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# Catch me if you can! Novel aspects of cadmium transport in mammalian cells

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**Abstract** Cadmium ($Cd^{2+}$) is a nonessential divalent metal ion that causes toxicity in multiple organs in humans. In order for toxicity to occur $Cd^{2+}$ must first enter cells by utilizing transport pathways for essential metals. This review focuses on studies in which $Cd^{2+}$ transport was directly demonstrated by electrophysiological, radiotracer or $Cd^{2+}$-sensitive fluorescent dye techniques. The chemistry of $Cd^{2+}$ and metal ions in general is addressed in the context of properties relevant for transport through membrane proteins, such as hydration energy. Apart from transport by the ZIP transporters SLC39A8 and SLC39A14, which is not topic of the review, uptake of free $Cd^{2+}$ has been demonstrated for the $Fe^{2+}/H^{+}$ cotransporter divalent metal transporter 1. Moreover, the multiligand endocytic receptors megalin and cubilin take up cadmium-metallothionein complexes via receptor-mediated endocytosis. The role of ATP binding cassette transporters in $Cd^{2+}$ efflux from cells is also discussed. Both the multidrug resistance-associated protein 1 and cystic fibrosis transmembrane conductance regulator are likely to transport cadmium–glutathione complexes out of cells, whereas transport of free $Cd^{2+}$ by the multidrug resistance P-glycoprotein remains controversial. Finally, arguments for and against $Cd^{2+}$ transport by $Ca^{2+}$ channels are presented. Most N- and L-type $Ca^{2+}$ channels are closed at resting membrane potential (with the exception of CaV1.3 channels) and therefore unlikely to allow significant $Cd^{2+}$ influx under physiological conditions. CaV3.1 and CaV3.2 T-type calcium channels are permeated by divalent metal ions, such as $Fe^{2+}$ and $Mn^{2+}$ because of considerable "window" currents close to resting membrane potential and could be responsible for tonic $Cd^{2+}$ entry. TRPM7 and the mitochondrial $Ca^{2+}$ uniporter are other likely candidates for $Cd^{2+}$ transporters, whereas the role of Orai proteins, the store-operated calcium channels carrying $Ca^{2+}$ release-activated $Ca^{2+}$ current, in $Cd^{2+}$ influx remains to be investigated.

**Keywords** Calcium channels · ABC transporters · Standard enthalpy of hydration · 24p3/NGAL/lipocalin-2 receptor · Epithelial transport

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## Introduction

### Organ toxicity of Cd

One of the major targets of Cd-induced damage is the kidney where Cd accumulates in the proximal tubule.

Up to 7% of populations exposed to $Cd^{2+}$ develop renal dysfunction (Jarup and Akesson 2009; Nordberg 2009). It is associated with a general transport defect of the proximal tubule that mimics the *de Toni-Debré-Fanconi-Syndrome* (Bernard et al. 1979) with proteinuria, aminoaciduria, glucosuria and phosphaturia (for review, see Wedeen and De Broe 1998). Other diseases associated with Cd exposure are pulmonary emphysema, diabetic and renal complications, deregulated blood pressure, immunosuppression and bone disorders (the Itai–Itai disease) (ATSDR 2005; Jarup and Akesson 2009; Nordberg 2009). Most importantly, environmental exposure to Cd increases total and non-cardiovascular mortality (Nawrot et al. 2008).

### $Cd^{2+}$ carcinogenesis

In 1993 the International Agency for Research on Cancer (IARC), which is part of the World Health Organization, classified Cd and Cd compounds as group-1 human carcinogens, having concluded that there was sufficient evidence of cadmium being carcinogenic to humans and animals (IARC 1993). The evidence for carcinogenicity in humans is supported by substantial recent epidemiologic evidence indicating that Cd induces cancer in many organs in humans, including the lung and the kidneys (Pesch et al. 2000; Hu et al. 2002; Hengstler et al. 2003). Moreover, in a prospective population-based study on environmental exposure to Cd and risk of cancer (Nawrot et al. 2006) demonstrated that chronic environmental Cd exposure is associated with development of lung cancer. Even though the carcinogenicity and toxicity of Cd has long been recognized, accumulating epidemiological evidence suggests significant toxicity even on long-term low exposure conditions and affecting more people than previously thought (see in particular Satarug and Moore 2004; Jarup and Akesson 2009, for review).The present consensus is that a direct mutagenic effect of Cd is weak (Waalkes 2003), but is presumably sufficient to induce tumors if combined with other pro-carcinogenic effects of Cd, such as formation of ROS and/or interference with anti-oxidative enzymes, inhibition of DNA repair enzymes, deregulation of cell proliferation, interference with the balance between pro and anti-apoptotic mechanisms, and disruption of cell adhesion (Waisberg et al. 2003).

### Biology of $Cd^{2+}$ toxicity

Cd plays no role as a trace element and is therefore a toxic-only element with no known physiological function, with one exception: in the absence of $Zn^{2+}$, $Cd^{2+}$ is used by some diatoms in the active site of carbonic anhydrase (Lane and Morel 2000). The major toxic form of Cd in eukaryotic cells is the $Cd^{2+}$ ion. Although $Cd^{2+}$ is not a Fenton metal and therefore not capable of exerting redox reactions in biological systems, it may induce formation of reactive oxygen species (ROS) by depletion of endogenous redox scavengers, inhibition of antioxidative enzymes, inhibition of the mitochondrial electron transport chain, and/or displacement of redox active metals, such as $Fe^{2+}$ or $Cu^{2+}$, from their carrier proteins and thereby cause mitochondrial damage and trigger apoptosis, to name few of the mechanisms underlying $Cd^{2+}$ toxicity (reviewed in Thévenod 2009). $Cd^{2+}$ can also substitute for $Ca^{2+}$ in cellular signaling or for $Zn^{2+}$ in many enzymes and transcription factors which may account for some of the biological effects of the $Cd^{2+}$ ion.

## Physico-chemical characteristics of $Cd^{2+}$: is there more to it than old wine in new skins?

In order to better predict the behavior of transport of free $Cd^{2+}$ by ion channels and transporters in mammalian organisms some physico-chemical properties of metal ions need to be considered with a particular emphasis on (1) their enthalpy of hydration and (2) their classification in "soft" and "hard" metal categories. Metal ions interact with water molecules to form water shells around the ion, a process called solvation. This ionic hydration energy is a significant barrier for metal ions crossing membrane protein pores, e.g., in ion channels, by slowing the dehydration process (Hille 1992). Generally speaking, cations have higher hydration energies (are more hydrated) than the anions of the same negative charge, and the energies are highest for small ions (e.g., $Mg^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Zn^{2+}$) and for ions with large ionic charge. The standard enthalpy of hydration $\left(\Delta H^{o}_{hydration}\right)$ reflects the residence time of water molecules near an ion (Edsall and McKenzie 1978; Marcus 1985) and is inversely proportional to the "water substitution rate" around an ion (Diebler et al. 1969). This concept may

help to understand the process of permeation in ion channels, where ions move by frequent replacements of water molecules in the inner shell with dipolar groups of the channel pore. The relatively high $\Delta H^{o}_{hydration}$ respective low water substitution rate of water molecules around divalent metals, such as $Ni^{2+}$, $Co^{2+}$, $Mg^{2+}$, $Mn^{2+}$ and $Cd^{2+}$ is one factor that may contribute to their differential behavior as "blockers" in contrast to "permeators", such as $Ca^{2+}$, $Sr^{2+}$ or $Ba^{2+}$.

The separation of metals into "hard" and "soft" classes is based on thermodynamic measurements of metal-ion/ligand complex stability (Jacobson and Turner 1980; Nieboer and Richardson 1980). Based on these criteria, as a rule of thumb free metal ions can be divided into 3 groups: hard, soft and borderline. "Hard" or "class A" metals include alkaline earth metals, lanthanoids and aluminum, to name a few. They do not readily share their electron density and bind mainly by electrostatic interaction with electron donors such as $F^-$ or O. "Soft" or "class B" metals include transition metals, such as $Au^+$, $Cu^+$, $Ag^+$ or $Hg^{2+}$. Those metals have a polarizable electron cloud. Thus, they participate more easily in electron sharing and bind easier via covalent interactions. Soft cations bind to inorganic anions, such as $I^-$ and $SCN^-$, or in organic molecules to sulfur (sulfhydryl, disulfide, thioether), or nitrogen (amino, imidazole, histidine, nucleotide base). Borderline cations are able to form stable complexes with ligands, but they may also display some degree of "softness". Among the latter are divalent cations, such as $Pb^{2+}$, $Cu^{2+}$ and $Cd^{2+}$ whereas $Mn^{2+}$, $Zn^{2+}$ and $Ni^{2+}$ have a higher degree of "hardness" and $Co^{2+}$ and $Fe^{2+}$ show intermediate features.

## $Cd^{2+}$ transport pathways: a focus on likely candidates and popular but unproven candidates

To develop toxicity $Cd^{2+}$ ions present in solutions must cross cellular membranes via proteinous pathways (such as receptors, transporters and pores) due to their hydrophilicity. $Cd^{2+}$ may be taken up from solutions across cell membranes by active (energy-dependent) or passive (facilitated diffusion) transport. Energy-dependent mechanisms include ATPase mediated efflux systems and receptor-mediated endocytosis (RME) of metal complexes whereas passive transport involves channels and carriers. Once in- or outside the cell, exchange to stronger ligands may take place, thereby preventing back diffusion and forming a kinetic trap for the metal. The similar chemical properties of $Cd^{2+}$ and essential metals determine its entry into mammalian cells. Target cells can only be damaged by $Cd^{2+}$ if they possess transport pathways or receptors with an affinity for the toxic metal, i.e., either free ions or as complexes with a carrier. To describe this process the term "ionic and molecular mimicry" has been coined (Clarkson 1993; Bridges and Zalups 2005). In fact, it has been suggested that transporters and receptors for free and complexed forms of essential metals, such as $Ca^{2+}$, $Fe^{2+}$, $Zn^{2+}$ or $Cu^{2+}$, mediate uptake of $Cd^{2+}$. Because of its ability of complex formation with organic molecules $Cd^{2+}$ is also likely to be transported by pathways for transport of organic molecules rather than as a free ion. Nevertheless, transporters for free essential metal ions have recently been identified that can also carry free $Cd^{2+}$. For a recent assessment on $Zn^{2+}$ transporters for which direct evidence for $Cd^{2+}$ uptake into cells has been provided I would like to refer to the review by D. W. Nebert and coworkers (He et al. 2009), which gives an update of $Cd^{2+}$ transport by ZIP8 (SLC39A8) and ZIP14 (SLC39A14) transporters in kidney, intestine and testis ((Dalton et al. 2005; He et al. 2006; Girijashanker et al. 2008; Liu et al. 2008). A $K_m$ of ZIP8 for $Cd^{2+}$ of $\sim$0.5 μM was determined using $^{109}Cd^{2+}$ (Liu et al. 2008) which indicates that ZIP8 is a candidate for high-affinity $Cd^{2+}$ transport mediating $Cd^{2+}$ toxicity in mammalian cells (together with DMT1/NRAMP2/SLC11A2; see below). The known mammalian copper influx carriers Ctr1/2 and efflux ATPases ATP7A/B have been reviewed recently (Kaplan and Lutsenko 2009). No evidence is currently available to suggest that $Cd^{2+}$ is a substrate of any of the copper transporters, which is most likely due to their use of monovalent $Cu^+$ as a substrate.

This review focuses on novel aspects of eukaryotic $Cd^{2+}$ transport by uptake pathways for $Fe^{2+}$. Moreover, the role of ATP binding cassette transporters (pumps and channels) in $Cd^{2+}$ efflux will be discussed. Finally, "pros" and "cons" of $Cd^{2+}$ transport by $Ca^{2+}$ channels will be presented. The review of the literature will include transporter studies in which $Cd^{2+}$ transport was directly demonstrated by

electrophysiological techniques (patch-clamp) or studies using $^{109}Cd^{2+}$ or $Cd^{2+}$-sensitive fluorescent dyes rather than studies that merely correlated development of $Cd^{2+}$ resistance with up-regulation of transporter proteins to deduce evidence for $Cd^{2+}$ transport. The aim of this "conservative" approach is to emphasize the significance of proven $Cd^{2+}$ transporters for $Cd^{2+}$ toxicity rather than elusive speculations. $Cd^{2+}$ is known to trigger a variety of pro-and anti-apoptotic signaling cascades (see (Thévenod 2009) for a recent review on the topic) which elicit activation of transcription factors and target genes, particularly survival genes. Hence up-regulation of certain transporters may be a consequence of $Cd^{2+}$ resistance to counteract and/or interfere with pro-apoptotic signaling pathways and could be independent of $Cd^{2+}$ transport.

## $Fe^{2+}$ uptake pathways: a likely entry route for $Cd^{2+}$

The majority of iron in mammalian organisms is bound to proteins involved in oxygen transport and electron transfer as well as iron binding-proteins, such as transferrin (Tf), lactoferrin and ferritin (Jurado 1997; Bullen et al. 2005; Zasloff 2007). Under iron-limiting conditions, such as occur during infection of the host, microorganisms (e.g., bacteria and yeast) synthesize and secrete "siderophores" (Miethke and Marahiel 2007). These very high-affinity iron-binding compounds (Neilands 1981; Verma et al. 1991) scavenge iron from the host iron-binding proteins. To combat this microorganism salvage process, host cells, above all immune cells but also hepatocytes, intestinal enterocytes and renal tubular cells, express and secrete a protein, lipocalin 24p3 (also known as lipocalin-2 or uterocalin in mice and neutrophil-gelatinase-associated lipocalin [NGAL] in humans), which captures the bacterial siderophore and thereby prevents bacterial iron uptake (Goetz et al. 2002; Flo et al. 2004; Jayaraman et al. 2005; Mori et al. 2005; Playford et al. 2006). Then host cells take up the NGAL/lipocalin-2:siderophore:iron complex via RME and thereby induce siderophore depletion from body fluids. Two cell surface receptors of very different molecular structure mediate RME in epithelial cells, megalin/cubilin (Hvidberg et al. 2005) and a novel receptor (24p3R) for lipocalin 24p3, a protein that was originally referred to as brain organic cation transporter (Devireddy et al. 2005).

### Megalin and cubilin

Megalin and cubilin are multiligand, endocytic receptors with significant physiological functions (Christensen and Birn 2002). Both receptors are expressed primarily in polarized epithelial cells, but also in neurons. With a few exceptions, they are located in luminal plasma membranes of epithelial cells. Megalin and cubilin are structurally very different, and each binds distinct ligands with different affinities, serving independent functions in some tissues. However, the receptors are also co-expressed in several tissues, where they interact for function. Indeed, internalization of several cubilin ligands is strongly facilitated by megalin. Megalin and cubilin are highly expressed in the apical plasma membrane and the early stages of the endocytic pathway in the renal proximal tubule (PT), where they are responsible for tubular clearance of filtered proteins (Verroust et al. 2002; Christensen and Gburek 2004). Following binding to these receptors, ligands are internalized into coated vesicles and delivered to early and late endosomes. Whereas the receptors are recycled to the apical membrane, the ligands are transferred to lysosomes for protein degradation.

Though it has been commonly assumed that Tf with a molecular weight of 78 kDa is above the filtration cut-off for glomerular sieving theoretically it can pass through the glomerular filter (see Smith and Thévenod 2009 for a review). Moreover, apical PT uptake of Tf has been demonstrated in vivo in rats (Ohno et al. 2005) and in the rat PT cell line WKPT-0293 Cl.2 (Abouhamed et al. 2006) suggesting that PT cells reabsorb filtered Tf-bound iron via RME. Most importantly, compelling experimental evidence by Kozyraki and collaborators (Kozyraki et al. 2001) support glomerular filtration of Tf and its subsequent reabsorption by megalin and cubilin mediated RME. This observation is of utmost importance for the understanding of uptake of $Cd^{2+}$ complexed to metallothionein (CdMT), a major form of $Cd^{2+}$ present in the blood circulation (Andersen 1984). CdMT is easily filtered by the glomerular sieve because of its molecular weight of $\sim$7 kDa and may be reabsorbed by the PT by a mechanism analogous to Tf uptake. CdMT has been suggested to play a

major role as a source of $Cd^{2+}$ in nephrotoxicity induced by chronic exposure to $Cd^{2+}$ (Andersen 1984; Klaassen et al. 1999; Erfurt et al. 2003; Klaassen et al. 2009). In fact, several reports have recently demonstrated that CdMT partly utilizes the same RME pathway as Tf for uptake by renal PT cells that is mediated by megalin and cubilin.

Indirect evidence had suggested that megalin is implicated in the uptake of CdMT complexes: In rats, PT uptake of CdMT and $\beta$2-microglobulin is mutually inhibitory (Bernard et al. 1987), and megalin is a receptor for $\beta$2-microglobulin in renal PT cells (Orlando et al. 1998). This hypothesis has been subsequently corroborated by studies demonstrating binding of MT to megalin (Klassen et al. 2004). Subsequently, we studied megalin expression and dependence of CdMT internalization and cytotoxicity on megalin in a renal PT cell model (WKPT-0293 Cl.2 cells) derived from the S1-segment of the rat kidney PT (Wolff et al. 2006). First, expression of megalin and cubilin was detected and second, internalization of fluorescent AlexaFluor®488-coupled MT could be demonstrated, which was concentration-dependent, saturating at about 15 μM. At this concentration MT uptake could be significantly attenuated by ~30% by 1 μM of the receptor-associated protein (RAP) used as a competitive inhibitor of CdMT binding to megalin and cubilin. Consistently, cytotoxicity by CdMT (50 μM $Cd^{2+}$:7 μM MT) was significantly reduced by the presence of RAP, by a functional anti-megalin antibody which competes with megalin ligands, or by the cubilin-specific ligand, apo-transferrin (Wolff et al. 2006). Hence renal PT CdMT uptake and cell death are mediated, at least in part, by megalin and cubilin. The significance of megalin/cubilin mediated RME for CdMT toxicity in PT cells was further confirmed by investigating the role of the small GTPase ARF6 (ADP-ribosylation factor 6), which is involved in internalization of ligands by RME and endocytic vesicular trafficking in rat PT cells (Maranda et al. 2001; El-Annan et al. 2004). Transfection of a dominant-negative form of ARF6 prevented recycling of megalin to the cell surface. Accordingly, RME of CdMT was reduced as evidenced by a decrease of AlexaFluor®488-coupled MT uptake and CdMT toxicity in PT cells (Wolff et al. 2008).

Though megalin and cubilin are also expressed in intestinal epithelial cells (and other epithelia; Kozyraki et al. 2001; Bose et al. 2007) uptake of Tf and other iron-binding proteins via these receptors has not been investigated so far and their role in intestinal CdMT uptake remains also to be investigated.

## The 24p3/NGAL/lipocalin-2 receptor

The moderate contribution of megalin/cubilin in CdMT uptake in cultured PT cells could be accounted for by the fact that their megalin expression is low compared to native kidney. Still 50–70% of Alexa Fluor® 488 conjugated MT uptake and CdMT mediated cell death is not blocked by megalin or cubilin ligands (Wolff et al. 2006), suggesting that other pathways may mediate CdMT uptake in the PT cell line and in vivo, such as megalin- and cubilin-independent, clathrin-mediated endocytosis or fluid-phase endocytosis pathways (Carvou et al. 2007). Moreover, additional receptors could contribute to CdMT RME and toxicity in the PT, such as the recently cloned neutrophil-gelatinase associated lipocalin-2 (24p3/NGAL) receptor, which binds mouse 24p3 (a eukaryotic protein which delivers iron to cells by binding siderophores) with a $K_d$ of ~100 pM and is expressed in a variety of tissues, including kidney, intestine and liver (Devireddy et al. 2001, 2005). Its distribution within each of these organs, however, remains to be investigated. Overexpression of 24p3 receptor in HeLa cells induces binding and uptake of NGAL (Devireddy et al. 2005), yet the specific expression of this receptor in NGAL target cells and the requirement for this receptor to mediate NGAL responses in vitro or in vivo remain to be determined. Coincidently, megalin binds NGAL of human origin, but with a much lower affinity ($K_d$ of ~60 nM) and mediates its cellular uptake (Hvidberg et al. 2005). As a first step towards investigating the potential role of this endocytic pathway in CdMT cytotoxicity, we have demonstrated by RT-PCR that WKPT-0293 Cl.2 cells express both the lipocalin-2 receptor as well as its lipocalin-2 ligand (N.A. Wolff, W.-K. Lee & F. Thévenod, unpublished observations). In addition, co-incubation with recombinant rat lipocalin-2 (500 pM) partially restores cell viability of WKPT-0293 Cl.2 cells exposed to CdMT (20 μM $Cd^{2+}$:2.9 μM MT) for 24 h. This concentration is much lower than the $K_d$ of ~60 nM observed for binding of NGAL to megalin (Hvidberg et al.

2005), suggesting that lipocalin-2 competes with CdMT for binding to the lipocalin-2 receptor. These observations are currently being investigated in more detail in my laboratory.

## Divalent metal transporter 1 (DMT1, DCT1, NRAMP2, SLC11A2)

In 1997, Hediger and colleagues identified the first mammalian iron transporter protein DMT1 (or divalent cation transporter DCT1 as it was termed by the authors because of its ability to transport a variety of divalent transition metals including $Fe^{2+}$) by expression cloning (Gunshin et al. 1997). DMT1 is a ferrous ion ($Fe^{2+}$) transporter that is energized by the $H^+$ electrochemical potential gradient and ferric ion ($Fe^{3+}$) is excluded (Gunshin et al. 1997; see, however (Mackenzie et al. 2006) for a discussion of $H^+$-independent $Fe^{2+}$ transport). Soon after, Fleming et al. discovered that Belgrade rats (b/b) and *mk* mice carry a DMT1 G185R mutation that leads to deficiency in $Fe^{2+}$ uptake and causes microcytic anaemia, confirming that DMT1's major function is $Fe^{2+}$ transport (Fleming et al. 1998; Su et al. 1998). Humans with DMT1 mutations at different intron or exon sites, such as an E399D substitution, a R416C substitution or a G212V substitution also exhibit hypochromic microcytic anemia, which confirm the crucial role of DMT1 function in $Fe^{2+}$ homeostasis amongst various mammalian species (Iolascon and De Falco 2009).

At the tissue level, DMT1 is ubiquitously expressed, most notably in the proximal duodenum, red blood cells, macrophages, but also in the kidney and brain (Gunshin et al. 1997; Lee et al. 1998). The sites where DMT1 is most critical for iron homeostasis are the intestinal tract (where it is clearly the major transporter for iron entry) and the red blood cell precursor (where it is essential for full hemoglobinization). Immunostaining studies indicate that DMT1 expression is strongest on the brush border of the apical pole of mature villous enterocytes where the expression of DMT1 is tightly regulated by body iron status and is consistent with the role for DMT1 in luminal $Fe^{2+}$ uptake (Canonne-Hergaux et al. 1999; Knopfel et al. 2005; see Mackenzie and Hediger 2004 for a review). In macrophages, DMT1 is restricted at the phagosomal membrane where red cells are engulfed (Gruenheid et al. 1999; Jabado et al. 2002). In the kidney the majority of DMT1 signal resides in the renal cortex and comparably weaker staining is evident in the medullary regions (Ferguson et al. 2001; Canonne-Hergaux and Gros 2002). Staining in the cortex stems from the presence of DMT1 in PT and collecting ducts. In contrast to distal convoluted tubules, which exhibit apical DMT1 immunostaining, only structures within the cells of rat PT are stained where immunogold labelling and electron microscopy pinpointed the intracellular DMT1 staining to late endosomes and lysosomes (Abouhamed et al. 2006; see also Smith and Thévenod 2009) for a discussion of discrepancies to the distribution of DMT1 in mouse PT). From these data it was concluded that DMT1 may be involved in movement of iron across late endosomal and lysosomal membranes (Abouhamed et al. 2006). Other groups have also detected intracellular DMT1 staining in other cells (Tabuchi et al. 2000, 2002; Lis et al. 2004).

DMT1 is expressed in the plasma membrane, typically in enterocytes, where it mediates Tf-independent $Fe^{2+}$ absorption into the organism which is coupled to the pumping of a proton (Gunshin et al. 1997). This mechanism is also operative in airway epithelial cells where DMT1 clears iron and other metals from the air and may thereby participate in detoxification of metals associated with air pollution particles (Wang et al. 2002; Ghio et al. 2007). Alternatively, when DMT1 is located intracellularly it is involved in the TfR pathway of iron acquisition (it is highly expressed in erythrocyte precursors because of their requirement for iron). DMT1 is localized to intracellular endosomes (and lysosomes) that are formed during endocytosis of the Tf–TfR complex. Vacuolar-type ATPases acidify the endosome/lysosome which causes dissociation of the metal. This will, in turn, activate DMT1 in the endosomal/lysosomal membrane to co-transport the metal ion along with $H^+$ into the cytosol (Gruenheid et al. 1995; Jabado et al. 2002).

DMT1 appears to be a key transporter involved in $Cd^{2+}$ toxicity. Gunshin et al. (1997) demonstrated that, in addition to $Fe^{2+}$, a broad range of transition metals $Cd^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Cu^{2+}$,, $Co^{2+}$, $Ni^{2+}$ and $Pb^{2+}$) can evoke inward currents in *Xenopus* oocytes expressing DMT1. Only $Fe^{2+}$ transport was demonstrated in this study using a radiotracer assay. Nevertheless, like inhibition of uptake, evoked currents demonstrate DMT1 reactivity with other metals but do not

demonstrate that they are actually transported. A more compelling study by Bannon et al. used DMT1 knockdown in Caco-2 cells which significantly inhibited $^{55}Fe^{2+}$ and $^{109}Cd^{2+}$ uptake without effect on uptake of $Pb^{2+}$ (Bannon et al. 2003). Moreover, radiotracer assays in oocytes and transfected HEK293 cells have established that DMT1 is also capable of transporting $Cd^{2+}$, $Mn^{2+}$ and $Zn^{2+}$ (Okubo et al. 2003; Mackenzie et al. 2006). $Zn^{2+}$ is a weak substrate (Mackenzie et al. 2007). Its $K_i$ for inhibition of $Fe^{2+}$ transport is about 50 μM whereas the $K_{0.5}$ for $Fe^{2+}$, $Cd^{2+}$, $Mn^{2+}$, $Co^{2+}$ range between 1-5 μM (B. Mackenzie, personal communication). The $K_m$ for $Cd^{2+}$ is ~1 μM (Okubo et al. 2003). Interestingly, $Cd^{2+}$ appears to be even transported more effectively by DMT1 than $Fe^{2+}$ (Garrick et al. 2006). The lower $\Delta H^{o}_{hydration}$ of $Cd^{2+}$ compared to $Fe^{2+}$ could account for the better transport rate of $Cd^{2+}$ by DMT1 though it is not clear whether the solvated metal ion is transported or dehydration is necessary for transport to occur.

Consequently, free $Cd^{2+}$ may be taken up from the gut lumen into enterocytes via DMT1-mediated transport (Kwong and Niyogi 2009). The DMT1 gene expresses several DMT1 mRNA variants. One of these variants carries a conserved iron-responsive element (IRE) ("IRE form"), whereas the other one lacks it ("non-IRE form"; Lee et al. 1998). Because the IRE form is up-regulated in the duodenum by iron deficiency (Gunshin et al. 1997; Canonne-Hergaux et al. 1999), a link between $Cd^{2+}$ uptake (and toxicity) and DMT1 expression has been proposed under conditions where iron homeostasis is disrupted. For instance, diet-induced iron deficiency in rats led to increased DMT1 mRNA expression in duodenum, kidneys and liver which was associated with increased $Cd^{2+}$ uptake from the gastrointestinal tract and an increased $Cd^{2+}$ burden of various tissues, including intestine, kidneys, liver, testes, brain and blood (Park et al. 2002). Moreover, upregulation of DMT1 in the duodenum of pregnant rats to accommodate the increased demand for iron by the fetus was associated with increased intestinal absorption of $Cd^{2+}$ and its accumulation in liver and kidneys of pregnant animals (Leazer et al. 2002). Furthermore, iron overload due to hereditary (hypotransferrinemia) increased iron intestinal absorption in mice resulted in increased duodenal $Cd^{2+}$ uptake (Raja et al. 2006). All these studies in experimental animals indicate that disruption of homeostasis increase the sensitivity to $Cd^{2+}$ because of increased expression of DMT1 which may apply to humans as well (Kippler et al. 2009).

In the rat kidney PT, DMT1 is expressed in late endosomes and lysosomes, but not in the plasma membrane (Abouhamed et al. 2006). Filtered CdMT rather than free $Cd^{2+}$ is therefore likely to be the source for DMT1-mediated $Cd^{2+}$ transport. In cultured PT cells endocytosed CdMT was found to co-localize with the endosomal/lysosomal markers rab5A and LAMP1, which suggests that CdMT must traffic through the endosomal/lysosomal pathway (Erfurt et al. 2003). Moreover, chloroquine, an inhibitor of acidic compartments, and the PI3-kinase blocker, LY-294002, which inhibits endocytosis, prevented uptake of CdMT and apoptosis of rat PT cells (Erfurt et al. 2003). Using the same cell culture model Abouhamed and coworkers demonstrated reduced death rates of rat PT cells induced by CdMT after RNAi knockdown of DMT1 (Abouhamed et al. 2007), suggesting that following degradation of the MT protein moiety in late endosomes and lysosomes $Cd^{2+}$ is extruded by DMT1 from these acidic compartments into the cytosol where free $Cd^{2+}$ triggers apoptosis of PT cells (Dorian et al. 1992a, b; Klaassen et al. 1999; Thévenod 2003). Recently, small molecule inhibitors of DMT1 have been identified which would be useful for testing this hypothesis provided they can gain access to lysosomes (Buckett and Wessling-Resnick 2009).

There is also some evidence indicating that $Cd^{2+}$ may be taken up by distal segments of the nephron both in vivo in rats (Barbier et al. 2004) and in cultured cells (Friedman and Gesek 1994; Olivi et al. 2001). Based on these data, it has been hypothesized that some of the $Cd^{2+}$ delivered to the luminal compartment of the distal nephron and collecting duct is taken up in an absorptive manner by DMT1. This hypothesis is supported in part by the immunolocalization experiments showing apical DMT1 localization in distal convoluted tubules (Ferguson et al. 2001). Additional support for this hypothesis comes in part from experiments demonstrating that $Cd^{2+}$ is a potent inhibitor of $Fe^{2+}$ uptake in cells derived from the distal nephron (Friedman and Gesek 1994; Olivi et al. 2001). Together, these findings provide indirect evidence suggesting that $Cd^{2+}$ may act as an ionic mimic of $Fe^{2+}$ at the site of the

luminal transporter, DMT1, in epithelial cells of the distal nephron and collecting duct.

Whether the $Fe^{2+}$ export transporter ferroportin 1 (FPN1; IREG1; MTP1), an iron-regulated transporter implicated in the basolateral transfer of iron from the duodenum and other tissues (placenta, kidney, liver, and testis) to the circulation (Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000), mediates $Cd^{2+}$ efflux from cells has not been demonstrated so far. Although a recent study showed increased $Cd^{2+}$ uptake in $Fe^{2+}$-deficient mice which was associated with increased expression of DMT1 and FPN1 in duodenum and other tissues (Kim et al. 2007), this is insufficient evidence to link FPN1 to $Cd^{2+}$ transport.

In summary, several physiological $Fe^{2+}$ (free $Fe^{2+}$ and iron-protein complexes) uptake and transport pathways have recently been identified that may play a previously unsuspected role in toxicity of CdMT complexes and/or the free $Cd^{2+}$ ion.

## ATP-binding cassette transporters: the ABC of $Cd^{2+}$ efflux?

### Multidrug resistance P-glycoprotein (MDR1, ABCB1)

Most members of the superfamily of ATP binding cassette (ABC) transporter proteins act as pumps carrying substrates as diverse as large hydrophobic drugs, small anions, or peptides across membranes at the cost of ATP hydrolysis. P-glycoproteins (ABCB1) can confer multidrug resistance by actively extruding structurally unrelated, hydrophobic amphiphilic and cationic drugs from the cells (Ambudkar et al. 2003; Gottesman and Ling 2006). In epithelial cells of the gastrointestinal tract, liver, kidney, and capillaries of the brain, testes, and ovaries, ABCB1 acts as a barrier to the uptake of xenobiotics, and promotes liver and renal excretion of drugs and xenobiotics into the bile and urine. Polymorphisms in the ABCB1 gene may affect the pharmacokinetics of many commonly used drugs, including anticancer drugs (Sauna et al. 2007). It has been known for almost 20 years that $Cd^{2+}$ induces upregulation of the multidrug transporter P-glycoprotein ABCB1 (Chin et al. 1990; Murakami et al. 1991). Moreover, decreased toxicity to $Cd^{2+}$ was demonstrated in kidney PT cells over-expressing ABCB1 (Thévenod et al. 2000). Subsequent studies in OK and LLC-PK1 cells have demonstrated increased $^{109}Cd^{2+}$ steady-state accumulation (incubation with 1 μM $Cd^{2+}$ for 15–30 min) upon application of various ABCB1 inhibitors, such as verapamil, vinblastine, cyclosporine A or the functional antibody UIC2 (Endo et al. 2002). This was complemented by experiments using LLC-PK1 cells over-expressing ABCB1 where reduced $Cd^{2+}$ accumulation was demonstrated. In a subsequent study ABCB1-dependent trans-epithelial transport was determined by applying 1 μM $^{109}Cd^{2+}$ from the apical or basolateral cell side and by measuring transepithelial transport at 37°C for 60 min (Kimura et al. 2005). However, these data should be taken with caution as they assess ABCB1-dependent $Cd^{2+}$ efflux indirectly and therefore may also measure ABCB1-independent trans- and paracellular transport pathways, which may also contribute to $Cd^{2+}$ fluxes. For instance, it has been previously shown that $Cd^{2+}$ application to the basolateral cell side of LLC-PK1 monolayers decreases transepithelial resistance by 60% within 3 h resulting in increased permeability of the monolayer (Zimmerhackl et al. 1998). In addition, extracellular $Cd^{2+}$ is known to disrupt adherens junctions by displacing $Ca^{2+}$ from E-cadherin (Prozialeck et al. 2002; Thévenod et al. 2007).

In contrast Achard-Joris and coworkers assessed the role of multidrug resistance (MDR) proteins in $Cd^{2+}$ resistance and transport by expressing human *ABCB1*, *Lactococcus lactis LmrA*, and *Oenococcus oeni OmrA* in an *Escherichia coli tolC* mutant strain which proved to be hypersensitive to $Cd^{2+}$ (Achard-Joris et al. 2005). Both the human and bacterial MDR genes conferred $Cd^{2+}$ resistance to *E. coli* up to 0.4 mM concentration. Quantification of intracellular $Cd^{2+}$ concentration by atomic absorption spectrometry showed a reduced $Cd^{2+}$ accumulation in cells expressing the MDR genes. $Cd^{2+}$ accumulation was reduced in LmrA, OmrA and ABCB1 overexpressing bacteria at 0.4 mM extracellular $Cd^{2+}$, but only in bacteria overexpressing lmrA and omrA at 0.25 mM $Cd^{2+}$, which indicates that at 0.25 mM the major efflux pathways for $Cd^{2+}$ are ABCB1-independent. Inside-out membrane vesicles of *L. lactis* over-expressing *LmrA* displayed an ATP-dependent $^{109}Cd^{2+}$ uptake (50 μM) that was stimulated by 625 μM glutathione (GSH), suggesting that $Cd^{2+}$ is transported by LmrA as a complex with GSH or ATP. $^{109}Cd^{2+}$ transport mediated by ABCB1 was not tested

in inside-out vesicles, most likely because ABCB1-mediated $^{109}Cd^{2+}$ transport is not detectable at this concentration. These results differ from the studies of Endo and Kimura where 1 μM $Cd^{2+}$ was used (Endo et al. 2002; Kimura et al. 2005).

In an attempt to clarify the role of ABCB1 in $Cd^{2+}$ transport, we have investigated $^{109}Cd^{2+}$ efflux as a function of ABCB1 expression (W.-K. Lee, B. Torchalski and F. Thévenod, in preparation). A substrate of ABCB1, rhodamine $123^{+}$ (5–100 μM), showed increased efflux in MDCK cells permanently overexpressing ABCB1 (Pastan et al. 1988) compared to the parental cell line and was abolished by 0.1–1 μM of the ABCB1 blocker PSC833. However, both accumulation and efflux of 10 μM $^{109}Cd^{2+}$ were neither affected by the expression level of ABCB1, nor by PSC833 or the functional antibody UIC2. These data indicate that $Cd^{2+}$ efflux is not mediated by ABCB1 and that ABCB1-dependent resistance to $Cd^{2+}$ toxicity occurs independently from $^{109}Cd^{2+}$ efflux. However, we do not exclude that $Cd^{2+}$ at high concentrations is transported by bacterial MDR transporters as a complex with GSH or ATP, as suggested by Achard-Joris et al. (2005).

### Multidrug resistance-associated protein 1 (MRP1, ABCC1)

The multidrug resistance-associated protein 1 (MRP1) is a high-affinity transporter of the cysteinyl leukotriene C(4) and is responsible for the systemic release of this cytokine in response to an inflammatory stimulus. The substrate specificity of MRP1 and other members of the MRP family is extremely broad and includes many organic anion conjugates of structurally unrelated endo- and xenobiotics (Deeley et al. 2006; Kruh et al. 2007). MRP1 and other members of this branch of lipophilic anion transporters are expressed in various epithelial tissues, such as liver and kidney, as well as in endothelia of the blood–brain barrier and in neurons (Silverman 1999). It is likely that MRP family members are efflux pumps for $Cd^{2+}$ and similarly to YCF1, which resembles the human MRP1 (63% amino acid similarity), transport $Cd^{2+}$ as $Cd^{2+}$–GSH complexes (Tommasini et al. 1996). YCF1 encodes an MgATP-energized glutathione S-conjugate transporter that is responsible for vacuolar sequestration accumulation of $Cd^{2+}$-GSH complexes in *Saccharomyces cerevisiae* (Tommasini et al. 1996; Li et al. 1997). In fact, the study by Tommasini et al. (1996) demonstrated GSH transport by MRP1, but $Cd^{2+}$-GSH transport has not been proven so far.

### Cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7)

The cystic fibrosis transmembrane conductance regulator (CFTR) belongs to the ABC family of integral membrane proteins. It is mainly located in the apical membrane area of salt transporting tissues, such as secretory epithelia and exocrine glands, where it functions as a cAMP-dependent $Cl^{-}$ channel required to control ion and fluid homeostasis on epithelial surfaces (Riordan 2008). Mutations in the CFTR gene cause cystic fibrosis (CF), the most common autosomal recessive disease in the Caucasian population. Additional roles have been attributed to CFTR, e.g., as a "conductance regulator" via autocrine signaling through CFTR-mediated release of ATP, which controls the activity of outwardly rectifying $Cl^{-}$ channels, in particular those involved in volume regulation (Hryciw and Guggino 2000; Sabirov and Okada 2005). In addition, recent studies indicate that, similar to other members of the ABC protein family, CFTR may mediate GSH export from cells (Kogan et al. 2003). In fact, a defect in this GSH transport function of CFTR may contribute to the pathology of CF (Childers et al. 2007). In addition to exocrine epithelia, CFTR has been located in the mammalian kidney, particularly in the apical pole of proximal (PT) and distal tubule (DT) cells (Crawford et al. 1991; Jouret et al. 2007).

Evidence for CFTR interaction with $Cd^{2+}$ is scarce. $Cd^{2+}$ is known to react with cysteine residues. This may account for the ability of $Cd^{2+}$ (0.1 mM) to affect the gating properties of CFTR channels of inside-out patches or of membrane vesicles incorporated in planar bilayer experiments (Harrington et al. 1999). $Cd^{2+}$ decreased both the opening and closing rates of the channel irreversibly suggesting covalent modification. Moreover, oxidizing agents mimicked the effect of $Cd^{2+}$ whereas reducing agents had opposite effects on CFTR channel properties. The authors concluded that regulation of the redox state of the channel may be a physiological mode of control of CFTR channel activity (Harrington et al. 1999). In contrast 0.3 mM $Cd^{2+}$ applied to intact colonic epithelia did not affect CFTR-mediated $Cl^{-}$ secretion

(O'Donnell et al. 2000). This suggests that the redox sensor of CFTR may be located at an intracellular domain of the channel protein. Similar conclusions can be drawn from the study of Annereau et al. (2003) which showed inhibition of the ATPase activity of a recombinant CFTR construct (Gly-404–Lys-830 region of human CFTR) containing the first nucleotide-binding fold and regulatory domain by 1 mM $Cd^{2+}$. These observations have been recently extended in a CFTR molecule mutated at the signature sequence LSGGQ which defines the adenosine triphosphate (ATP)-binding cassette transporter superfamily (Wang et al. 2009). The data suggested that $Cd^{2+}$ (10 μM) can reversibly activate gating CFTR from inside-out patches in the absence of ATP by metal bridge formation between yet to be identified cysteine residue(s) and engineered aspartate or cysteine in the signature sequence. This study is in line with the previous ones because it provides evidence for cysteine residues near NBD1 to which $Cd^{2+}$ can bind. However, it is also different from the previous work because it shows reversible activation of ATP-independent gating by $Cd^{2+}$ binding to a mutated NBD1 (Annereau et al. 2003) as opposed to irreversible inhibition of ATP-dependent gating and ATPase activity by $Cd^{2+}$ (Harrington et al. 1999). From these data it can be concluded that high concentrations may favor covalent reactions of $Cd^{2+}$ with cysteine residues (Harrington et al. 1999; Annereau et al. 2003) whereas this may not be necessarily the case for low micromolar $Cd^{2+}$ concentrations (Wang et al. 2009).

Whereas these studies suggest direct actions of $Cd^{2+}$ with cytosolic domains of CFTR but do not assume that $Cd^{2+}$ is actually transported by CFTR, L'Hoste et al. (2009) have recently suggested such a mechanism. They showed that exposure of mouse PT cells expressing CFTR to 5 μM extracellular $Cd^{2+}$ triggers CFTR-like $Cl^-$ currents within a few minutes. These $Cd^{2+}$-induced currents differ from typical CFTR $Cl^-$ currents in one respect: they are not cAMP-dependent, but are rather activated by the extracellular signal-activated protein kinase (ERK1/2). $Cd^{2+}$-induced activation of $Cl^-$ currents in CFTR-expressing PT cells is associated with increased ROS formation and depletion of cellular GSH content due to CFTR-mediated efflux of GSH which triggers apoptotic cell death. Strikingly, all the described effects are not set in motion if CFTR is not present. The authors conclude that $Cd^{2+}$ triggers CFTR activation, which modulates cellular ROS levels via efflux of the ROS scavenger GSH and/or $Cd^{2+}$-GSH complexes to ensure that apoptosis of PT cells occurs. In this model, CFTR is proposed to extrude $Cd^{2+}$ as a complex with GSH, similarly as has been described for the prototypical ABC transporter yeast cadmium resistance factor (YCF1) (Li et al. 1997) and suggested for ABCB1 (Achard-Joris et al. 2005). In contrast to the study of Harrington et al. (1999) where the changes of the redox state of the cell (induced by $Cd^{2+}$) affect CFTR function, the study by L'Hoste et al. implies that CFTR (which is activated by $Cd^{2+}$) modulates ROS levels and hence the cellular redox state via CFTR-mediated efflux of GSH and/or $Cd^{2+}$-GSH complexes (L'hoste et al. 2009, 2010). The study by L'Hoste et al. (2009) differs from other studies because it postulates that $Cd^{2+}$ activates the CFTR $Cl^-$ channel indirectly via activation of the ERK1/2 signaling pathway. In fact, Wang et al. (2009) saw no effect of 10 μM $Cd^{2+}$ on channel gating of wild-type CFTR in the absence of ATP because CFTR channels can only be opened by ATP after they have been phosphorylated by protein kinase A (Riordan 2008). It would not be surprising if extracellular $Cd^{2+}$ turned out to elicit these ERK1/2-dependent processes by binding to a $Ca^{2+}$-sensing receptor that is expressed in the apical membrane of PT cells (Smith et al. 1989; Riccardi et al. 1998; reviewed in (Thévenod 2009). Activation of the $Ca^{2+}$-sensing receptor is known to activate ERK signaling (Ward et al. 2002; Hobson et al. 2003).

In summary, whereas high concentrations of free $Cd^{2+}$ may covalently modify intracellular domains of CFTR (possibly at cysteine residues) and thereby inhibits CFTR gating and ATPase activity, low micromolar $Cd^{2+}$ concentrations have either no effect on gating in the absence of ATP or activate channel gating indirectly to allow efflux of GSH, and possibly $Cd^{2+}$–GSH complexes, similarly as has been described for other ABC transporters.

## $Ca^{2+}$ channels: "I know that I know nothing"

Because $Ca^{2+}$ and $Cd^{2+}$ have similar ionic radii permeation of $Ca^{2+}$ channels by $Cd^{2+}$ has been suggested as a mechanism of entry in many different tissues, in particular in excitable tissues (Shafer 2000). Many reports have postulated $Cd^{2+}$ uptake

by L- and N-type voltage-dependent calcium channels (VDCCs) in excitable and non-excitable cells, including macrophages and hepatic cells (see for example (Blazka and Shaikh 1991; Hinkle et al. 1992; Hinkle and Osborne 1994; Souza et al. 1997; Limaye and Shaikh 1999; Misra et al. 2002). However, the physiological and toxicological relevance of these observations is difficult to assess. First, transcripts for L-type $Ca^{2+}$ channels have been identified in the rat kidney distal tubule (Yu et al. 1992), but their role in $Ca^{2+}$ transport has remained elusive (van de Graaf et al. 2007). Moreover, whereas whole liver expresses transcripts for L-type $Ca^{2+}$ channels, hepatocytes do not (Hughes et al. 1993). Finally, no stringent evidence for $Cd^{2+}$ transport has been provided for VDCCs by electrophysiological and/or radiotracer techniques. On the contrary, $Cd^{2+}$ potently blocks VDCC current (mainly N- but also L-type $Ca^{2+}$ channels) with an $IC_{50} \geq 0.3$ μM (Thévenod and Jones 1992). Formally, $Cd^{2+}$ can be permeant (Lansman et al. 1986; Swandulla and Armstrong 1989; Chow 1991; Thévenod and Jones 1992), since block can be relieved either by strong non-physiological depolarization or strong hyperpolarization. These driving forces may be required for dehydration in order for $Cd^{2+}$ to pass the primary binding/blocking site of the selectivity filter (covalent modification can be excluded because block can be relieved by voltage changes) but experimental evidence for this hypothesis is lacking. There is also evidence for more superficial binding sites, as recently described for $Ni^{2+}$ in CaV3.1 (α1G) T-type $Ca^{2+}$ channels (Obejero-Paz et al. 2008). And some ions, notably $Zn^{2+}$ and $Cu^{2+}$, selectively inhibit the CaV3.2 (α1H) T-type $Ca^{2+}$ channel by binding to an extracellular site near the S3–S4 linker of domain I (Kang et al. 2006, 2009).

The crucial question is whether $Cd^{2+}$ influx through VDCCs is significant under physiological conditions. The open probability of VDCCs is strongly voltage-dependent. Hence most VDCCs are open over a very narrow voltage range and closed at or near resting membrane potential. Yet it has been recently realized that the traditional view of L-type calcium channels being high-voltage-activating does not hold for all L-type $Ca^{2+}$ channels. In fact, analyses of CaV1 clones, which encode L-type $Ca^{2+}$ channels, in heterologous expression systems has revealed that CaV1.3 channels have low activation thresholds near the resting membrane potential (for review, see Lipscombe et al. 2004). However, whereas these channels are strongly inhibited by $Cd^{2+}$ (see above) no attempt has been yet made to demonstrate permeation by $Cd^{2+}$ (or other divalent metals) under physiological conditions. In contrast, the CaV3.1 (α1G) T-type calcium channel may be a good candidate for a VDCC permeated by divalent metal ions under physiological conditions. This channel exhibits considerable "window" currents close to resting membrane potential, so it could also mediate tonic divalent metal ion entry (Talavera and Nilius 2006). T-type $Ca^{2+}$ channels are blocked by $Cd^{2+}$ (Lacinova et al. 2000; Diaz et al. 2005) and hence are candidates for $Cd^{2+}$ permeation, as having considerable "window currents". Indeed, $Mn^{2+}$ and $Fe^{2+}$ are both "blockers" and "permeators" of CaV3.1 (α1G) T-type $Ca^{2+}$ channels at voltages near resting membrane potential. In preliminary reports Jones and coworkers (Lopin et al. 2007; Jamieson et al. 2008) showed block of CaV3.1 (α1G) T-type $Ca^{2+}$ channels by $Fe^{2+}$ and $Mn^{2+}$, but these metal ions carried detectable whole-cell inward currents at millimolar concentrations and in the absence of extracellular $Ca^{2+}$. The authors estimated that CaV3.1 (α1G) channels can mediate net $Fe^{2+}$ entry of ~20 ions per open channel per second at −60 mV and pH 7.2, in 1 μM $Fe^{2+}$ (with 2 mM extracellular $Ca^{2+}$; Lopin et al. 2007). Similar results were obtained with $Mn^{2+}$, except that inward currents were larger than with $Fe^{2+}$ (Jamieson et al. 2008). Since α1G channels exhibit a significant 'window current' in that voltage range ($P_{open} \sim 1\%$), these results suggested that α1G channels are a possible pathway for $Fe^{2+}$ entry into cells, at clinically relevant concentrations. These transport rates are low by channel standards, but not too different from dedicated $Fe^{2+}$ transporters (S.W. Jones, personal communication). $Mn^{2+}$ has also been shown to permeate CaV3.2 (α1H) $Ca^{2+}$ channels (Kaku et al. 2003). The $\Delta H^{o}_{hydration}$ of $Cd^{2+}$ (−1,807 kJ/mol) is similar to that of $Mn^{2+}$ (−1,841 kJ/mol) or $Fe^{2+}$ (−1,946 kJ/mol) suggesting that $Cd^{2+}$ may permeate CaV3 channels under physiological conditions in tissues expressing these channels. CaV3 $Ca^{2+}$ channels are expressed in excitable cells, such as neurons, heart, smooth and skeletal muscle and endocrine cells (Perez-Reyes 2003). Surprisingly, CaV3.1 is also found expressed in the distal nephron of the kidney (Andreasen et al. 2000), where it may be involved in $Ca^{2+}$ reabsorption (Leclerc et al. 2004).

CaV3.1 could account for some of the $^{109}Cd^{2+}$ transport observed by Friedman and Gesek (1994) in kidney distal tubule cells, but DMT1 could also have contributed to their $^{109}Cd^{2+}$ fluxes because of its apical expression in distal tubule cells and residual DMT1-mediated divalent metal transport at neutral pH (Mackenzie et al. 2006). The only indirect hint so far that CaV3.1 (α1G) channels may be involved in $Cd^{2+}$ transport and toxicity is a study by Leslie et al. (Leslie et al. 2006) in MTI/II (−/−) cells with acquired $Cd^{2+}$ resistance where expression of CaV3.1 was reduced thus suggesting that decreased expression of this T-type $Ca^{2+}$ channel protects cells from $Cd^{2+}$ exposure by limiting $Cd^{2+}$ uptake. Hence $Cd^{2+}$ transport by CaV3.1 channels still needs to be tested and is predicted to be of a similar magnitude as for $Mn^{2+}$ or $Fe^{2+}$.

$Cd^{2+}$ could also permeate other $Ca^{2+}$ channels, such as store-operated $Ca^{2+}$ channels (SOCs) triggered by hormones and neurotransmitters, which are responsible for a non-voltage-gated $Ca^{2+}$ current called ICRAC ($Ca^{2+}$ release-activated $Ca^{2+}$ current; for review, see Potier and Trebak 2008). Hoth and Penner (1993) demonstrated that ICRAC, which was induced by inositol-1,4,5 triphosphate-mediated emptying of internal $Ca^{2+}$ stores in mast cells, was inhibited by $Cd^{2+}$ in a voltage-independent manner. The concentration–response relationship for $Cd^{2+}$ could be described by a Michaelis–Menten function with an apparent $K_D$ of 0.24 mM and a Hill coefficient of 1. Reversibility of $Cd^{2+}$ block was not tested. Unlike VDCCs in which $Sr^{2+}$ and $Ba^{2+}$ behave as good permeators, these divalent cations also blocked ICRAC. Consequently, ICRAC is highly $Ca^{2+}$-selective which is different from VDCCs (Parekh and Penner 1997). Metal classification or $\Delta H^{o}_{hydration}$ cannot account for the inhibitory potency of divalent cations described by Hoth and Penner (1993). Interestingly, though $Mn^{2+}$ also potently blocks ICRAC (Hoth and Penner 1993), SOC channels can be permeated by $Mn^{2+}$ (this property of $Mn^{2+}$ has been subsequently introduced as a technique to determine activation of SOC channels as "$Mn^{2+}$ quenching of Fura-2 fluorescence"; Bird et al. 2008) suggesting that some of the blocking divalent metal ions may also permeate the channel (Fasolato et al. 1993). Hence it cannot be excluded that $Cd^{2+}$ both blocks and permeates CRAC channels. Orai and Stim proteins are the long sought after molecular entities underlying store-operated calcium influx where Orai proteins are the SOCs carrying ICRAC (Feske et al. 2006; Prakriya et al. 2006; Vig et al. 2006; Yeromin et al. 2006; Zhang et al. 2006; reviewed in Deng et al. 2009). Given that mutations at the outer loops of CRAC channels (there are three aspartates) can affect $Gd^{3+}$ and $Ca^{2+}$ affinity, it seems plausible that divalent ions would coordinate there first. Whether or not they then permeate is currently unknown (R. Penner, personal communication).

$Ca^{2+}$ permeable cation channels differing from VDCCs and SOCs may also mediate the uptake of $Cd^{2+}$, e.g. maitotoxin-stimulated nonselective cation channels (Olivi and Bressler 2000) or 2-aminoethoxydiphenyl borate sensitive $Ca^{2+}$ and $Mg^{2+}$ permeable cation channels, as described by Levesque et al. (2008). They provided evidence to suggest that TRPM7 may be responsible for $Cd^{2+}$ uptake in human osteoblast-like MG-63 cells. TRPM7 is a member of the melastatin-related subfamily of TRP channels and represents a protein that contains both an ion channel and a kinase domain (Penner and Fleig 2007). It is ubiquitously expressed and the only ion channel known that is essential for cellular viability. It is permeable to $Ca^{2+}$ and $Mg^{2+}$, but also conducts essential metals such as $Zn^{2+}$, $Mn^{2+}$, and $Co^{2+}$, as well as $Ni^{2+}$, $Cd^{2+}$, $Ba^{2+}$, and $Sr^{2+}$ (Monteilh-Zoller et al. 2003). The channel is constitutively open but strongly downregulated by intracellular levels of $Mg^{2+}$, MgATP and other Mg-nucleotides (reviewed in Penner and Fleig 2007). The function of the kinase domain is not completely understood, but may involve autophosphorylation of TRPM7 as well as phosphorylation of other target proteins such as annexin and myosin IIA heavy chain. Based on these properties, TRPM7 is currently believed to represent a ubiquitous homeostatic mechanism that regulates $Ca^{2+}$ and $Mg^{2+}$ fluxes based on the metabolic state of the cell. Physiologically, the channel may serve as a regulated transport mechanism for these ions that could affect cell adhesion, cell growth and proliferation, and even cell death under pathological stress such as anoxia (Penner and Fleig 2007). Though TRPM7 may be an interesting candidate for $Cd^{2+}$ mediated toxicity and death the ability of $Cd^{2+}$ to permeate TRPM7 is the lowest of all divalent metal ions tested (Monteilh-Zoller et al. 2003).

The mitochondrial $Ca^{2+}$ uniporter (MCU) located in the inner mitochondrial membrane (MCU) is a highly selective $Ca^{2+}$ channel that binds $Ca^{2+}$ with extremely high affinity ($K_D \leq 2$ nM; Kirichok et al. 2004). The relative divalent ion conductance of the MCU was $Ca^{2+} \approx Sr^{2+} >> Mn^{2+} \approx Ba^{2+}$. Unfortunately, $Cd^{2+}$ was not tested. We have shown that $Cd^{2+}$ is taken up by kidney cortex mitoplasts using the $Cd^{2+}$-sensitive fluorescent indicator FluoZin-1 (Lee et al. 2005a). $Cd^{2+}$ uptake occurred most likely via the MCU because MCU inhibitors ($La^{3+}$, ruthenium red and Ru360) prevented $Cd^{2+}$-induced swelling of energized kidney cortex mitochondria (Lee et al. 2005a). Further experiments demonstrated that $Cd^{2+}$ in the matrix was found to activate aquaporin-8 water channels and a $K^+$ uniporter of the inner mitochondrial membrane to induce osmotic swelling (Lee et al. 2005a, b; reviewed in Lee and Thévenod 2006). Hence $Cd^{2+}$ may permeate the MCU directly but more direct evidence, e.g., by patch-clamp techniques and/or $^{109}Cd^{2+}$ radiotracer experiment would substantiate these observations.

**Table 1** $Cd^{2+}$ transport pathways in mammalian cells

| | Transported $Cd^{2+}$ moiety | Mechanism of transport | References |
|---|---|---|---|
| *Proven $Cd^{2+}$ transport pathways* | | | |
| Megalin/cubilin (receptor) | CdMT | Receptor-mediated endocytosis | Klassen et al. (2004), Wolff et al. (2006) |
| DMT1/SLC11A2 | $Cd^{2+}$ | $Cd^{2+}/H^+$ cotransport | Gunshin et al. (1997), Bannon et al. (2003) Okubo et al. (2003), Garrick et al. (2006) |
| ZIP8/SLC39A8 | $Cd^{2+}$ | $Cd^{2+}/2HCO_3^-$ cotransport | Dalton et al. (2005), He et al. (2006), Liu et al. (2008) |
| ZIP14/SLC39A8 | $Cd^{2+}$ | $Cd^{2+}/HCO_3^-$ cotransport (?) | Girijashanker et al. (2008) |
| *Likely $Cd^{2+}$ transport pathways* | | | |
| MRP1/ABCC1 | CdGSH | ATP-dependent efflux | Tommasini et al. (1996) |
| CFTR/ABCC7 | CdGSH | ATP-dependent efflux (?) | Kogan et al. (2003), L'Hoste et al. (2009) |
| TRPM7 | $Cd^{2+}$ | Channel | Monteilh-Zoller et al. (2003), Levesque et al. (2008) |
| CaV3.1-3 T-type $Ca^{2+}$ channels (VDCCs) | $Cd^{2+}$ | Channel | Lacinova et al. (2000), Diaz et al. (2005), Lopin et al. (2007), Jamieson et al. (2008), Kaku et al. (2003) |
| Mitochondrial $Ca^{2+}$ uniporter | $Cd^{2+}$ | Channel | Lee et al. (2005a, b) |
| *Less likely $Cd^{2+}$ transport pathways* | | | |
| ICRAC (SOCs) | $Cd^{2+}$ | Channel | Hoth and Penner (1993) |
| L- and N-type $Ca^{2+}$ channels (VDCCs) (with the possible exception of CaV1.3 channel) | $Cd^{2+}$ | Channel | Thévenod and Jones (1992), Lansman et al. (1986), Swandulla and Armstrong (1989), Chow (1991) |
| *Unclear or controversial $Cd^{2+}$ transport pathways* | | | |
| MDR1/ABCB1 | $Cd^{2+}$/CdGSH/ CdATP | ATP-dependent efflux | Endo et al. (2002), Kimura et al. (2005), Achard-Joris et al. (2005) |

*CdMT* cadmium–metallothionein complex, *CdGSH* cadmium–glutathione complex, *VDCCs* voltage dependent $Ca^{2+}$ channels, *ICRAC* $Ca^{2+}$ release-activated $Ca^{2+}$ current, *SOCs* store-operated $Ca^{2+}$ channels. References support but do not prove $Cd^{2+}$ transport. For further details, please refer to the text

## Conclusions and outlook

The screening of the literature for stringent experimental evidence (using electrophysiological, radiotracer techniques, fluorescent dyes combined with molecular biology) for $Cd^{2+}$ ion transport by membrane carriers, channels, ATPases and receptors only yields convincing proof for the $Fe^{2+}$ transporter DMT1 (and ZIP transporters that are not the topic of this review). $Cd^{2+}$ may also be taken up as a complex with MT by RME involving megalin and cubilin, and possibly other mechanisms of RME. So far, no experimental data have proven $Cd^{2+}$ efflux by mammalian ABC transporters, either as free $Cd^{2+}$ or $Cd^{2+}$-complexes, though the latter are likely to be transported by MRPs and CFTR. Obviously, more experimental evidence using electrophysiological techniques and radiotracers is necessary to prove the contribution of $Ca^{2+}$ channels in $Cd^{2+}$ transport though certain more likely candidates have been spotted, such as CaV3 channels, TRPM7 (Table 1), and perhaps CaV1.3 channels. A plethora of publications on $Cd^{2+}$ "transport" is available, but major confusion arises from correlative studies investigating $Cd^{2+}$ resistance and expression levels of transporter proteins. Clearly, to sort the wheat from the chaff a "conservative" approach is necessary. Proof of $Cd^{2+}$ transport can only be claimed by providing experimental evidence for vectorial transport of $Cd^{2+}$ across cellular membranes. This review aims to enlighten researchers to focus their future research on likely candidates for $Cd^{2+}$ transport in order to pin-point strategies for prevention and/or therapy of $Cd^{2+}$ toxicity and carcinogenesis.

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