graph showing growth of yeast strain DDY4/pAR1 under iron limiting conditions](image)

**Figure 2.11** Growth of yeast strain \textit{DDY4/pAR1} under iron limiting conditions. The yeast iron transport mutant strain DDY4 [32] was transformed with the \textit{DMT1} containing plasmid pAR1 (filled squares) or with the control expression vector pUG35 (open squares) and growth of the two strains was compared in minimal medium containing 1 mM EDTA and increasing concentrations of FeCl₃. The maximal growth rate of each strain under iron excess conditions (EDTA 1 mM, FeCl₃ 1.25 mM) was defined as 100% and growth of the same cells in limiting concentrations of FeCl₃ was expressed in relation to it. Shown is a representative experiment.
2.4 Discussion

We have cloned a member of the Nramp gene family in the green microalga *C. reinhardtii*, named DMT1 for divalent metal transporter 1. The cloned cDNA displays characteristics typical of *C. reinhardtii*, that is a GC content of over 60% and a polyadenylation signal 13 base pairs upstream the polyadenylation site. Analysis of the genomic sequence which was recently released (Doe Joint Genome Institute, California, [52]), showed that the *DMT1* gene consists of 13 exons and 12 introns, with the introns reaching more than double the length of the coding sequence. This is rather uncommon for *C. reinhardtii*, where intronic sequence equals the coding sequence length [108].

The deduced amino acid sequence reached 513 amino acids and contained structural features common for the Nramp family. Firstly, the DMT1 protein was predicted to comprise 11 transmembrane domains (TMs). In agreement to that it was shown by topological analysis that the *E. coli* MntH forms 11 TMs, with a cytoplasmic N-terminus and a periplasmic C-terminus [28, 29]. For other Nramp homologues a number of 10-12 TMs was predicted [18, 57, 105]. Secondly, the highly conserved “consensus transport motif” (CTM) found in every Nramp homologue is also present in DMT1 within the same region (between TM8 and TM9). This motif was suggested to be involved in the transport mechanism of Nramps, since it is similar to the prokaryotic binding-protein-dependent transport signature (EAA region of prokaryotic ABC transporters) and to the pore region of K⁺ channels [16]. The EAA region is found in the hydrophobic subunits of the ABC transporters and most likely interacts with the ATPase subunit [12, 85, 93]. For the Nramp proteins an interaction with an ATPase subunit in order to facilitate transport by ATP hydrolysis seems unlikely, since uptake of cations was shown to be coupled to H⁺ import [57]. Nevertheless, the consensus sequence described in [131] for the CTM motif might indicate an important function in the Nramp protein but distinct to the function of the EAA region in bacterial ABC transporters. Regarding voltage gated K⁺-channels, the similarity to Nramps is restricted to the pore region sequence [16, 136], which is thought to be responsible for ion selectivity [60]. Since the CTM of Nramps is predicted in a cytoplasmic loop, it seems unlikely that the motif should have a similar function as in K⁺ channels.

Putative N-glycosylation sites, a third feature shared by Nramp proteins, were predicted for DMT1 (by the programme NetNGlyc 1.0; Gupta, R., Jung, E., and Brunak, S. manuscript in preparation), yet with rather low probability, at the N-terminal region before the first TM (2
The Nramp homologue of C. reinhardtii sites) and between TM8 and TM9 (1 site, Fig. 2.2). All three putative sites are localized intracellularly in contrast to other Nramps. Gunshin et al. [57] predicted N-glycosylation sites between TM7 and 8 for rat DCT1, and Vidal et al. [132] showed experimentally that mouse Nramp1 is extensively glycosylated, with two possible N-glycosylation sites between TM6 and 7. Posttranslational modification, such as N-glycosylation, can be important for proper protein targeting and stability in the membrane or for regulation of function.

Strikingly, the algal DMT1 protein sequence showed higher similarity to prokaryotic than to eukaryotic members of the Nramp family. A scenario for the evolution of Nramps which includes horizontal gene transfer events between eu- and prokaryotes was proposed by Cellier et al. [19]. In spite of such events the investigated eukaryotic Nramps in that study clustered exclusively in one group and they all showed higher homology to each other than to prokaryotic homologues. The DMT1 protein seems to be an exception as it clusters within the prokaryotic group of Nramps (Fig. 2.3). Likewise, it was shown for the globin protein in Chlamydomonas eugametos that it is more similar to globins from ciliates and Nostoc than to eukaryotic homologues, and gene transfer events between the Nostoc ancestor and a common ancestor to Paramecium, Tetrahymena and Chlamydomonas were considered as an explanation [91]. In addition, regarding only the N-terminus of DMT1, it stands out that it possesses only features of prokaryotic Nramps, in contrast to other eukaryotic homologues (Fig. 2.5). This unique position of C. reinhardtii DMT1 – a “prokaryotic” protein in a eukaryotic organism – among Nramp homologues might prove useful for further studies on the phylogenetic evolution of Nramps.

Since the N-terminus of DMT1 is longer than that of any other homologue considered in this study, it seems possible that it plays some important role in the function or regulation of the protein, although no familiar protein motif seems to be present. Tabuchi et al. [123] studied the function of H. sapiens Nramp1 and Nramp2 by exchanging the N-termini of the two proteins. Complementation experiments showed that the native N-terminus is essential for a fully functional Nramp1 or Nramp2. This is an interesting result, considering that the N-terminus of some Nramps (e.g. E. coli) is extremely short.

Heterologous expression in a manganese transport mutant of S. cerevisiae was utilized to investigate the function of the DMT1 protein. A deletion mutant for SMF1 – the yeast Nramp homologue – was transformed with a moderate level expression plasmid containing DMT1 and tested for growth under metal depleted conditions. Growth impairment in medium with the divalent metal chelator EGTA was partially restored in cultures expressing C. reinhardtii DMT1, suggesting a role for DMT1 as a divalent metal ion transporter. This
The Nramp homologue of *C. reinhardtii* finding was affirmed by the fact that the strain expressing *DMT1* was less tolerant to increasing MnCl₂ concentrations than the strain with the control plasmid. These results demonstrate the ability of DMT1 to transport manganese ions into the yeast cell.

In order to check the ability of DMT1 to transport other divalent metal ions, we performed growth impairment experiments with the same yeast strains as above (*smfl/pAR1, smfl/pUG35*). Addition of CdCl₂ to the growth medium resulted in growth impairment for both yeast strains, but the strain expressing DMT1 responded to lower concentrations, that way displaying greater sensitivity against the metal. Cadmium was also described to pass the cell membrane via the Nramp transporter in *Arabidopsis thaliana* [127] and Kehres et al. [72] showed that Mn²⁺ uptake by the Nramp homologue MntH of *E. coli* and *S. typhimurium* was inhibited strongly by Cd²⁺. Interestingly, Cd²⁺ inhibited also the Mn²⁺ uptake by the ABC transporter SitABCD in *Salmonella enterica*, a transporter which showed a similar specificity profile as the MntH of *E. coli* and *S. typhimurium* [71]. In contrast, MntH was inhibited only weakly by Cu²⁺, an element which was transported by DMT1 as was shown by the increased sensitivity of the *smfl* yeast mutant strain expressing the algal Nramp, compared to the strain with the control vector. Apparently, this was not true for ZnCl₂ were both strains showed the same growth behaviour after addition of increasing ZnCl₂ concentrations. In order to confirm whether zinc was indeed not transported by the DMT1 protein, *DMT1* was expressed in the yeast strain ZHY3 (*zrt1/zrt2*) which shows defects in zinc transport. The phenotype of ZHY3, namely reduced growth in medium with the metal chelator EDTA, was not complemented by *DMT1* expression. Thus, both experiments – the zinc sensitivity test with the *smfl* mutant and the complementation test with the *zrt1/zrt2* mutant – lead to the conclusion that DMT1 was not able to transport zinc ions into the yeast cells. Concerning zinc, different results were obtained for its transport by Nramp homologues. MntH of *E. coli* and *S. typhimurium* were not inhibited considerably by zinc in manganese uptake [72], whereas Supek et al. [121] mention inhibition of Smf1 manganese import by zinc. In rat, it was shown that zinc inhibited iron and manganese uptake via the Nramp homologue DCT1 (DMT1, Nramp2) [57], but Sacher et al. [113] could not show transport of ⁶⁵Zn²⁺ via DCT1 or Smf1p. Thus, Zn²⁺ might bind at the same site on the transporter protein as the other metal ions but without entering the cell.

For a further characterization, *DMT1* was expressed in the yeast strain DDY4 (*fet3/fet4*) in which the high and low affinity iron transport systems are impaired. The phenotype of growth impairment under iron limiting conditions was partially complemented by expression of *DMT1*, indicating a role for the DMT1 protein in iron transport. This is
consistent with the finding that Nramp1, Nramp3 and Nramp4 in *A. thaliana* are also involved in iron transport [30, 127]. Recently, components of a high affinity iron transport system similar to the *S. cerevisiae* Frt1p/Fet3p iron assimilation pathway were described in *C. reinhardtii* [61, 78]. This does not disagree with our results, considering that generally there are several uptake systems present in the cell, mediating high and low affinity transport of the same substrate. An efficient transport system for iron is mostly important for the cell as this element is essential for many vital processes. However, under iron excess conditions, the high affinity system can be shut down and a low affinity system can take on the uptake of iron. Accordingly, DMT1 might play a role in low affinity iron assimilation when the high affinity system is down regulated.

In conclusion, we have identified, cloned and characterized a divalent metal transporter in an alga – the DMT1 of *C. reinhardtii* – which must be placed among prokaryotic Nramp homologues regarding sequence similarity. Furthermore, we elucidated the function of the protein by complementation experiments in several yeast mutants and showed that the specificity of the transporter was quite broad, including manganese, cadmium, copper and iron but not zinc transport.

**Acknowledgements**

We would like to thank Dr. J. H. Hegemann for the pUG35 vector, Dr. D. Eide for yeast strains and the Kazusa DNA Research Institute for the *C. reinhardtii* EST clone. We are indebted to Dr. A. J. B. Zehnder for support, helpful discussions and constant interest in the project.
3.1 Further verification that DMT1 mediates growth improvement under metal limiting conditions

The DMT1 coding sequence was cloned in the pUG35 vector for moderate expression (see Materials and methods chapter 2). The MET-25 promoter of the vector can be repressed by addition of 1mM methionine into the culture medium. In order to further confirm that the effects observed on the growth rate of the smfl yeast mutant arise from DMT1 expression, a complementation assay was performed where the promoter was switched off. For this, minimal medium agar plates with EGTA and/or methionine were spotted with dilutions of yeast strains smfl/pUG35 (control) and smfl/pARl (DMT1). Methionine itself had no negative effect on the growth of the strains, whereas EGTA reduced growth of the control strain (Fig. 3.1A). The DMT1 expressing strain grew better than the control on EGTA but not when methionine was added. In addition, a western blot (see Materials and methods chapter 2) showed that the DMT1 protein is not expressed in the presence of 1mM methionine (1B). Thus, the complementation observed for strain smfl/pARl (chapter 2) was due to DMT1 expression.

Figure 3.1
A) Complementation experiment (spot assay). smfl/pUG35: yeast mutant smfl transformed with the empty pUG35 vector; smfl/pARl: yeast mutant smfl transformed with the DMT1 containing plasmid pARl; Met: methionine. 10μl culture of increasing dilutions were spotted from left to right.
B) Western blot of yeast protein extracts with anti-HA antibody. pARl: yeast mutant smfl transformed with the DMT1 expression plasmid pARl; pUG35: yeast mutant smfl transformed with the empty pUG35 vector; pARl+met: 1mM methionine was added to the culture medium before protein extraction.
3.2 Study of DMT1 mutants

During the cloning procedure of DMT1 (see materials and methods chapter 2) point mutations were introduced into the amino acid sequence resulting in four different plasmids. The plasmid harboring the correct sequence was designated pAR1, the plasmids with amino acid substitutions were named pAR1mut, pN1 and pN2HA (Fig. 3.2 and Tab. 3.1).

```
1 MVLGRLFARSEASAAPALADYNNEGNGSTIDTSVVAVEVEASGSPPSSATVR
51 GDSTLNEPLLEGFKDRGELPSLPEASLSFPEESAPWWRKFAFMCLGF
101 LISVGYMPGWATDLAGGSFGYTLLFVVLCSLCAFMQLYLSSLKLGIA
151 SDRDLAQACRDAYHPVNLWVVAEEIAAATDLAEVVGCAIAFNLLGI
201 PLWAGVLTAADVIILLVAEARS*FRFLEILVALTALISACFIYELVKAQ
251 PDMGKVMRGYLFSKEIIKNDMLYLATGILGATVMPHNLYLHSSVISQTRA
301 YPRTTYARTIAKILGLVDSTLSLLVAFVINSSILIVAAAFHYATPPLED
351 IAIDTDAYELLASSLSKGASAIFGVALLAAGQMNSTITGTLTSGQIVMEGF
401 LSFKMRPWLRRIITTRGAAIVPAATVAAMGREGVSGQLLVLSQVILSLTLP
451 FAVFPLVHFTSSRKYICNFANRWFASVIAWLLFLFISALNVNVLVQSAIL
501 GSFGGGLGHRAAV*
```

pN1 (G97S, D353G, W473R)
pN2HA (A137T, G467S)
pAR1mut (S223G, L246Q, S263F, F341L)

Figure 3.2 Point mutations in DMT1. Regular amino acids correspond to plasmid pN1, bold to pN2HA, and italics to pAR1mut. Residues absolutely conserved throughout evolution are marked by a grey box with a black line. Residues highly conserved are in a grey box without a black line and residues partially conserved are in a white box with black line. Residues not at all conserved are circled. *This amino acid is conserved as a glycine in all organisms except DMT1 where it is replaced by a serine.
Expression levels of the mutated proteins were checked by western blotting (see Materials and methods chapter 2). Protein levels of pN1 were almost similar to the wildtype, while pAR1mut showed weaker expression. Protein stability was severely affected in pN2HA and no protein was detected (Fig. 3.3A, total protein content was checked by coomassie blue staining of a gel ran in parallel, not shown).

In agreement to this observation, pN2HA was not able to rescue growth of an smfl yeast mutant on medium with 15mM EGTA (Fig. 3.3B). Also no complementation was achieved in the smfl yeast mutant strain transformed with the pAR1mut plasmid which was tested in medium with varying concentrations of EGTA (Fig. 3.3C). This can be attributed to the decreased protein stability in comparison to the wildtype. In contrast, pN1 was functional and complemented the mutant phenotype on medium with 15mM EGTA (Fig. 3.3B).

Thus, the introduced mutations in pAR1mut and pN2HA affected protein stability, whereas the mutations in pN1 allowed the expression of a functional protein. Sequence alignment with other Nramp homologues (not shown) revealed that G97, which is substituted by S in pN1, is conserved in all Nramps (Tab. 3.1). Whether this is a functional conservation has to be tested with further amino acid substitutions. It is notable that evolution insisted on using glycine at this position although substitution with serine in DMT1 did not affect function. However, this amino acid could also be involved in regulation or specificity of the protein, which would not be detected in this experimental setup.

The substituted amino acid residues described here do not correspond to the ones described for Smf1p and Nramp2 in chapter 1.4.2.

---

**A**

<table>
<thead>
<tr>
<th>pUG35</th>
<th>pAR1</th>
<th>pN1</th>
</tr>
</thead>
<tbody>
<tr>
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<td>[ ]</td>
<td>[ ]</td>
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</table>

**B**

<table>
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<th>15mM EGTA</th>
</tr>
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<tbody>
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<td>[ ]</td>
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**C**

![Graph showing relative growth against EGTA concentration]
Figure 3.3
A) Western blot of smfl yeast strain protein extract. pUG35: empty vector; pAR1: plasmid with correct DMT1; pN1, pAR1mut and pN2HA: plasmids with mutated DMT1.
B) Spot assay. Cultures of smfl yeast mutant transformed with empty vector (pUG35), and plasmids with mutated DMT1 (pN1, pN2HA) were spotted on minimal medium agar plates with and without the metal chelator EGTA.
C) Growth curve of smfl yeast mutant with empty vector (pUG35, squares), correct DMT1 (pAR1, circles) and mutated DMT1 (pAR1mut, triangles) in increasing concentrations of the metal chelator EGTA. Maximal growth rate of each strain in minimal medium without EGTA was defined as 100% and growth of the same cells in different concentrations of EGTA was expressed in relation to it.

Table 3.1
A: Overview of the DMT1 mutants

<table>
<thead>
<tr>
<th></th>
<th>pN1</th>
<th>pN2HA</th>
<th>pAR1mut</th>
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<td>expressed and functional</td>
<td>G97S, D353G</td>
<td>A137T, G467S</td>
<td>S223G, L246Q</td>
</tr>
<tr>
<td>not expressed</td>
<td>W473R</td>
<td>G467S</td>
<td>S263F</td>
</tr>
<tr>
<td>low expression, not functional</td>
<td>no</td>
<td>partial</td>
<td>absolute as G</td>
</tr>
</tbody>
</table>

*conservation* refers to the degree of conservation of this particular amino acid residue throughout evolution.

B: Graphical representation of the DMT1 protein. Black bars symbolize the predicted transmembrane regions of the protein. Mutated amino acids are indicated below the corresponding TM. CTM: consensus transport motif.
3.3 Role of DMT1 in Zn-mediated reduction of Mn toxicity

In chapters 2.3.5 and 2.3.6 I showed that Zn is not transported by DMT1. In order to further investigate a possible interaction between DMT1 and Zn I tested growth of the yeast strain smfl expressing DMT1 in medium with various manganese to zinc concentration ratios (Fig. 3.4). Growth in medium without additional metal was defined as 100% (Fig. 3.4, Mn 0, Zn 0). Addition of 100 μM MnCl2 resulted in a 10% growth reduction, as was already shown in figure 2.8A. When the medium was supplemented with 1000 μM ZnCl2 (MnCl2 to ZnCl2 ratio of 1:10), the inhibitory effect of Mn$^{2+}$ was almost entirely suppressed. Already a MnCl2 to ZnCl2 ratio of 1:1 (100 μM MnCl2, 100 μM ZnCl2) led to a slightly better growth, indicating that addition of Zn had a protective effect against Mn toxicity.

![Figure 3.4 Competition between zinc and manganese for DMT1. The yeast manganese transport mutant strain smfl (Y16272) was transformed with the DMT1 containing plasmid pAR1 and growth under different conditions was tested. Growth in minimal medium without any additional metal was defined as 100% and growth of the same cells under different metal concentrations was expressed in relation to it. Mn: MnCl2, Zn: ZnCl2. The graph is derived from two independent experiments.](image-url)
Three possible explanations for this effect are imaginable. a) Zn enters the cell via other transporters than DMT1 (e.g. by the Zrt1p), accumulates intracellularly and protects the cell against Mn toxicity from inside, e.g. by competing with Mn for protein active sites. b) Zn prevents Mn from entering the cell by blocking the binding sites of the DMT1 transport protein. c) Zinc prevents Mn from entering the cell by some other unspecific way. To differentiate between the first and the other two alternatives the metal content of the yeast cells can be measured by inductively coupled plasma mass spectrometry (ICP-MS). If the Mn concentration in the yeast cells grown in excess Mn is equal to the Mn concentration in the yeast cells grown in Mn and Zn, then Zn does not prevent Mn from entering the cell and the protective mechanism of Zn may be localized inside the cell (a). In contrast, if less Mn accumulates in the second culture, then Mn import is either inhibited directly by Zn binding to the DMT1 transporter (b), or indirectly by interactions of the two metals at the cell surface or in the medium (c). Distinguishing between b and c may be accomplished by comparing the yeast strain expressing DMT1 (smf1/DMT1) with the mutant carrying only the empty vector (smf1/pUG35) in terms of adsorbed metal concentration. Again, using ICP-MS as a measuring tool, one can differentiate between intracellular metal concentration and the concentration of metals which only adsorbed to the cell surface. The adsorbed amount of metals is calculated by subtracting the intracellular concentration (determined after washing the cells with the metal chelator EDTA and thereby removing all metals at the cell surface) from the total metal concentration. If the adsorbed Zn reaches higher concentrations in the smf1/DMT1 strain than in the control, then Zn probably binds to the DMT1 protein and thereby hinders Mn from entering the cell. If the concentration is equal for both strains, then the reduction of Mn toxicity after Zn addition may not be an effect specific of DMT1 expression.
Chapter 4

Experiments on the regulation of DMT1

Most characterized Nramps are regulated at the transcriptional level. Only the yeast SMF1 and SMF2 homologues are regulated at the level of protein stability by the Bsd2p protein (see 1.4.2 Nramp regulation). A search in the C. reinhardtii genomic data base for sequences similar to BSD2 produced no hits. BLAST search in the non redundant data base revealed putative BSD2 sequences only in Neurospora crassa, Magnaporthe grisea and Schizosaccharomyces pombe (not shown). Thus, this regulatory pathway may be restricted to fungi or depend on factors other than Bsd2p in other organisms. We decided to start investigating DMT1 regulation by studying the mRNA synthesis under different conditions.

4.1 Materials and methods

4.1.1 RNA isolation

Total RNA of C. reinhardtii cw15arg7 cells was extracted with Trizol Reagent (GibcoBRL) according to supplier’s instructions. Briefly, 25 ml of culture were centrifuged (3345 x g, 4min, 4°C) and the pellet resuspended in 1 ml Trizol. Insoluble material (proteins, polysaccharides) was removed by centrifugation (12000 x g, 10 min, 4°C). 200 μl of dichloromethane were added and the tube shaken vigorously by hand for 15 sec. After centrifugation (12000 x g, 15 min, 4°C) the aqueous (upper) phase was transferred to a fresh tube. 500 μl isopropanol were added. Centrifugation (12000 x g, 10 min, 4°C) followed incubation at RT (10 min) and the supernatant was removed. The pellet was washed with 1 ml ethanol 75%, dried at RT and dissolved in 50 μl RNase free water.
4.1.2 Culture conditions

1200 ml TAP medium (see Materials and methods chapter 2) supplemented with 50 mg/l arginine were inoculated with 50 ml preculture and divided into two flasks (2 l). After overnight incubation at 25°C under constant illumination and agitation, total RNA was isolated when cultures reached a density of approximately 5x10^6 cells/ml. In order to induce DMT1 transcription, the culture was treated with EGTA. Samples were taken at time point 0 (no treatment) and at time points 1, 2, 3, 6, 9 and 23 (hours after addition of 1mM EGTA to the flask, control was left untreated).

4.1.3 In-vitro-transcription

To generate an RNA-probe for hybridization in-vitro-transcription was performed with the Riboprobe System-SP6/T7 from Promega. Plasmid pAR1 (harboring DMT1) was digested with Clal and XhoI. A band of 1 kb corresponding to part of the DMT1 sequence was excised from the agarose gel and after purification it was ligated to vector pGEM7 Zf+. As a control plasmid pCS2.1 (kindly provided by Michel Goldschmidt-Clermont, University of Geneva, Switzerland) harboring an EcoRI fragment of the gene of subunit of ribulose bisphosphate carboxylase/oxygenase (RbcS2) was digested only with EcoRI (due to lack of other appropriate restriction sites). After purification the 750 bp long fragment was ligated to pGEM7 Zf+. In order to check orientation the new plasmid was digested with BamHI. Both new plasmids were linearized with Clal and purified for transcription by polymerase T7. For in-vitro-transcription 1 µg linearized DNA was reversely transcribed in the presence of 10mM DTT, 20u RNasin ribonuclease inhibitor, 1x digoxigenin-UTP RNA labeling mix (Roche) and 20u T7-polymerase. The reaction was incubated 2h at 37°C. To test labeling efficiency a dot blot was performed where labeling efficiency of DMT1 and RbcS2 was compared to a control (Actin RNA probe, digoxigenin-labeled, Roche).
4.1.4 RNA gel and Northern blot

Two combinations of different protocols for RNA gels and northern blots were tested: 1) RNA was denatured for 1h at 50°C (2 μl Na-phosphate buffer 100mM pH7, 3.5 μl glyoxal 6M, 5 μl DMSO, RNase free water ad 19 μl). After 0.5h ethidium bromide was added to the tube. For loading on the 1% agarose gel RNA was mixed with 0.1 vol loading buffer (50% sucrose and bromophenol blue). 10mM Na-phosphate buffer was the running buffer. 2) RNA was denatured for 10 min at 65°C (1:2 in 50% deionized formamide, 6% formaldehyde, 1xMOPS, 10% glycerol, 0.05% bromophenol blue). The 1% agarose gel contained 1xMOPS, 1.85% formaldehyde and ethidium bromide. 1xMOPS was the running buffer (10xMOPS: 41.8g 3-Morpholinopropane sulfonic acid (MOPS) (0.2M), 6.8g Na-acetate (0.05M), 20ml EDTA 0.5M (0.01M), RNase free water ad 1 liter, to pH7 with NaOH pellets, autoclaved (buffer turns yellow) and stored in the dark). The gel was washed after electrophoresis with 20xSSC (3M NaCl, 0.3M Na-citrate, pH7) in order to remove formaldehyde which might inhibit transfer. Both gel types were run at 40V for 3h.

Blotting was performed either with the VacuGene™XL vacuum blotting system from Pharmacia Biotech according to supplier’s instructions (3h), or by capillarity following standard protocols (overnight). The membrane used was a positively charged nylon membrane from Roche.

4.1.5 Hybridization

Prehybridization (2h) and hybridization (overnight) of the membranes were performed at 68°C. Probe concentration was approximately 100 ng/ml hybridization solution (EasyHyb Roche, or Ultrahyb Ambion). The membranes were washed after hybridization (low stringency wash: RT 2xSSC, 0.1%SDS; high stringency wash: 68°C 0.1xSSC, 0.1%SDS). Detection was performed with Anti-Digoxigenin-AP (1:10000) and CSPD as a substrate according to supplier’s instructions (Roche). Films were exposed for varying amounts of time, depending on the signal strength.
4.2 Results

4.2.1 Detection of RbcS2 and DMTL mRNA

DIG-labeled probes for RbcS2 and DMTL were generated and labeling efficiency was controlled by a dot blot (Fig. 4.1). Total C. reinhardtii RNA was loaded onto an agarose gel and blotted by capillary transfer. Hybridization was performed with 100 ng probe/ml hybridization solution. A band corresponding to the mRNA of RbcS2 was visible (Fig. 4.2, for an explanation of each blot see below). However, no signal was detected by the DMTL probe. In case that the DMTL mRNA is transcribed at a very low rate the cultures were treated with 1mM EGTA to induce transcription. Still, no signal was detected even after use of the Ultrahyb buffer (Ambion) which is designed to increase sensitivity of the hybridization (not shown).

Figure 4.1 A: Agarose gel with DMTL labeled RNA probe. B: Dot blot of DMTL probe compared to a labeled control.

4.2.2 Comparison of electrophoretic and transfer conditions

Four different blots were prepared in order to test which experimental conditions provide the best results:

<table>
<thead>
<tr>
<th>Blot Nr.</th>
<th>Gel</th>
<th>Blotting method</th>
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<tbody>
<tr>
<td>1</td>
<td>Na-phosphate</td>
<td>Vacuum</td>
</tr>
<tr>
<td>2</td>
<td>Formaldehyde</td>
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<tr>
<td>3</td>
<td>Na-phosphate</td>
<td>Capillary</td>
</tr>
<tr>
<td>4</td>
<td>Formaldehyde</td>
<td>Capillary</td>
</tr>
</tbody>
</table>

Hybridization and detection conditions were identical for all blots. Blots 1 and 3, and 2 and 4 were visualized on the same film. Comparison of the blots shows that the capillary blot
DMT1 Regulation

was more efficient than vacuum blotting. Also Na-phosphate gels seem to give sharper bands than formaldehyde gels.

![Figure 4.2 Comparison of electrophoretic and transfer conditions. For blots 2 and 4: 1=1.79, 2=4.47, 3=8.93, 4=17.86, 5=26.79, 6=35.72, 7=44.65 µg total C. reinhardtii RNA. For blots 1 and 3: 1=1.45, 2=3.6, 3=7.27, 4=14.5, 5=21.8, 6=29, 7=36.4 µg total RNA. Northern blots were hybridized with the RbcS2 probe.](image)

4.3 Discussion

For murine Nramp1, tomato Nramp1 and E. coli and S. typhimurium MntH, regulation by transcriptional activators was shown (see 1.4.2 Nramp regulation). As a first step to study regulation of DMT1 expression we decided to check whether the DMT1 gene is transcribed differentially at varying culture conditions. Firstly, all the methods were optimized using an RbcS2 probe (the Rubisco gene is constitutively expressed and used as a control for expression studies). A DMT1 probe was generated under the same conditions but no signal was detected on the blot. Since DMT1 is a metal transporter, it is quite possible that its expression is induced when the metal conditions are below optimum. Therefore, EGTA was added to the medium prior to RNA extraction. However, the DMT1 mRNA was not visible. It could be that the transcript is very low abundant and thus below detection limit of this method. Membrane embedded proteins cannot be expressed to levels as high as cytoplasmic proteins, since this would interfere with membrane integrity.
Chapter 5

Transition metal transport in the green microalga *Chlamydomonas reinhardtii* – Genomic sequence analysis

Uptake and export systems play a major role in transition metal homeostasis. The objective of this study was to identify potential metal transport mechanisms in the green microalga *Chlamydomonas reinhardtii*. We concentrated on the four major transition metal transporter families found in plants and other organisms: the ZIP-, CDF- and Nramp-family, and the CPx-ATPases. Using the information available for these protein families we performed a comparative sequence analysis in the recently released genome of *C. reinhardtii*. By this approach we were able to identify members of all four transporter families (four ZIPs, one CDF, two CPx-ATPases, and five Nrpamps). These findings advance our current knowledge of the metal transport processes present in *C. reinhardtii*. In addition, by subsequent in silico splicing of the genomic sequence we obtained cDNA sequences which led to the identification of ESTs (expressed sequence tags) in the *C. reinhardtii* EST database. These identified ESTs will be valuable for the cloning and characterization of several metal transporters utilized by the alga.

5.1 Introduction

Transition metal ions play a significant role in cellular physiological processes. Along with their broad range of action, which makes them indispensable for cell survival, toxic effects of the same elements demand mechanisms that guarantee a subtly regulated metal homeostasis. A sophisticated system of uptake, storage and detoxification is at play that not only supplies the cell with essential metals, but also maintains the desired concentration balance of the various metallic elements. Research on metal transport has concentrated mainly on prokaryotic, but also on mammalian and plant systems. A vast pool of molecular data is available regarding uptake and efflux of transition metals in several organisms. Interestingly,
up to now algae have not been prominent in such research. Yet, like plants, this organism group is especially dependent on a precise regulation of metal balance. The photosynthetic process requires manganese for water splitting, and oxidative stress resulting from photosynthesis must be antagonized by metalloenzymes. In algae, general metal homeostasis has been studied at the molecular level [67, 77, 92], but information about metal transport is mainly based on physiological data [47, 63, 120]. Only recently, two metal transport systems have been identified and cloned in *C. reinhardtii* (multicopper ferroxidase Fox1/Flp, [61, 78] and iron permease Ftr1, [78]; divalent metal ion transporter DMT1, Rosakis A. and Köster W. in preparation). Two other proteins, transferrin in *Dunaliella salina* [41] and H43 in *C. reinhardtii* [112], were suggested to be involved in iron uptake.

In order to shed more light on the nature of the transition metal acquisition network in algae, we have conducted a search for potential homologues belonging to gene families known to be involved in metal transport (Fig. 5.1). For this, we performed a thorough sequence analysis of the genome of the model organism *Chlamydomonas reinhardtii*. *C. reinhardtii* is the first alga for which the entire genomic sequence is available (Doe Joint Genome Institute, California, [52]).
Figure 5.1 Schematic representation of four transition metal transporter families with approximate positions of conserved motifs. ZIP-family [46]: The histidine rich variable region and the conserved residues essential for IRT1 function are indicated. CDF-family [46]: The (HX)n motif and the metal binding motif are indicated. CPx-ATPases: A. thaliana PAA1 as an example [118]. The metal binding, phosphorylation and ATP binding domain, and the CPx motif are indicated. Nramp-family: For most Nramps 10 or 12 transmembrane domains are predicted (dark grey), but C. reinhardtii DMT1 is predicted to have 11 (light grey). The conserved transport motif (CTM) for C. reinhardtii DMT1 is indicated.
5.2 Materials and methods

ESTs (expressed sequence tags), which have the advantage of harboring the cDNA instead of the genomic sequence, are only partially available, with the published sequence often limited to the untranslated regions. This hampered identification of potential genes in an EST library, and similarity search directly in the C. reinhardtii EST database gave no results. Therefore, homologous genes from other organisms were firstly identified in the recently released C. reinhardtii genome (http://genome.jgi-psf.org/chlre1/chlre1.home.html [52]). The matrix for BLAST was BLOSUM62, a filter for low complexity was applied and an ungapped alignment was performed. Subsequently, corresponding ESTs were searched in the C. reinhardtii database (http://www.kazusa.or.jp/en/plant/chlamy/EST/blast.html; Kazusa DNA Research Institute, Japan, [4, 5]). For EST search we used the original genomic sequence as well as the sequence resulting after in silico splicing (http://opal.biology.gatech.edu/GeneMark/eukhmm.cgi, Eukaryotic GeneMark.hmm, Borodovsky M. and Lukashin A. unpublished) and translation. Still, it was not always possible to assign EST clones to selected sequences. This may be due to gaps in the genomic sequence, sequencing mistakes which would lead to false splicing, or truncation of the genomic sequence on the respective scaffold. Since the scaffolds are randomly numbered, it is not possible to reconstruct a sequence by stringing them together. The putative genes identified were provisionally named by the family name followed by the number of the sequence scaffold to which they belong. Only non-redundant ESTs are given.

Alignments were performed by ClustalW 1.8 (http://searchlauncher.bcm.tmc.edu/) (Tab. 5.1).
Table 5.1 Parameters for ClustalW 1.8 alignments

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5.3 Results

Table 5.2 gives a summary of all results.

In addition to our search as described in the materials and methods section, we performed a text search in the JGI website (http://genome.jgi-psf.org/cgi-bin/searchGM2.cgi?db=chlre1&dName=chlre) with the words Nramp, CDF, CPx-type ATPase, and ZIP. For Nramp and CDF no new sequences were identified by this search (Smith-Waterman Hits, all available databases). For CPx-type ATPase and ZIP there were also other hits besides the sequences identified by us, but with smaller scores. These were not investigated further in this study.
## Table 5.2 Overview of *C. reinhardtii* clones and putative transition metal transport systems

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\(^a\)only non-redundant ESTs are given

\(^b\)identical to ESTs BE761354 and BG844651 identified by [78] as a putative copper transporting ATPase
5.3.1 The ZIP-family (zrt, irt-like protein)

The ZIP family includes members from plants, protozoa, insects, mammals and fungi, as well as from eubacteria and archaea [37, 38, 45, 49, 51, 138] (and reviewed in [46, 56]). In the Arabidopsis thaliana genome 18 ZIP members were identified. IRT1 (iron regulated transporter 1) was the first ZIP identified [37] and is considered as the major iron uptake system from soil. Four other ZIP proteins in A. thaliana, ZIP1-4, may play a role in zinc transport [53]. Most ZIP proteins were predicted to possess eight transmembrane domains (TMs) with extracytoplasmic amino- and carboxyterminal ends [56] (Fig. 1). A particular feature of many ZIPS is a long cytoplasmic loop between transmembrane domains III and IV (called the “variable region”, because its sequence is not notably conserved, [56]), which accommodates many histidine residues. Since histidines are often involved in metal binding, this loop is a potential metal binding site of the proteins. In IRT1, residues important for function were mapped in TMs IV and V [110] (Fig. 5.1). ZIPs are divided into 4 subfamilies, according to sequence similarity [46].

We performed similarity searches with members of each of the 4 subfamilies (LIV-1, gufA, ZIP I and ZIP II, [46]). Similarity search in the C. reinhardtii genome with the A. thaliana ZIP1 (Acc. no. AAC24197), which belongs to subfamily ZIP I, revealed the existence of at least two genes possibly coding for proteins of the ZIP family. The sequence for ZIP77 is located in scaffold 77 contig 3 and for ZIP735 in scaffold 735 contig 2. EST AV630909 corresponds to ZIP735. No ESTs were found for ZIP77. Translation of the DNA sequence after in silico splicing yielded a good alignment for ZIP735 with S. cerevisiae Zrt1p and A. thaliana ZIP1 (Fig. 5.2). Translated ZIP77 contains regions which do not fit very well, possibly due to errors in the splicing. The high degree of conserved amino acid residues, including the four that are essential for function of IRT1 (His197, Ser198, His224, Glu228, [110]), places these putative genes among the ZIP family.

For the gufA subfamily, zupT from Escherichia coli (Acc. no. P24198, [51]) was used as an input. No similarities were found in the EST database, but two hits in the genomic database seem to correspond to ZIP homologues. Scaffold 162 and 1381 harbor sequences which are more similar to ZupT, especially at the second half of the amino acid sequence, than to ZIP1. ZupT, ZIP162 and ZIP1381 possess His224 and Glu228. Only ZupT and ZIP162 possess His197 as well, but the Ser198 is replaced by Asn.

We aligned 8 amino acid sequences of 8 ZIP homologues or putative homologues and generated a similarity tree for better visualization (Fig. 5.3). ZIP735 and ZIP77 are
Transition metal transport in C. reinhardtii positioned closer to *A. thaliana* ZIP1, while ZIP162 and ZIP1381 are more similar to *E. coli* ZupT.

The nucleic acid sequence of EST AV635915 is identical to part of the putative ZIP1381 DNA sequence. Interestingly, the in silico spliced ZIP1381 bears more similarity to ZupT on amino acid sequence level than the EST (not shown). It is possible that an intronic sequence is still present in the EST which was spliced out in silico. Another EST which matches ZIP1381 is AV389034.

We did not find a homologue in the LIV-1 subfamily when we searched with *S. cerevisiae* YIL023c (Acc. no. NP_012241) and with *Schizosaccharomyces pombe* (Acc. no. T39242) as an input. Search for ZIP II members produced also no hits. Here, a search was performed with *Homo sapiens* hZIP1 (NP_055252), *H. sapiens* hZIP2 (Acc. no. AAF35832) and *Caenorhabditis elegans* (Acc. no. T29592) hypothetical protein. In summary, *C. reinhardtii* possesses representatives of at least 2 ZIP subfamilies.
Figure 5.2 Alignment of *S. cerevisiae* Zrt1p, *A. thaliana* ZIP1 and two putative ZIP transporters in *C. reinhardtii*. Bars indicate the approximate position of transmembrane regions predicted for Zrt1p and ZIP1 [46]. Residues found to be essential for function of IRT1 [110] are shaded grey in the consensus sequence.
5.3.2 The CDF-family (cation diffusion facilitator)

CDF proteins constitute a family of metal cation transporters identified in bacteria, archaea and eukaryotes (reviewed in [46]). Certain members of the family are thought to function in uptake while others catalyze efflux. In the yeast *Saccharomyces cerevisiae* at least 5 CDFs have been identified. Zrc1p is involved in zinc and cadmium resistance [69]. The *A. thaliana* CDF homologue ZAT1 is expressed in all organs and is also involved in zinc resistance. Overexpression in plants can lead to enhanced zinc resistance and accumulation in roots, showing that zinc must be transported into a compartment (vacuole) in the root cells [129]. CDFs are divided into the 3 groups CDF I, CDF II and CDF III [46]. Subfamily I includes mostly eubacterial and archaeal proteins, while in II and III pro- and eukaryotic proteins are distributed equally. Both the N- and the C-terminus are predicted to be cytoplasmic with 6 transmembrane domains between them (Fig. 1). A long loop region is found between TMs IV and V, frequently containing a histidine-rich motif (HX)n (where n=3-6 and X often S or G). This loop is thought to act in metal binding as was shown for ZAT1 [11].
*A. thaliana* ZAT1 belongs to subfamily CDF III and produced one hit (scaffold 820) in the *C. reinhardtii* genome database. After in silico splicing and translation, the alignment with *S. cerevisiae* Zrc1p (Acc. no. NP_013970) and *A. thaliana* ZAT1 (Acc. no AAD11757) shows that CDF820 possesses several conserved residues found in proteins of the CDF family (Fig. 5.4). The putative metal binding motif \((H-D/E-X-H-X-W-X-L-T-X8-H)\) at the C-terminal tail is also conserved. The loop described for CDFs between transmembrane domains IV and V which contains the motif \((HX)n\) is also found in CDF820. Errors in the assignment of splice junctions at this region are possible since the loop is much longer than that of the other homologues. Only one EST was identified for CDF820, but with several base mismatches and only partial alignment (AV395632).

In subfamily CDF I, scaffold 2650 showed similarity to a putative protein of *A. thaliana* (Acc. no. CAB67634), which was included in the CDF family in the review of Gaither and Eide [46]. However, a two sequence alignment between ZAT1 and the putative protein CAB67634 showed that the metal binding motif is not conserved in CAB67634. Since this protein has not been functionally characterized yet, a role as a metal transporter for CDF2650 must be interpreted with caution. Notably, CDF2650 did not appear as a hit in the text search in the JGI website. No homologues were identified in subfamily CDF II with CzcD from *Bacillus subtilis* (Acc. no. NP_834116) and ZitB from *E. coli* (Acc. no. Q8X400) as input. Thus, at least 1 of the 3 CDF subfamilies is represented in the *C. reinhardtii* genome.
### Figure 5.4 Partial alignment of *C. reinhardtii* CDF820 with *A. thaliana* ZAT1 and *S. cerevisiae* Zrc1p.

The putative metal binding motif (H-D/E-X-H-X-W-X-L-T-X8-H) is boxed, with the conserved amino acids indicated by a star above the sequence. Transmembrane domain V is indicated by a line above the sequence [46].

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Transmembrane domain V is indicated by a line above the sequence.

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82
5.3.3 The family of CPx-type ATPases

CPx-type ATPases have been identified in a wide range of organisms and are assumed to transport essential and potentially toxic metal cations (e.g. Cu$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Pb$^{2+}$). They belong to the class of P-type ATPases, which pump charged substrates across biological membranes at the expense of ATP (reviewed in [135]). Plant CPx-ATPases homologues are PAA1 [118, 122], PAA2, RANI and HMA5. PAA1 is thought to be localized in the chloroplast inner envelope membrane and to supply the Cu/Zn superoxide dismutase with copper ions via a stromal metallochaperone [118]. The divalent metal cations P-type ATPases were defined as CPx-ATPases due to a conserved intramembranous cysteine-proline-cysteine/histidine/serine (CPx) motif which is thought to function in metal cation transduction [119]. Other conserved motifs are the ATP-binding motif GDGxNDx and the phosphorylation motif DKTGTLT, found in the large cytoplasmic domain between transmembrane domains VI and VII. These two motifs are conserved amongst all P-type ATPases. The metal binding motif GMTCxxC is found in one or several copies at the N-terminal part of the protein (Fig. 5.1).

tBLASTn with A. thaliana CPx-type ATPase PAA1 (Acc. no. Q9SZC9) showed that at least two potential homologues exist in C. reinhardtii (Fig. 5.5). CPx1184 is located in scaffold 1184, contigs 2, 3 and 4. It is shorter than expected, possibly due to false splicing which causes a premature stop. One EST was identified for this putative gene (AV636551). CPx89 is located in scaffold 89 contigs 3 and 4. Part of CPx89 DNA sequence is identical to the ESTs BE761354 and BG844651 previously identified as a copper transporting ATPase [78]. We identified several ESTs for CPx89: AV387187, BE129393, BE761353 and BE761354. Both putative CDF homologues possess motifs conserved in CPx-ATPases (CPx, ATP-binding, phosphorylation, metal binding).
Figure 5.5 Alignment of PAA1 and RAN1 from *A. thaliana* with *C. reinhardtii* CPx1184 and CPx89. Black lines indicate functional domains [118] and stars above the sequence indicate the conserved GMxCxxC motif. The CPx motif is marked by a white bar above the sequence.
5.3.4 The Nramp-family (natural resistance associated macrophage proteins)

Nramps are highly conserved metal transporting proteins with a broad specificity. They are widely distributed and occur in many organisms from bacteria to man [18, 72, 79, 82, 103, 123, 127]. In the *A. thaliana* genome several Nramp homologues were found. AtNramp1, 3 and 4 contribute to manganese, iron and cadmium transport as was shown by yeast complementation assays [30, 127]. Previous to the release of the genomic sequence, we had cloned and functionally characterized an Nramp homologue in *C. reinhardtii* (DM1, Acc. no. AF515631, Rosakis A. and Köster W. in preparation). Nramps are predicted to consist of 10 or 12 transmembrane domains, while DMT1 from *C. reinhardtii* is predicted to possess 11 (Fig. 5.1). A remarkable feature of Nramps is the presence of a highly conserved motif in the cytoplasmic loop between transmembrane domains VIII and IX, often referred to as the “consensus transport motif” (GQNSTITGTLSGQIVMEGF for DMT1). The function of this motif is not clear yet.

A search for Nramp homologues in the genome of *C. reinhardtii* revealed another four putative Nramp genes apart from the cloned DMT1 (scaffold_489, contig 3, base pairs 30332:34793). Nramp579, located in scaffold 579 contig 7, codes most probably for a complete open reading frame (ORF). The putative amino acid sequence was determined by in silico splicing and translation. Some regions were corrected by hand after comparison with the DMT1 sequence because the program had chosen the false reading frame. Alignment with DMT1 showed that Nramp579 possessed the most conserved regions of Nramps over the largest part of the sequence (Fig. 5.6). The N-terminus is not possible to determine as it is the most variable part of the Nramp proteins and the correct reading frame cannot be defined. Two ESTs were identified identical to Nramp579 (BE724331, BE337524). BLAST with Nramp579 showed that the potential partial amino acid sequence of this Nramp homologue is more similar to prokaryotic than to eukaryotic Nramps (not shown), as already shown for DMT1 (Rosakis A. and Köster W. in preparation).

Other sequences similar to Nramps are located in scaffold 3557 contigs 1 and 2, scaffold 672 contig 1 and scaffold 2132 contig 1. Gaps and truncation of the genomic sequence in the scaffolds do not allow identification of the complete sequences of these putative homologues and we cannot exclude the possibility that Nramp672 and Nramp2132 or Nramp672 and Nramp3557 belong to the same ORF. Only for Nramp3557 one EST (AV628920) was identified.
Alignment of the 5 *C. reinhardtii* Nramp homologues shows that the sequence motifs conserved in most Nramps are also present: DPGN in Nramp579, 2132 and 3557; MPH in Nramp579, CTM-motif in Nramp579 and 672 (Fig. 5.6).

**5.3.5 Further genes involved in metal transport**

The green microalga *Dunaliella salina* was the first organism outside the animal kingdom in which a transferrin homologue was identified [40]. Transferrins are iron binding proteins involved in iron transport. Hints that transferrins exist also in other algae are given by kinetic characterization and antibody detection [41]. In *C. reinhardtii* we did not identify any genomic or EST sequence which bears similarity to plasma membrane bound transferrin from *D. salina* (Acc no. T10729). Further, we looked into the COPT family of copper transporters recently identified in *A. thaliana* [70, 116]. Five homologues are present in the genome of the thale cress (COPT1, AF466373; COPT2, AF466370; COPT3, AF466371; COPT4 AF466372; COPT5, AF466374). With each one of them we performed similarity search (tBLASTn and BLAST) in the EST as well as in the genomic library of *C. reinhardtii*. The search produced no results, indicating that specific copper transport in *C. reinhardtii* may be mediated by another protein family.

**Figure 5.6** Alignment of *C. reinhardtii* DMT1 and putative Nramp homologues. The potential sequence for Nramp579 was derived from the genomic DNA by in silico splicing and translation and some correction by hand. Other sequences were derived by in silico splicing and translation. The alignment was performed with ClustalW1.8 and corrected by hand. Identical residues are highlighted black and the CTM motif is boxed. In the consensus sequence stars mark identical residues.
Transition metal transport in C. reinhardtii

consensus

DMT1

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5.4 Discussion

In order to obtain more molecular information on the transport processes for transition metals in algae, we performed a comparative sequence analysis in the genome of the green microalga *Chlamydomonas reinhardtii*. By BLAST search we identified putative members in four transporter families: four ZIPs, one CDF, two CPx-ATPases, and five Nramps. We present alignments of the newly identified sequences with already characterized homologues from *A. thaliana* and *S. cerevisiae*, which show that the sequence motives typical for these protein families are also represented in the putative *C. reinhardtii* homologues. By this, the probability that the *C. reinhardtii* sequences encode proteins functional in metal transport is increased. Since the precise sequence of the identified open reading frames cannot be confirmed at this point, alignments must be interpreted with caution, serving mainly as a good indication for the existence of potential metal transporter homologues. Still, we now have an overview on some of the main metal transport systems in *C. reinhardtii*. With the raw or in silico spliced genomic sequences we performed a search in the EST database of *C. reinhardtii* and identified ESTs corresponding to the putative metal transporters (Tab. 5.2).

It is not surprising that the alga may possess less homologues than the plant *A. thaliana*, since in multicellular organisms we often find many homologues of the same family expressed in different tissues. It is interesting to speculate that the Nramp family might be represented by five homologues, since specificity of these transporters is generally considered to be quite broad. The reason for this redundancy may be found in a different localization or expression pattern of these potential proteins, as is the case in *S. cerevisiae*. In the yeast the Nramp homologue Smf1p is localized in the plasma membrane, while Smf2p is expressed in the membrane of intracellular vesicles and Smf3p in the vacuolar membrane [84, 86, 103].

The results of this study advance our current knowledge of the metal transporter network present in *C. reinhardtii* and facilitate cloning of selected metal transporter genes for further investigation and characterization. Where EST clones were identified, the cloning procedure would be based on RT-PCR (reverse transcription-polymerase chain reaction) and RACE (rapid amplification of cDNA ends). Otherwise, amplification using genomic DNA or hybridization of a cDNA library might be the method of choice.

Acknowledgement

The genomic sequence data were produced by the US Department of Energy Joint Genome Institute, http://www.jgi.doe.gov/ and are provided for use in this publication only.
Chapter 6

Conclusions and perspectives

Algae are by far the most important oxygen producers on earth and it is of great interest to find out how these photosynthetic organisms handle varying metal concentrations in their environment. The lack of molecular data regarding transition metal transport in algae gave the impulse for this work. *Chlamydomonas reinhardtii* is a green microalga suitable for laboratory work and served as a model organism in the study of metal transport systems at the molecular level. It should be pointed out here that, although *C. reinhardtii* is quite versatile in laboratory work, the major drawback – the lack of a reliable gene knock-out system – hampers research considerably. Thus, after identification and cloning of DMT1 I studied the function of the protein in a heterologous system by yeast complementation assays, which is a well accepted method to study function of plant transport proteins [22].

DMT1 acted as a metal transporter with broad specificity. In the native organism, the alga itself, specificity may be narrower, provided by regulation of gene expression. Thus, study of the regulation may prove useful in order to further characterize the role of the protein in the cell. My preliminary results from northern blot experiments – aiming to elucidate potential transcriptional regulation of the gene – showed that the DMT1 transcript is not abundant and not detectable in the hybridization system, in contrast to the transcript of the control gene which was detectable. Treatment of the cultures with the metal chelator EGTA and sampling at several time points after the treatment did also not allow detection of the DMT1 mRNA.

It was shown that growth of *C. reinhardtii* in iron depleted medium (-Fe) was equal to growth in medium with combined iron and copper deficiency (-Fe/-Cu, where Cu was 0 μM) [78]. Since the high affinity iron uptake system FOX1/FLP-FTR1 requires copper for function, it is plausible that a backup iron uptake mechanism must exist when copper is limited [78]. I expect the backup for the FOX1 system to be rather a high affinity system, since growth is not compromised at all when Cu (and thus FOX1) is absent [78]. Measurements of the metal content of algal cells subjected to different metal conditions suggested that an unspecific metal uptake system is functioning in iron uptake when copper
Conclusions and perspectives

(and thus FOX1) is limiting [61]. However, the authors referred to a low affinity system, as was shown by iron uptake measurements, and not to a high affinity system. Thus, clarification is needed concerning the affinity of the suggested backup iron uptake system, by measuring growth and iron uptake activity in the same experiment. DMT1, being able to transport iron, as was shown by the complementation of the yeast strain deficient for high affinity iron transport (fet3/fet4), might function as this backup iron uptake system. Whether it displays a high or low affinity towards divalent metal ions, needs to be demonstrated. DMT1 also has a broad specificity, a feature described for the alternative iron uptake system [61]. However, it might be more sensible if the backup uptake system was specific. That way iron assimilation under combined iron and copper limitation would be more efficient without increasing the cellular concentration of other metals as well. Silencing of DMT1 by RNA interference (RNAi) and subsequent growth and uptake experiments would definitely give much information on its actual role in the cell. A role as a transition metal ion uptake system with low affinity and low specificity is by all means possible.

The eventuality of five Nramp homologues present in a unicellular organism (which cannot be explained by tissue specific expression) is at least unusual, especially considering the broad specificity which is described for most Nramps. Only two homologues have been identified so far in humans and three in S. cerevisiae. While one would expect all C. reinhardtii Nramps to have the same function, i.e. metal transport, the role of each homologue in the cell might be quite different. Localization experiments with the putative homologues may shed some light on this question. Directed gene silencing of each homologue, e.g. by RNAi, is a promising alternative to yeast complementation assays, with the advantage of studying the protein function in the native organism. Yet, the method is inappplicable for generation of mutated proteins. Thus, identification of specific sites on the Nramp protein essential for function, specificity and regulation might be better performed in a “cleaner” system, like S. cerevisiae where the genetic background can be defined and the gene of interest precisely targeted without the drawbacks of multiple insertions or deletions. Alternatively, the homologues could be compared in their function by overexpression in yeast, followed firstly by complementation assays and secondly by metal uptake assays and competition experiments. Thereby, differences in the affinity of each protein may be detected giving a lot of information on their role together with the localization experiments. Regarding the identified members of other transporter families, the characterization procedure could follow a similar route.
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