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SLC30A10: A novel manganese transporter

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Homozygous mutations in *SLC30A10* cause familial parkinsonism associated with manganese (Mn) retention. We recently identified SLC30A10 to be a cell surface-localized Mn efflux transporter and demonstrated that parkinsonism-causing mutations block its intracellular trafficking and efflux function. In *C. elegans*, SLC30A10 over-expression protected against Mn-induced lethality and dopaminergic neurotoxicity, consistent with results in mammalian systems. Here, we present new data about SLC30A10 function in *C. elegans*. SLC30A10 expression did not protect worms against $ZnSO_4$toxicity, suggesting that SLC30A10 does not mediate Zn export in *C. elegans*. Furthermore, while a blast search identified 5 potential SLC30A10 homologs in worms (*cdf-1, cdf-2, ttm-1* and *toc-1*; sequence identity <35%), knock-down of these genes showed a tendency of increased survival after Mn exposure (although only *ttm-1* was statistically significant), suggesting that the worm homologs may function differently.

Manganese (Mn) Induced Toxicity

Mn is an essential metal present at high levels in the environment. It is required for many enzymatic activities (such as superoxide dismutase, arginase and pyruvate carboxylase) across different species.[1] Mn is abundant in the food chain and environment. It is available from whole grains, legumes, rice and nuts, as well as chocolate, tea, leafy green vegetables and some fruits like blueberries, but at smaller amounts.[2] Although essential at trace amounts, elevated cellular levels of Mn are cytotoxic. The primary cause of Mn toxicity is exposure to elevated Mn under occupational settings. Industrial workers, such as miners, welders and smelters, and individuals working in and living around ferroalloy plants are at higher risk of Mn poisoning, secondary to inhalation of Mn-containing dusts or fumes.[3] Given that Mn is primarily excreted by the liver into bile[4] and the competitive nature of Mn with iron (Fe),[5] patients with liver disease and iron deficiency may be highly susceptible to Mn toxicity without exposure to elevated Mn[6,7] In addition, premature neonates receiving total parenteral nutrition (TPN, containing Mn) via intravenous TPN administration are also at heightened risk for Mn exposure.[8]

In cells, especially neurons, high levels of cytosolic Mn can result in oxidative stress,[9,10] mitochondrial dysfunction and DNA fragmentation,[11] autophagy dysregulation,[12] protein misfolding,[13] endoplasmic reticulum (ER) stress and apoptosis.[14] Mn toxicity in the nervous system leads to the development of an irreversible and progressive parkinsonian syndrome, termed manganism, which is characterized by an array of symptoms analogous to idiopathic Parkinson disease (PD), including a variety of psychiatric and motor disturbances. Notably, however, T1-weighted magnetic resonance imaging (MRI) shows excessive levels of Mn accumulation preferentially in the basal ganglia, especially in the globus pallidus.[15,16] This is distinct from PD, which primarily targets the substantia nigra pars compacta (SNpc).[17] In addition, patients with manganism do not clinically respond to levodopa.[18] Currently, there are limited treatments available for manganism patients.

Identification of SLC30A10

In 2008, Tuschl et al.[19] reported a new form of familial parkinsonism in a 12-year old girl with hypermanganesaemia, liver cirrhosis, an extrapyramidal motor disorder and polycythaemia. Her older brother died with the same symptoms. The Mn concentration (>3000 nmol/L) in her blood was 10 fold higher than normal. Genotyping of 2 known putative Mn transporters, SPCA2 and SERCA3, however, revealed that they were not the responsible genetic factors. Four years later, Tuschl *et al* and Quadri *et al* identified additional patients who presented with the same clinical picture and further demonstrated that all affected patients carried homozygous mutations in the gene coding for *SLC30A10*.[15,20] Interestingly, while all the patients exhibited 10–20 fold increase in blood Mn levels concomitant with Mn deposition in the basal ganglia; they had not been exposed to high Mn-containing environment, suggesting the Mn accumulation was due to a primary defect in Mn metabolism.[15,20] In humans, SLC30A10 is expressed at high levels in the liver and the central nervous system.[20] Several mutations that cause familial parkinsonism have been identified in

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SLC30A10, including L89P, Δ98–134, Δ105–107, T196P and Q308Stop.[15,20] Further, expression of wildtype (WT) *SLC30A10* in Mn sensitive Δ*pmr1* yeast cells rescued growth in high Mn-containing media, indicating that SLC30A10 may function to protect cells from Mn-induced toxicity.[15]

Exploring the Role of SLC30A10 in Mn Homeostasis and Detoxification

Based on sequence similarities, SLC30A10 is one of the 10 solute carrier family 30 (SLC30) transporters (ZnTs, Zn transporters), belonging to a large superfamily of transporters referred to as the cation diffusion facilitators (CDFs). ZnT-1 to ZnT-8 are known Zn transporters. ZnT1 functions primarily as an exporter on the cell membrane and ZnT-2 to ZnT-8 are localized on the membrane of intracellular organelles to transport cytoplasmic Zn into various compartments.[21] There is no evidence to support a role for ZnT-9 in transporting Zn and it appears to function as a cofactor of nuclear receptors.[21] SCL30A10 (or ZnT-10) was predicted to be a Zn transporter.[22]

After mapping out SCL30A10 mutations in the familial parkinsonian patients, we became interested in its function in rescuing Mn-induced toxicity. Using a combination of mechanistic and functional studies in cell culture (HeLa and AF5 cells), *Caenorhabditis elegans* (*C. elegans*) and mouse primary midbrain neurons, we showed that SLC30A10 functions primarily as an exporter at the cell membrane to transport cytoplasmic Mn ions across the membrane to the extracellular space, thus reducing intracellular Mn levels and protecting against Mn-induced toxicity.[23] Further, we discovered that the disease-causing SLC30A10 mutants (L89P, Δ98–134, Δ105–107, T196P and Q308Stop) failed to traffic to the cell surface, and instead, were trapped in the ER.[23] Importantly, our results revealed that these mutant proteins also failed to mediate Mn efflux.[23] In *C. elegans* exposed to 100 and 200 mM of $MnCl_2$, expression of WT-SLC30A10 significantly increased worm survival rate when compared with control worms, while the disease-causing mutant L89P had no effect.[23] Given the neurotoxicity of Mn, the function of SLC30A10 in neurons was also investigated. Note that in *C. elegans*, Mn specifically targets dopaminergic (DAergic) resulting in their neurodegeneration.[10] Using green fluorescent protein (GFP)-labeled DAergic neurons, we found that expression of WT-SLC30A10 prevented Mn-induced DAergic neurodegeneration while expression of the L89P mutant had no protective effect.[23] This phenotypic result was confirmed by basal slowing response, a behavior specifically mediated by DAergic neurons.

SLC30A10 is a Mn Specific Transporter

Mn measurement assays in HeLa cells, performed using inductively coupled plasma mass spectrometry, revealed that cells expressing SLC30A10-WT contained significantly lesser intracellular Mn compared to transfection control cells.[23] Further, a pulse-chase assay performed in HeLa cells demonstrated that, compared to transfection control cells, expression of SLC30A10-WT increased Mn efflux while the Δ105–107 mutant had no effect.

As SLC30A10 was first predicted to be a Zn transporter, here we queried whether this protein is able to transport Zn. To test this hypothesis, we exposed *C. elegans* expressing WT or L89P SLC30A10 to $ZnSO_4$ and analyzed their survival rate.[23] As shown in **Figure 1**, worms expressing WT or L89P SLC30A10 showed analogous sensitivity to $ZnSO_4$ with a 50% lethal dose (LD_{50}) of 1.4 mM compared to control worms, showing an LD_{50} of 1.8 mM (**Fig. 1**). Consistent with our previous findings, expression of WT SLC30A10 in HeLa cells did not protects against Zn-induced cell death with no significant difference in intracellular Zn levels between WT SLC30A10 and transfection control cells.[23] Similarly, expression of WT SLC30A10 did not affect intracellular Cu levels in HeLa cells.[23]

These results indicate that SLC30A10 facilitates intracellular Mn export, but not Zn or Cu; thus, SLC30A10 may be the first Mn specific transporter. As SLC30A10 specifically transports Mn, its function might not be replaced by other shared metal transporters, such as the divalent metal transporter (DMT-1) and transferrin, to name a few. This may explain why patients carrying SLC30A10 mutations have >10 fold increase in Mn blood levels when compared with controls.

Functional Role of *C. elegans* Homologs of Human SLC30A10

A Basic Local Alignment Search Tool (BLAST) search in Wormbase.org protein database using SLC30A10 protein

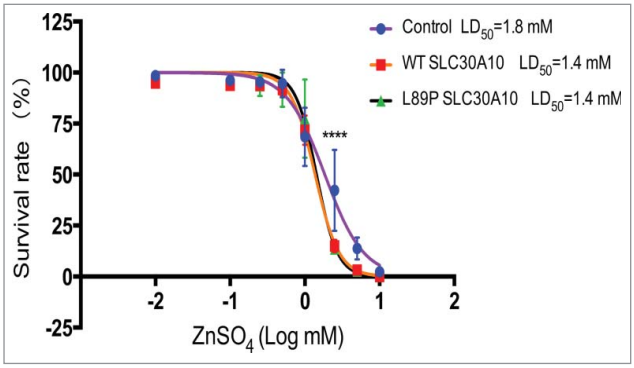
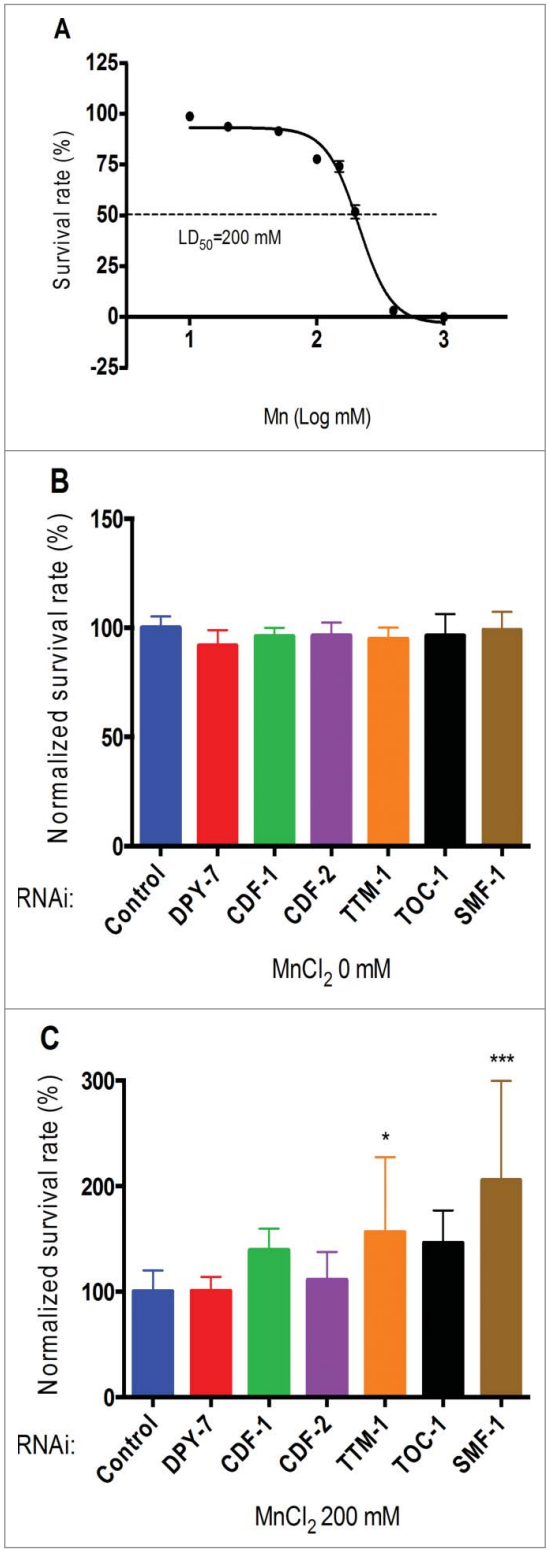


Figure 1. SLC30A10 is not a Zn transporter. Worms were synchronized at larva 1 (L1) stage and then exposed to 0, 0.01, 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 mM of $ZnSO_4$ in 85mM of NaCl for 1 hour, respectively. ~30 treated worms were put on a NGM plate seeded with food. One day later, the living worms were scored and the survival rate was calculated. The data were analyzed using GraphPad Prism 6. Multiple group comparison at the same time was done using 2 way ANOVA with the Dunnett's multiple comparison test. Values are Mean ± SD (N = 3). The interaction (treatment by strain) P value = 0.0335. There are no significant differences between 3 strains except at 2.5 mM $ZnSO_4$, control worms showed a higher survival rate than WT and L89P SLC30A10 worms (***$P < 0.0001$).

sequence found 5 potential SLC30A10 homologs in worms: CDF-1, CDF-2, Y105E8A.3, TTM-1 and TOC-1. However, the homology is low with CDF-1 shares the highest sequence identity to human SLC30A10 (~34%) and TOC-1 the lowest (~23%). CDF-1 and CDF-2 are known Zn transporters.[24,25]



TTM-1 and TOC-1 were predicted to regulate Zn homeostasis, while Y105E8A.3 has an unknown function. First, we established a Mn dose-dependent lethality curve of larva stage 4 (L4) worms in a RNAi sensitive strain GR1373 [*eri-1*(*mg366*)] (**Fig. 2A**). Treatment with 200 mM $MnCl_2$ for 1 hour induced ~50% death in this strain (LD_{50}≈200 mM). Next, we investigated whether knocking down the known primary nematode Mn transporter (SMF-1; the homolog of mammalian DMT-1) affected the lethality in *C. elegans*. DPY-7 served as a negative control, since knocking down of this gene results in an easily visible dumpy phenotype, but does not affect the sensitivity to Mn. Knock down of SMF-1 did not affect worm development and lethality in the absence of Mn exposure (**Fig. 2B**). When exposed to Mn, SMF-1 knockdown worms showed a significant increase in survival rate compared to control worms (**Fig. 2C**). The result was consistent with previous findings that SMF-1 is the primary Mn importer in *C. elegans*.[26] The worm SLC30A10 homologs (except Y105E8A.3) were knocked down using corresponding RNAi bacteria. All RNAi bacteria were from the Ahringer RNAi library (Source BioScience). Knocking down these genes did not affect the development of worms and had no effect on lethality, in the absence of Mn exposure (**Fig. 2B**). Interestingly, a trend toward elevated survival rate was noted in the knockdown worms in the presence of Mn (**Fig. 2C**), arguing against their role in exporting Mn. Among the 4 homologs, only *ttm-1* showed significantly increased survival after Mn exposure. *ttm-1* encodes 2 transcripts, *ttm-1a* and *ttm-1b*. Roh et al. showed that TTM-1B is induced by high dietary Zn and regulates Zn excretion from intestinal cells into the lumen of the gut, however, TTM-1A is not Zn regulated and its function remains unknown.[27] The RNAi bacteria we used here specifically targeted *ttm-1a*, but not *ttm-1b*. These results indicated a novel role of TTM-1A to transport Mn intracellularly. Notably, *ttm-1* encodes an ortholog of SLC30A4, which is a known Zn transporter (ZnT-4).[21] Pups die of Zn deficiency when they consume milk from mice carrying homozygous lethal milk mutation in ZnT-4.[28] SLC30A4 and SLC30A10 both belong to the SLC30 family of solute carriers, and they have 33% sequence identity. Our results raise the possibility that SLC30A4 (ZnT-4) may be capable of facilitating Mn influx, but this has yet to be systematically tested.

Figure 2. *C. elegans* putative SLC30A10 homologs appear to assume different function compared to human SLC30A10. A RNA interference (RNAi) sensitive strain GR1373 [*eri-1*(*mg366*)] and a RNAi bacteria feeding method[30] was applied to knock down target genes. Worms were synchronized at L1 stage and fed on corresponding RNAi bacteria to L4 stage, followed by Mn exposure. The control worms were fed with bacteria carrying an empty vector. (**A**). Lethality curve of Mn for GR1373 L4 worms. (**B**). Knocking down of all genes did not affect development and survival in the absence of Mn. (**C**). Knocking down of SMF-1 and TTM-1 significantly increased survival in the presence of Mn exposure. For each treatment, 30 worms were analyzed. All survival rates were normalized to the survival rate of the corresponding control. The data were analyzed using GraphPad Prism 6. Multiple group comparison at the same time was done using one way ANOVA with the Dunnett's multiple comparison test. Values are Mean ± SD (N = 3). *, $P < 0.05$; ***, $P < 0.001$.

Conclusions

Using *C. elegans* as an animal model, we showed that worms expressing WT and L89P SLC30A10 responded in an analogous fashion to $ZnSO_4$ treatment. Combined with our previous cell culture results, our findings indicated that SLC30A10 is likely not a Zn transporter. In addition, our RNAi results showed that *C. elegans* might not have a SLC30A10 homolog or that the putative homologs assume different function from human SLC30A10. Surprisingly, we found that *ttm-1*, the ortholog of human ZnT-4, might function to facilitate Mn influx.

Mn is an essential metal that is required for various cellular enzymatic activities. However, an overload of Mn may lead to a parkinsonian disorder. Mn homeostasis is tightly regulated by a number of transporters. To date, several Mn importers have been identified, including the divalent metal transporter 1 (DMT1), transferrin receptor (TfR), Zn transporters-ZIP8 and ZIP14, Dopamine transporter (DAT), Ca channels, choline transporter and Citrate transporter.[29] Ferroportin and SLC30A10 are the only 2 transporters localized on cell membrane that facilitate Mn export. Importantly, among these transporters, only SLC30A10 is Mn specific. Given its specificity, SLC30A10 may prove an optimal pharmacological target for regulating intracellular Mn concentrations. A small molecules screen identifying enhancers of Mn efflux activity of SLC30A10 may be of benefit to individuals exposed to high levels of Mn.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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