Molecular components of vertebrate Mg\textsuperscript{2+}-homeostasis regulation

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Abstract. Over the past decades, the clinical relevance and biological significance of Mg\textsuperscript{2+} have been thoroughly documented. Although multiple Mg\textsuperscript{2+}-transport pathways have been biophysically characterized, the molecular identity of the postulated components of Mg\textsuperscript{2+}-homeostasis regulation in vertebrates remain undefined. Recent advances in the fields of genetics, genomics and proteomics, and novel technologies such as cDNA microarrays have allowed for substantial progress in this area. The mitochondrial Mrs2 protein was the first human Mg\textsuperscript{2+} transporter characterized as such, and an important element for future analyses of the role of mitochondria in managing intracellular Mg\textsuperscript{2+}. Several molecules with Mg\textsuperscript{2+}-transport capabilities have been identified through a screen designed to find genes upregulated under hypomagnesic conditions. This includes SLC41A1 and 2, ACDP2 and MagT1. Finally, the elucidation of the molecular cause underlying two different hereditary diseases leading to hypomagnesemia resulted in the cloning and characterization of claudin 16 (paracellin-1), and TRPM6. Whereas claudin 16 plays a crucial role in paracellular Mg\textsuperscript{2+} transport, TRPM6 is involved in the transcellular pathway. TRPM6 and its closest relative TRPM7 are both puzzling ion channel-kinase fusions, and perhaps the most unexpected newly identified players in the regulation of Mg\textsuperscript{2+}-homeostasis in vertebrates.

Key words: Mrs2, ACDP2, SLC41A1 and 2, Paracellin-1, Claudin 16, MagT1, TRPM6, TRPM7

Mg\textsuperscript{2+} is the most abundant divalent cation in living cells, with a total cellular concentration between 14 and 20 mM. Virtually every biological process requires Mg\textsuperscript{2+}, not only as an essential cofactor for hundreds of enzymes, but also because it is crucial for the maintenance of the active conformation of macromolecules, for the regulation of lipid- and phosphoinositide-derived second messengers, for charge compensation, and for the regulation of various transporters and ion channels [1]. Furthermore, Mg\textsuperscript{2+} is an important modulator of intracellular free Ca\textsuperscript{2+} concentration and pH, which are major determinants of cell contraction, secretion, motility, and proliferation. Numerous publications have reported the clinical aspects of Mg\textsuperscript{2+}-homeostasis. Mg\textsuperscript{2+} regulates smooth muscle relaxation, dilates coronary arteries and peripheral vessels, exerts antiarrhythmic effects and can play a role in various thrombogenic conditions. Many diseases such as cardiac arrhythmia, diabetes and chronic alcoholism are associated with diminished levels of plasma and parenchymal Mg\textsuperscript{2+}. Hypomagnesemic disorders frequently arise in critically ill patients as a consequence of failure of the kidneys often secondary to medication, predisposing these patients to life-threatening effects [2]. Additionally, several genetic diseases are described which result in a deregulation of Mg\textsuperscript{2+}-homeostasis [3]. Mg\textsuperscript{2+} is also essential for the normal functioning of many components of the immune response. In an experimental model of hypomagnesemia in the rat, just a few days of Mg\textsuperscript{2+}-low nutrition are sufficient to elicit a clinical inflammatory syndrome, including the activation of leukocytes and macrophages, and increased levels of their correlates, such as cytokines and reactive oxygen species [4]. The detrimental effects of experimental
severity Mg²⁺-deficiency on the immune system are well known, and it has also been shown to affect the humoral response [5]. Despite the clinical relevance and biological significance of Mg²⁺, our knowledge about the molecular mechanisms and components involved in regulating Mg²⁺-homeostasis at the level of the whole organism, organs, or single cell was very limited until a few years ago. This review aims at highlighting some of the recent advances in this quickly moving field by providing an overview of molecules linked to the regulation of Mg²⁺-homeostasis in vertebrates (summarized in table 1).

Mitochondrial Mg²⁺-homeostasis: Mrs2

Mrs2 was firstly identified in 1987 in a screen aiming at isolating nuclear genes suppressing RNA splicing defects in mitochondrial introns of yeast mutants [6]. In a subsequent study, disruption of the MRS2 gene in yeast was confirmed to affect all excisions of group II introns in mitochondrial RNAs [7]. Beyond this splicing phenotype, MRS2 deficient yeasts are also respiration deficient and therefore unable to grow on non-fermentable substrates such as glycerol (petite or pet phenotype), possibly due to the significant reduction in cytochromes observed in Δmrs2p mitochondria. This holds true even in strains where all affected mitochondrial introns have been deleted, suggesting that Mrs2p fulfills supplementary functions beyond RNA-splicing regulation. Several years later, Mrs2p was found to exhibit short regions of homology to the bacterial Mg²⁺ transporter CorA [8], and to share a similar overall membrane topology with two predicted TM spans. Experimentally, the potential role of Mrs2p as a Mg²⁺-transporter was further suggested by the partial complementation of the pet phenotype of Mrs2-deficient yeasts by a CorA version fused to the mitochondrial N-terminal leader sequence of Mrs2p, ensuring CorA’s proper insertion into the mitochondrial inner membrane. In this same publication, the mitochondrial Mg²⁺ concentration was markedly reduced, pointing out the probable involvement of Mrs2 as a major component of mitochondrial Mg²⁺-homeostasis regulation. The existence of an Mg²⁺ specific mitochondrial transporter has been controversially discussed in the past. In an elegant study using isolated mitochondria from wt and MRS2 deficient yeasts, and monitoring matrix Mg²⁺-levels [Mg²⁺]m with the fluorescent indicator dye Mag-fura, it was shown that the rapid increase in [Mg²⁺]m observed upon elevation of the external Mg²⁺ levels (up to 25% in the first second) is almost abrogated in the Δmrs2 mitochondria, but significantly elevated upon Mrs2p overexpression [9]. The authors of this study concluded that all their observations are “most compatible with Mrs2p constituting a channel” and that Mrs2p is a crucial and maybe even the sole component of the “rapid high capacity [mitochondrial] Mg²⁺ influx system” they identified. The motor of this Mg²⁺-influx pathway appears to be the mitochondrial membrane potential ΔΨ, since pharmacological agents inhibiting the F₁F₀ ATP synthase or the ADP/ATP translocase lead to a substantial decrease of the Mg²⁺-influx. Importantly, ΔΨ itself is not affected in yeast mutants lacking Mrs2p.

A recent structure function relationship analysis performed in yeast revealed the importance of conserved motifs in the middle part of the protein in mediating Mg²⁺-homeostasis regulation, either by directly participating in the multimerization of Mrs2p to form functional channels (coiled coil region), or in the gating of the channel [10]. Future studies will help determine how Mrs2p is tied into the larger context of cellular Mg²⁺-homeostasis regulation in yeast.

Beyond MRS2, one other MRS2 like gene was found in yeast called LPE10 that has been shown to share many of Mrs2p’s structural and functional attributes, although its physiological role is less well defined [11]. In plants, Mrs2 related genes are strongly represented with 15 homologues identified in the genome of Arabidopsis thaliana [12]. Mammalian genomes contain one single representative of the Mrs2 family. RT-PCR studies conducted in mouse tissues have shown Mrs2 to be widely expressed, albeit at a comparatively low level. The human Mrs2 protein (hsaMrs2p) is also localized in the mitochondrial subcompartment and is capable of partially complementing the mitochondrial Mg²⁺-deficiency in a Δmrs2 yeast strain, resulting in an improved growth behavior on non-fermentable substrate (pet phenotype), as well as of partly rescuing mitochondrial splicing of group II introns [13]. Thus, the human Mrs2 homologue appears to be able to mediate the influx of Mg²⁺ into yeast mitochondria, suggesting that it is fulfilling similar functions in human cells. The discovery of Mrs2 as a crucial route of entry of mitochondrial Mg²⁺ is an important step towards understanding the role of these organelles in regulating cellular ion homeostasis. The deletion of MRS2 in yeasts also unmasked a much slower, but clearly detectable Mg²⁺-entry pathway that was sufficient to restore steady state [Mg²⁺]m to about 35% of wildtype levels, and might ensure the survival of the ΔMRS2 yeasts. The identity of this other potential transporter system is currently unknown.
Table 1. Main features of molecularly identified vertebrate Mg²⁺ transporters.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structural features/ Biophysics</th>
<th>Tissue distribution</th>
<th>Comments</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Acdp2</td>
<td>Can carry a range of divalent cations, including Mg²⁺, Co²⁺, Mn²⁺, Sr²⁺, Ba²⁺, Cu²⁺, and Fe²⁺, only Zn²⁺ effectively inhibits. Mg²⁺-transport voltage dependent, Na⁺ or Cl⁻ independent, ( K_m = 0.5 \text{mM} )</td>
<td>Widely expressed, but strongest in brain and absent in skeletal muscles.</td>
<td>Gene upregulated in renal epithelial cells under hypomagnesic conditions. Acdp proteins are over 50% homologous to the bacterial CorC transporter.</td>
<td>[26, 30]</td>
</tr>
<tr>
<td>MagT1</td>
<td>38 kDa, 5TM spans, whereby first C-terminal TM predicted to be cleaved. Channel-like properties and selective towards Mg²⁺, ( K_m = 0.21 \text{mM} ). Ni²⁺, Zn²⁺, and Mn²⁺ can inhibit Mg²⁺ currents.</td>
<td>Widely expressed, mRNA highest in liver, kidney and colon, lower in lung, brain, intestine, and spleen.</td>
<td>Gene upregulated in renal epithelial cells under hypomagnesic condition. No homologies to known bacterial transporters, but distantly related to the yeast oligosaccharide transferase complex OST3/OST6.</td>
<td>[25]</td>
</tr>
<tr>
<td>Mrs2</td>
<td>2TM spans. Rapid increase in [Mg²⁺]ᵣ observed upon elevation of external Mg²⁺ is almost abrogated in yeast Δmrs2 mitochondria.</td>
<td>Ubiquitous. Located in the inner mitochondrial membrane.</td>
<td>- Motor is the mitochondrial membrane potential ΔΨ. - CorA and Ahr1p related. - Anm12 yeast phenotype can be partially rescued by CoxA</td>
<td>[7-9]</td>
</tr>
<tr>
<td>Panceelin-1 (Claudin 16)</td>
<td>4TM spans with two extracellular loops and both termini located in the cytoplasm. Permeability profile unclear.</td>
<td>Kidney</td>
<td>Tight junction protein that regulates paracellular Mg²⁺ transport. Panceelin-1 mutations cause hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC). Dietary Mg²⁺ supplementation does not help.</td>
<td>[32]</td>
</tr>
<tr>
<td>SLC41A1</td>
<td>10 TM? Non-selective divalent cation transporter with a preference for Mg²⁺, ( K_m = 0.75 \text{mM} ). Carries Mg²⁺, Fe²⁺, Zn²⁺, Cu²⁺, Co²⁺ and Cd²⁺. Ca²⁺ is neither transported nor inhibiting Mg²⁺ transport.</td>
<td>Strongest in heart testes, followed by skeletal muscles, pancreas, adrenal gland and thyroid</td>
<td>Gene upregulated in renal epithelial cells under hypomagnesic condition. Related to the bacterial MgtE transporter family.</td>
<td>[20, 21]</td>
</tr>
<tr>
<td>SLC41A2</td>
<td>10 TM? Termini appear to be on opposite sides, rather than 1TM? Carries Mg²⁺, Ba²⁺, Ni²⁺, Co²⁺, Fe²⁺ and Mn²⁺, but not Zn²⁺, Cu²⁺, or Cd²⁺, inhibited by Ca²⁺, ( K_m = 0.34 \text{mM} ).</td>
<td>Widely expressed</td>
<td>Related to the bacterial MgtE transporter family. Overexpression in TRPM7-/- cells can partially compensate for their requirement in supplemental Mg²⁺. mRNA level not altered in the kidneys of hypomagnesic mice.</td>
<td>[22, 23]</td>
</tr>
<tr>
<td>TRPM6  (CHA2)</td>
<td>6TM spans, assumed to form tetramers. C-terminal alpha kinase domain. Unitary conductance of TRPM6 homomer 83.6 pS, 40.1 pS for TRPM7, and 65.5 pS for the TRPM6/7 heteromer.</td>
<td>Mainly in intestine and kidney (although wider distribution described, including heart and brain).</td>
<td>TRPM6 mutations cause Hypomagnesemia with Secondary Hypocalcemia (HSI). Leading to seizures and death unless supplemented with Mg²⁺. Assoicates with TRPM7 (in some studies TRPM7 required for TRPM6 functional surface expression).</td>
<td>[49, 50, 52, 53]</td>
</tr>
<tr>
<td>TRPM7  (LIRPC7, TRP-PLIK, CHA1)</td>
<td>6TM spans, assumed to form tetramers. C-terminal alpha kinase domain. Unitary conductance of TRPM7 homomer 40.1 pS, and 56.5 pS for the TRPM6/7 heteromer.</td>
<td>Ubiquitous.</td>
<td>TRPM7 deficiency in the DT40 B-cell line is lethal unless medium supplemented with 5-10 mM Mg²⁺. TRPM7 kinase not required for channel activation and Mg²⁺-sensitive gating.</td>
<td>[24, 40-42]</td>
</tr>
</tbody>
</table>
It is well accepted that mitochondria are major cellular Mg$^{2+}$ sinks, but the physiological significance and extent of these Mg$^{2+}$ stores are subject to discussion. [Mg$^{2+}$]$_i$ is thought to influence the activity of mitochondrial enzymes such as dehydrogenases, as well as cytochrome c oxidase, therefore directly impacting the mitochondrial respiratory rates [14]. The potential role of Mg$^{2+}$ as a signaling ion is being debated in the literature [15]. Several reports in various cellular systems support the notion that [Mg$^{2+}$]$_i$ can be rapidly increased in response to hormones [16, 17]. How mitochondrial Mg$^{2+}$ stores could take part in these events is unclear. The possibility that Mg$^{2+}$ serves as a mitochondrial volume sensor has also been discussed [18], but a study by Jung et al. failed to support this model [19]. As illustrated by the characterization of Mrs2, there is no doubt that mitochondrial Mg$^{2+}$-homeostasis is a regulated process, and future analyses will help defining the special place the mitochondrial subcompartment occupies in managing whole cell [Mg$^{2+}$], as well as cell metabolism.

**Relatives of the bacterial MgtE family:**

**SLC41A1, SLC41A2**

The SLC41 subfamily of human solute carriers currently includes three members that are distantly related to the bacterial MgtE Mg$^{2+}$-transporters. The first member of the family to be described, SLC41A1, was identified in 2003 through a bioinformatics screen designed to reveal novel transmembrane and secreted proteins [20]. Human SLC41A1 is a 56 kDa protein predicted by the authors of this original study to include ten transmembrane spans, the homology to the MgtE family being restricted to two of the ten segments. SLC41A1 gene expression appears to be widely distributed, but varies quantitatively in different human tissues. Northern blot analyses have shown a single 5kb SLC41A1 transcript that is represented strongest in heart and testis, slightly less in skeletal muscles, prostate, adrenal gland and thyroid, and weakest in the hematopoietic tissues bone marrow, lymph node, thymus and spleen, although it was clearly detectable in the T-lymphoid Jurkat cell line. In another study, mice that were fed a low-Mg$^{2+}$ diet upregulated the transcript levels of SLC41A1 in some tissues, including the kidney cortex and the heart, indicative of SLC41A1 involvement in Mg$^{2+}$-homeostasis regulation in vertebrates [21]. The biophysical characterization of SLC41A1 cloned from renal mouse distal convoluted tubule cells and heterologously expressed in Xenopus oocytes has confirmed that SLC41A1 is a functional transporter. Although it has a preference for Mg$^{2+}$, SLC41A1 should be regarded as a nonselective divalent cation transporter. Beyond Mg$^{2+}$, it could also play a role in the transport of Fe$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Co$^{2+}$ and Cd$^{2+}$. On the other hand, Ca$^{2+}$ is neither transported nor capable of inhibiting Mg$^{2+}$ transport. Given the limited level of overall similarity between the bacterial MgtE and SLC41A1 proteins, it is not surprising that the permeability profiles of these two transporters differ significantly. For example, as opposed to MgtE, SLC41A1 does not carry Co$^{2+}$, nor is its ability to transport Mg$^{2+}$ inhibited by Ni$^{2+}$.

Based on sequence homologies, the aforementioned bioinformatics screen led to the identification of two more human SLC41 proteins and their murine counterparts. Goytain and Quamme who performed the primary characterization of SLC41A1 mentioned above [21] conducted a similar series of electrophysiological experiments aimed at characterizing mouse SLC41A2 [22]. It was also found to be a transporter with a low ionic preference, although with a different cationic selectivity and inhibition profile [22]. SLC41A2 carries Mg$^{2+}$, Ba$^{2+}$, Ni$^{2+}$, Co$^{2+}$, Fe$^{2+}$, and Mn$^{2+}$, but not Zn$^{2+}$, Cu$^{2+}$, or Ca$^{2+}$. As opposed to SLC41A1, Ca$^{2+}$ inhibits SLC41A2-mediated Mg$^{2+}$-currents. Ionic uptake through SLC41A1 and SLC41A2 is voltage dependent with apparent Mg$^{2+}$-affinities of 0.75 and 0.34 mM respectively. SLC41A2 is widely expressed and its mRNA level does not seem to be altered in the kidneys of hypomagnesic mice. The human SLC41A2 protein is 70% similar to SLC41A1 and is thought to show the same 10 TM topology, though this structural prediction has been challenged by a recent study demonstrating that the C- and N- termini of SLC41A2 heterologously expressed in HEKs are on different sides of the plasma membrane, implying an uneven number of TM-spans (N- outside) [23]. This finding is compatible with hydrophobicity analyses conducted by the same group supporting a model where SLC41A2 consists of two five TM spans MgtE-like domains that are in the same orientation and connected by a supplementary span resulting in eleven TM segments total. Importantly, this work also provides the first experimental evidence that SLC41A2 participates in regulating cellular Mg$^{2+}$ homeostasis in vertebrates since its overexpression helps TRPM7 deficient DT40 B-cells overcome their requirement for supplementary extracellular Mg$^{2+}$ to support cell growth. The channel kinase TRPM7 is thought to be a crucial component of the vertebrate Mg$^{2+}$-homeostasis machinery and its deletion leads to decreased
[Mg\(^{2+}\)], and a severe cell proliferative defect that can be fully rescued by supplementing the medium with mM Mg\(^{2+}\) concentrations (see section 5) [24]. In contrast to the results obtained in *Xenopus* oocytes, it was not possible to detect SLC41A2 mediated currents in DT40 cells, suggesting either very low current amplitudes, or cell-type specific biophysical characteristics. On the other hand, Mg\(^{2+}\)-uptake was shown to be at least double in DT40 cells overexpressing SLC41A2, the only SLC41 protein natively expressed in DT40s, further supporting the concept of SLC41A2 functioning as a plasma membrane Mg\(^{2+}\)-transporter in vertebrates [23].

To this date, nothing has been published about SLC41A3, the third member of the SLC41 protein family. Future work will help define the functional features and biological role of the last member of this interesting group of molecules, which appears to include several functional human and murine Mg\(^{2+}\) transporters.

MagT1 and ACDP2

Both MagT1 and ACDP2 transcript levels are being upregulated under hypomagnesic conditions in human epithelial cells, as identified by Goytain and Quamme [25, 26]. This experimental approach is based on the finding that epithelial cells maintained under low Mg\(^{2+}\) concentrations adjust their rate of Mg\(^{2+}\)-transport to increase their Mg\(^{2+}\)-intake, and that transcriptional control is the main molecular mechanism underlying this adjustment.

MagT1: channel-like and selective towards Mg\(^{2+}\)

Using an oligonucleotide microarray, the MagT1 gene was one of 116 DNA fragments whose level was significantly increased in epithelial cells grown under low Mg\(^{2+}\) [25]. The corresponding protein is predicted to have a molecular weight of 38 kDa, including five transmembrane spans, whereby the first C-terminally located TM segment is predicted to be cleaved resulting in a mature MagT protein containing four TM spans. In contrast to other eukaryotic Mg\(^{2+}\)-transporters, such as Mrs2, SLC41 proteins, and Acdp family members, MagT1 does not show homologies to known prokaryotic Mg\(^{2+}\)-transporters, but is similar to various proteins of undefined function (such as the murine implantation protein, or a putative prostate cancer tumor suppressor protein), and surprisingly a distant relative of the yeast oligosaccharide transferase complex OST3/OST6 that mediates a crucial step of protein glycosylation in the endoplasmic reticulum [27]. Tissue distribution analyses in the mouse have shown the highest levels of MagT1 transcripts in liver, followed by heart, kidney and colon, and lower but clearly detectable amounts in lung, brain, intestine and spleen. Protein analyses have documented a good correlation with the mRNA tissue distribution pattern, except in the liver, where only little protein could be detected [25].

Biophysically, a striking difference to the SCL41A1/2 and ACDP2 transporters is the specificity of MagT1 towards Mg\(^{2+}\), which it carries with a Michaelis constant of 0.23 mM. A few cations including Ni\(^{2+}\), Zn\(^{2+}\), and Mn\(^{2+}\) are capable of inhibiting Mg\(^{2+}\) currents evoked by MagT1 when applied extracellularly at a comparatively high concentration of 0.2 mM, which significantly exceeds physiological levels. Ca\(^{2+}\) can neither be carried by MagT1, nor does it inhibit MagT1-mediated transport. Pharmacologically, it is interesting to note that MagT1-mediated Mg\(^{2+}\)-currents are inhibited by 10 μM of the dihydropyridine voltage-gated Ca\(^{2+}\)-blocker nitrindipine, but not by the same concentration of its analogue compound nifedipine.

In summary, MagT1 appears to display channel-like characteristics with a selectivity towards Mg\(^{2+}\). It is therefore a hopeful candidate as an important component of the vertebrate Mg\(^{2+}\)-homeostasis machinery.

Ancient conserved domain proteins, ACDPs

The human Acdp gene family was first identified in 2003 as a result of the characterization of a genomic region on chromosome 10q23-10q24 which has been linked to the genetic disorder Urofacial Syndrome (UFS). It is composed of four members that have been named ancient conserved domain proteins to reflect the finding that all the identified Acdp proteins have one domain in common which appears to be phylogenetically conserved from bacteria to man [28]. Northern blot analyses of human tissues have shown that ACDP1 has the most restricted expression profile, being only detectable in brain, and to a lesser degree in testis. ACDP2 is widely expressed, but strongest in brain and absent in skeletal muscles. ACDP3 mRNA levels appear highest in heart and spleen, but it is also noticeable that the transcript size varies depending on its location. Finally, ACDP4 shows a ubiquitous distribution with an emphasis on heart, and to a lower degree on kidney and placenta [29]. In a subsequent publication, the same authors reported the distribution of the mouse Acdp family members as being very similar to their human coun-
terral Mg^{2+}-levels in vertebrates was provided by a protein family might indeed be involved in regulating cellular Mg^{2+}-levels in vertebrates was provided by a recent study showing that Acdp2 overexpressed in Xenopus oocytes can carry a range of divalent cations, including Mg^{2+}, Co^{2+}, Mn^{2+}, Sr^{2+}, Ba^{2+}, Cu^{2+}, and Fe^{2+}, where only Zn^{2+} effectively inhibits transport [26]. Mg^{2+}-transport via Acdp2 was found to be voltage dependent, independent from Na^+ or Cl^- ions, and the Michaelis constant of the uptake to be around 0.5 mM. As mentioned previously, ACDP2 was one of the genes whose mRNA level was found to be increased upon Mg^{2+}-deprivation, reinforcing the notion that Acdp2 and possibly its homologues, ought to be added to the now growing list of vertebrate Mg^{2+}-transporters. It will be of great interest to characterize the other Acdp family members, but also to further define the potential role these puzzling molecules might play in modulating Mg^{2+} homeostasis in vertebrates through in vitro genetic deletion or overexpression studies.

Paracellin-1 (claudin 16) in gap junctions of the renal epithelium

Total body Mg^{2+} homeostasis in mammals is maintained by a careful balance between intestinal absorption and renal excretion and reabsorption. Patients suffering from a hereditary disease called hypomagnesemia with hypercalciuria and nephrocalcinosis (FHNC) experience massive renal Mg^{2+} and Ca^{2+} wasting, leading almost irremediably to end-stage kidney failure. As described in the next section, in contrast to TRPM6-deficient patients, the symptoms and gradual kidney damage caused by FHNC cannot be alleviated by intravenous or oral supplementation with Mg^{2+}, and kidney transplantation is often the only option at later stages of the disease. FHNC was suggested to originate from a defect in paracellular reabsorption in the kidneys, and in 1999, the gene responsible for this disorder was identified by positional cloning and named Paracellin-1 (PCLN-1) [32]. To date, more than 20 distinct mutations have been identified, which affect either the trafficking of PCLN-1, or its permeability [33]. The encoded protein is a member of the claudin family of tight-junction proteins that exhibit four transmembrane spans with two extracellular loops and both termini located in the cytoplasm. PCLN-1 has therefore been newly classified as claudin 16. Paracellular flux is thought to be the main renal Mg^{2+} reabsorption mechanism, and although it is impassable for water, it exhibits high conductance for Mg^{2+} and Ca^{2+}, probably involving PCLN-1. Based on these findings, it is tempting to hypothesize that PCLN-1 might form an Mg^{2+}-conducting pore. There is some discrepancy between results obtained upon overexpression of PCLN-1 in two different kidney epithelial cell lines. A recent study performed in the porcine proximal tubule LLC-PK1 cells suggests that PCLN-1 mediates mostly paracellular Na^+ permeation, and acts to generate and maintain the positive lumen potential that is the driving force for the Mg^{2+} and Ca^{2+} reabsorption [34], whereas in canine MDCK cells another group found PCLN-1 to decrease Na^+ permeability and to increase Mg^{2+} transport [35]. As described for other molecules relevant to Mg^{2+} homeostasis such as ACDP2 or MagT1, the human PLCN-1 promoter appears to be responsive to external Mg^{2+} concentrations, since the renal epithelial cell specific PLCN-1 gene expression correlates with the amount of Mg^{2+} offered in the growth medium [36]. As mentioned previously, multiple mutations in PLCN-1 appear to affect the proper delivery of the protein to the tight junctions [33]. At least one known mutation (T233R) impairs the interaction between PLCN-1 and the tight junction scaffolding protein ZO-1, resulting in the accumulation of PLCN-1 in the lysosomal compartment [37]. It was subsequently shown that protein kinase A (PKA) mediates the phosphorylation of PLCN-1 at Ser217, and that dephosphorylation or the S217A mutation leads to its dissociation from ZO-1 and its translocation into the lysosomes [35]. In conclusion, although the exact mechanism by which PLCN-1 influences paracellular Mg^{2+} and Ca^{2+} fluxes remains uncertain, there is no doubt that this molecule plays a central role in this process, as the severity of the disease caused by its alteration sadly documents.

The unexpected: the chimeric channel kinases TRPM6 and TRPM7

Discovery and primary biophysical characterization

Perhaps the most unexpected newly identified players in the regulation of Mg^{2+}-homeostasis in vertebrates are the two TRP (transient receptor potential) channel family members TRPM6 and TRPM7. These two molecules are structurally unique, as they are to date the only known fusions of an ion channel with a
kinase domain, a feature unique to the vertebrate TRPM6/7 proteins (figure 1). TRPM2, another member of the TRPM subfamily, is the only other example of an ion channel/enzyme fusion (“chanzyme”) [38], but instead of a kinase domain, TRPM2 is harboring an ADP-ribose hydrolase homologue at its C-terminus [39]. When TRPM7 and TRPM6 were originally cloned, it was not in a conscious effort to identify proteins involved in Mg2+-physiology. Alexey Ryazanov’s group had identified both genes seeking for homologues of eEF2kinase, and had named them CHAK1 (TRPM7) and 2 (TRPM6), standing for CHand AMP K inase [40]. Around the same time, TRPM7 was also isolated by two other laboratories; David Clapham and colleagues identified the TRPM7 kinase domain in a yeast-two-hybrid screen using the C2-domain of phospholipase C \( \beta \) PLC \( \beta \) as a bait [41], whereas we cloned TRPM7 (CHAK1, LTRPC7, TRP-PLIK) using a bioinformatics approach designed to identify novel potential ion entry pathways in human lymphocytes, with a particular focus on \( \text{Ca}^{2+} \)-signaling [42]. The biophysical characterization of heterologously expressed TRPM7 channels performed in these two studies differed in several aspects. Whereas Runnels et al. found that TRPM7 mediated nonselective, \( \text{Ca}^{2+} \)-permeant currents [41], we described TRPM7 as a \( \text{Ca}^{2+} \)- and \( \text{Mg}^{2+} \)-permeable, divalent cation specific channel, inhibited by intracellular \( \text{Mg}^{2+} \) [42]. Under physiological ionic conditions, TRPM7 carries preferentially \( \text{Mg}^{2+} \) and \( \text{Ca}^{2+} \), and is also expected to be involved in the transport of trace divalent cations such as \( \text{Ni}^{2+} \) or \( \text{Zn}^{2+} \) [43, 44].

The role of the covalently linked C-terminal kinase was also controversially discussed; Runnels et al. claimed that the kinase domain is essential for channel activation and gating [41], whereas we found that the kinase is not required for channel function, and does not fulfill the role of a gating domain [24, 42]. This latter model was confirmed by mutagenesis studies in which abrogating TRPM7’s phosphotransferase activity did not affect the activation of TRPM7 under conditions of low \( \text{Mg}^{2+} \) [24, 45]. The deletion of the entire kinase domain in human TRPM7 resulted in a partially active channel in our hands, although current amplitudes were markedly reduced to around 10% of wildtype levels [24]. Importantly, this Akinase version was also sufficiently biologically active to partially rescue the growth-deficient phenotype of a TRPM7-/- cell line. A slightly different construct generated from murine TRPM7 by Matsushita et al. was found to be totally inactive, which might originate from a difference in stability or trafficking properties between these two TRPM7\(-\)Akinase proteins [45]. The surface expression of the murine and human Akinase constructs has not been analyzed, but it was shown that hTRPM7\(A\)kin can associate with wildtype hTRPM7 and be cross-phosphorylated by it [46].

Native currents with properties similar to heterologously expressed TRPM7 were designated MagNuM for Magnesium-Nucleotide regulated Metal ion currents [42], or MIC for Magnesium Inhibited Cation [47] current, reflecting the inhibitory effect of intracellular \( \text{Mg}^{2+} \), as well as Mg-nucleotides, on TRPM7-mediated currents. These currents have been detected in a large number of different cell types, excitable and non-, confirming the ubiquitous distribution of TRPM7 already demonstrated at

![Figure 1](image_url)

**Figure 1.** Schematic representation of TRPM6 and TRPM7 homomers, and TRPM6/7 heteromer. Two subunits of each channel are represented as half a pore embedded in the lipid bilayer of the cell membrane. CCR = Coiled coil region.
mRNA level. A recent expression profile that included 22 TRP family members showed TRPM7 as the one molecule with the ubiquitously highest levels of transcript [48]. TRPM7's Mg2+ permeability and gradual inhibition by increasing [Mg2+] were the first clues suggesting a potential participation of TRPM7 in Mg2+-related cellular processes. As discussed in the next section, gene disruption approaches provided even more convincing evidence that TRPM7 might be the long sought for master regulator of Mg2+-homeostasis.

The biophysical characterization of homomeric TRPM6 was achieved by two different laboratories [49, 50], whereas another two groups including ours were not able to detect currents mediated by TRPM6, nor its presence at the cell surface, unless TRPM7 was co-expressed [46, 51]. There could be several reasons for this discrepancy, since any characteristic of the used expression constructs, cell lines, media components, or growth conditions might potentially affect trafficking and assembly of proteins. In these studies where TRPM6 could be measured, it was found to generate a current with similar properties than TRPM7, being a steeply outwardly rectifying conductance, permeability to Mg2+ and Ca2+, no voltage dependence, inhibition by intracellular Mg2+, and augmentation by acidic pH [49, 50]. On the other hand, some features were unique to TRPM6 such as a lower permeability to Ni2+, and a significantly larger unitary conductance of 83.6 pS, as compared to 40.1 pS for TRPM7, and interestingly, 56.5 pS for the TRPM6/TRPM7 heteromer [50]. In the same study, TRPM6 and TRPM7 and their heteromer were also distinguishable pharmacologically, as 2-APB at low concentrations was found to activate TRPM6, but to inhibit TRPM7. These criteria might prove useful in the future to determine if the TRPM6 and TRPM7 homomers, as well as their heteromers, are all present in vivo. To date, no cell line or tissue has been found that would contain TRPM6 alone, given the ubiquitous distribution of TRPM7, but it is conceivable that TRPM6 and TRPM7 might be spatially segregated inside the same cell. Functionally, it is important to mention that despite their resemblance, TRPM6 and TRPM7 cannot compensate for each other’s biological activity. TRPM6 deficient patients are hypomagnesemic although TRPM7 is ubiquitously expressed, and TRPM7+ cells cannot be rescued by TRPM6 overexpression [46].

Master regulators of Mg2+-homeostasis?

Although TRPM7’s permeability towards Mg2+ and the inhibition of I_{TRPM7} by Mg2+ were first clues suggesting its possible involvement in modulating cellular [Mg2+], at first, no further observation in a biological system was available in support of this hypothesis. In the DT40 avian B-cell line that allows the comparatively easy genomic disruption of genes of interest, we had demonstrated that TRPM7 was surprisingly an essential gene, although various signaling components of the B-cell receptor pathway, such as the IP3-receptors or PLCγ2 could be deleted without affecting the viability of the cells [42]. This was indicative of TRPM7 playing a role in cell maintenance processes beyond a putative BCR Ca2+-signaling function. Shortly after these findings were published, two different groups using a positional cloning approach identified various mutations in the TRPM6 gene as being most probably causal to a rare autosomal recessive disease called Hypomagnesemia with Secondary Hypocalcemia (HSH) [52, 53]. If not detected and treated early, HSH leads to severe seizures in affected infants, resulting in neurological damage and even death. Total relief of the clinical symptoms can be achieved by intravenous and subsequent life-long oral Mg2+ supplementation.

Although the hypocalcemia is completely alleviated by this treatment, these patients remain hypomagnesic, with serum Mg2+ levels around 0.5-0.6 mmol/L [54]. Early physiological characterization of HSH had determined that the primary defect lies in the intestinal Mg2+ absorption, although it was more recently confirmed that renal Mg2+ wasting also occurs [55]. The assumption is that the supplementary Mg2+ intake increases passive paracellular absorption, since the transcellular pathway is defective. This is in contrast to paracellin-1 deficient patients who cannot be helped by Mg2+ supplementation, since the passive paracellular pathway is defective and cannot be compensated for by the transcellular route. Most TRPM6 HSH mutations lead to truncated versions of the channel except for two notable exceptions. The S141L missense mutation in the N-terminal cytosolic portion of human TRPM6 disrupts the tetrameric assembly of the channel and its heteromultimerization with TRPM7 [51]. A recently described second missense mutation found in HSH patients leads to the P1017R amino acid exchange in the putative pore region of TRPM6 [56]. Importantly, this mutant exhibits a dominant negative effect on TRPM7 when co-expressed with it. Both corresponding mutations in TRPM7, S138L and P1040R show the same functional defects.

It was subsequently found that the TRPM7 deficient DT40 cells can also be rescued and their growth rate restored to wildtype level if provided with
many other ion channels are known to be regulated. The presence of a kinase domain at the C-terminal end of TRPM6/7 is particularly intriguing: whereas most TRPM family members lack a kinase domain, TRPM6 and TRPM7 kinase as a signaling module

The presence of a kinase domain at the C-terminal end of TRPM6/7 is particularly intriguing: whereas many other ion channels are known to be regulated by kinases “in trans” through phosphorylation, there is no other example of a kinase domain included in the same molecule than a pore. Noticeably, the closest TRPM7 homologue in the fruit fly Drosophila is missing the kinase domain, possibly indicating that its presence became a requirement to allow supplementary regulation levels in more complex organisms, and is an adaptation specific to vertebrates.

The kinase domain of TRPM6 as well as TRPM7 belong to the atypical family of eukaryotic protein kinases including eukaryotic elongation factor-2 kinase (eEF2-kinase, eEF2K), Dictyostelium myosin heavy chain kinases A, B, and C, and several mammalian putative protein kinases [61, 62]. eEF2-kinase, previously called Ca²⁺/calmodulin-dependent protein kinase III, has been discovered as the first member of this family. A detailed biochemical characterization of TRPM7 kinase concluded that given intracellular ion concentrations, among divalent metal ions, only changes in [Mg²⁺] can directly influence the enzymatic activity of TRPM7-kinase [63].

We have shown that TRPM7-kinase is not required for channel gating, but might play a role as a modulator of channel activity [24]. This modulator function could itself be regulated by interaction partners coordinating channel activity and cellular physiology, but there could also be another side to this dual kinase/channel concept: reversing the roles, one could speculate that channel activity is regulating kinase activity, which would subsequently regulate associated cytoplasmic molecules, establishing a novel kind of link between ion-entry and signal transduction events into the cell. In contrast to Ca²⁺-signaling events where the ion itself has messenger function, the ion-flow through the TRPM7 pore would be the signal transduced through the putative change in activity of the covalently linked kinase domain.

TRPM7 and TRPM6 can phosphorylate themselves (also associated subunits in trans), and although TRPM6 can phosphorylate TRPM7, the opposite appears not to be true, indicating that despite their 95% homology, these two kinases can exhibit different substrate specificity [46]. A Ser/Thr rich segment just upstream of the TRPM7 kinase domain contains two serine residues that were shown by mass spectrometry to be autophosphorylated (Ser1551 and 1567 in the mouse version), and are also phosphorylated inside the cell. The mutation of these two residues does not affect channel activity nor its inhibition by Mg²⁺ [45]. One or several further site(s) including phosphothreonine(s) remain uncharacterized since the serine 1551/1567 double mutant shows...
some residual phosphorylation [45], and phosphothreonines in TRPM7 can be detected radioactively as well as by a phosphothreonine specific antibody [46].

Two recent studies have now identified annexin I and the myosin IIA heavy chain as the first substrates of TRPM7-kinase [64, 65]. Annexin-I (ANXA1) belongs to a family of Ca\textsuperscript{2+} - and phospholipid-binding proteins, and has been described as an important endogenous modulator of inflammation. It contributes to various cellular processes such as cell migration, growth and differentiation, apoptosis, vesicle fusion, lipid metabolism, and cytokine expression. It is phosphorylated by TRPM7 on Ser5, but the physiological implication of this phosphorylation remains unclear [64].

In lower eukaryotes such as the cellular slime mold Dictyostelium, alpha kinases are long known to inhibit the formation of Myosin II filaments by heavy chain phosphorylation, and therefore control actomyosin relaxation. It has now been demonstrated that TRPM7 also interacts with cytoskeleton components in a Ca\textsuperscript{2+} - and kinase-dependent manner following activation with bradykinin in a mouse neuroblastoma cell line. Overexpressing TRPM7 at a comparatively low level in these cells led to an increase in [Ca\textsuperscript{2+}] (the potential role of Mg\textsuperscript{2+} was not investigated), as well as cell spreading and stronger cell adhesion [65].

Perhaps not surprisingly, since TRPM7 appears to be a crucial element of cellular physiology, its regulation is complex as documented by multiple studies. The interaction between TRPM7 kinase and PLC isoforms prompted several groups to analyze the effect of phosphoinositides on TRPM7, and PIP\textsubscript{2} hydrolysis was described as being inhibitory in one study [66], whereas another could not confirm this observation, but proposed that TRPM7 is modulated by cAMP and PKA, by a mechanism requiring an active TRPM7 kinase [67].

Recently, a different group has shown that inhibition of TRPM7 currents in response to PLC activation only takes place under low [Mg\textsuperscript{2+}], but that at physiological concentrations, TRPM7 currents are actually activated following PLC activation [68]. Despite this controversy, there is no doubt that the complexity of TRPM7’s architecture reflects the high level of biological malleability of this system, in accordance to a model of TRPM7 functioning as a master sensor and regulator of cellular ion homeostasis.

**Conclusion**

As we have depicted in this review, the past few years have led to exciting discoveries in the Mg\textsuperscript{2+} field. For a long time, this productive area of research thoroughly documented the role of this essential cation in Biology and Medicine, but suffered from a lack of identified molecular components involved in these well characterized processes, therefore limiting our capacity to intervene therapeutically in cases of deregulated Mg\textsuperscript{2+}-homeostasis. Thanks to the discoveries made by many research teams, substantial progress has been made, leading to the identification of several unique and fascinating molecules. Further characterization of Mg\textsuperscript{2+} transporters and ion channels listed here, as well as the probable identification of novel ones will continue to contribute to our growing body of knowledge about this crucial aspect of cellular biology.

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