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The Mucolipin-1 (TRPML1) Ion Channel, Transmembrane-163 (TMEM163) Protein, and Lysosomal Zinc Handling

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Abstract

Lysosomes are emerging as important players in cellular zinc ion (Zn^{2+}) homeostasis. The series of work on Zn^{2+} accumulation in the neuronal lysosomes and the mounting evidence on the role of lysosomal Zn^{2+} in cell death during mammary gland involution set a biological precedent for the central role of the lysosomes in cellular Zn^{2+} handling. Such a role appears to involve cytoprotection on the one hand, and cell death on the other. The recent series of work began to identify the molecular determinants of the lysosomal Zn^{2+} handling. In addition to zinc transporters (ZnT) of the solute-carrier family type 30A (SLC30A), the lysosomal ion channel TRPML1 and the poorly understood novel transporter TMEM163 have been shown to play a role in the Zn^{2+} uptake by the lysosomes. In this review, we summarize the current knowledge on molecular determinants of the lysosomal Zn^{2+} handling, uptake, and release pathways, as well as discuss their possible roles in health and disease.

Keywords

Mucolipidosis IV; lysosomes; zinc transport; SV31

Introduction

The central paradigm of Zn^{2+} handling involves its entry in cells via the plasma membrane transporters, chelation by the cytoplasmic proteins, and export into membrane-bound organelles via dedicated transporters (Falchuk et al., 1995; Sensi et al., 1997; Colvin et al., 2000; Balaji and Colvin, 2005). As commonly known, such organelles include components of the Golgi network, mitochondria, and the “secretory vesicles.” The observations in neuronal cells, in breast tissue, and in certain model system points out that lysosomes are

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also involved in the Zn^{2+} handling. Such evidence is based on Zn^{2+} accumulation in the lysosomes of cells exposed to Zn^{2+}, or in cells undergoing processes associated with large Zn^{2+} transitions, such as cell death at the onset of mammary gland involution and pathological events (Eichelsdoerfer et al., 2010; Kelleher et al., 2011; Seo et al., 2011; McCormick and Kelleher, 2012; Kukic et al., 2013; Cuajungco et al., 2014; Kukic et al., 2014). The lysosomal involvement in Zn^{2+} handling is interesting from several perspectives. First, it redefines the role of the lysosomes as purely digestive organelles. Second, it uncovers some interesting new biology pertaining to cell death and survival, especially in tissues in which the lysosomal involvement has been under-appreciated. Third, it shows the involvement of new regulatory circuits involving energy sensing, oxidative stress, and organellar biogenesis.

Zn^{2+} enters the lysosomes through endocytosis of Zn^{2+}-bound proteins, autophagy of Zn^{2+}-rich organelles, and from the cytoplasm via the zinc transporters located in the lysosomal membrane. Proteomic analysis of the lysosomal membrane suggested the presence of ZnT2 and ZnT4 transporters, which was confirmed using confocal microscopy and knockdown studies in several systems (Murgia et al., 1999; McCormick and Kelleher, 2012; Roh et al., 2012; Bostanci et al., 2014). Another pathway, a “maturation” of Zn^{2+}-rich secretory granules into the lysosomes has been shown in the mammary gland (Lopez and Kelleher, 2009; Seo et al., 2011; McCormick et al., 2014)**;** the presence of such a process in other tissues remains to be an interesting question. Whereas the Zn^{2+} entry into the lysosomes via endocytosis and autophagy appears to be a normal consequence of the lysosomal digestive function, its uptake from the cytoplasm into lysosomes is an emerging role for these organelles. Based on this evidence, we proposed a concept of the lysosomal Zn^{2+} sink, a process of absorption of Zn^{2+} from the cytoplasm into the lysosomes. This process could be especially important during abrupt changes in the cytoplasmic concentration of Zn^{2+}, as it would give the cells time to update their Zn^{2+} chelation and extraction capacities (Kukic et al., 2014) (Fig. 1).

An extremely attractive aspect of the lysosomal biology that sets it apart from the Golgi as a Zn^{2+} sink is its dynamic regulation by the recently discovered lysosomal gene network. Such a network regulated by transcription factor EB (TFEB), and its relatives TFE3 (transcription factor binding IGHM enhancer 3) and MITF2 (microphthalmia-associated transcription factor 2) was shows to relay the information from mechanistic target-of-rapamycin (mTORC1) to transcriptional activity of a number of genes coding for the lysosomal and autophagic proteins (Martina et al., 2014). Since mTORC1 is a signaling nexus responsible or gauging the cellular energy and the functional status of the lysosomes (Settembre et al., 2011; Martina et al., 2012), a possible role of the lysosomes as a cellular Zn^{2+} clearance pathway casts a new light on these organelles as key players. Indeed, if the lysosomes are a powerful transition metal buffering and extraction pathway, as was recently proposed, then perhaps the definition of the lysosomes and the lysosomal gene network might be amended to include the oxidative stress response element. The recent evidence that the lysosomal biogenesis is stimulated by the transition metal exposure supports this idea (Peña and Kiselyov, 2015).

The growing knowledge of the lysosomal role in Zn^{2+} handling coincides with the discovery of the lysosomal exocytosis and lysosomal biogenesis as driving factors in cellular detoxification. Upregulation of the lysosomal biogenesis stimulated by the TFEB overexpression has been linked to improving cellular phenotypes in *in vitro* models of several diseases including Pompe, Alzheimer's, Parkinson's, Huntington's, and hepatic SERPINA1 deficiency (La Spada, 2012; Decressac et al., 2013; Feeney et al., 2013; Spampanato et al., 2013; Polito et al., 2014). Such an improvement has been linked to increased removal of the storage bodies and misfolded protein aggregates. Lysosomal exocytosis has been proposed as a driving force behind such an improvement (Medina et al., 2011; Feeney et al., 2013). It is thus telling that TFEB overexpression and increased lysosomal exocytosis have been linked to improved removal of copper ions (Cu^{2+}) and Zn^{2+}, and suppressed oxidative stress in cells exposed to these metals (Peña et al., 2015; Peña and Kiselyov, 2015). On the contrary, suppression of the lysosomal exocytosis has been linked to increased oxidative stress in cells treated with Cu^{2+} or Zn^{2+}. It is important to note that beyond the "lysosomal" genes, the TFEB-responsive network (Settembre and Medina, 2015) incorporates genes involved in regulation of oxidative stress such as heme oxygenase 1 (HMOX1). Indeed, the TFEB-dependent upregulation of HMOX1 expression has been shown in response to Cu^{2+} exposure of cultured cells (Peña and Kiselyov, 2015). To further the argument for the interaction of the lysosomal biogenesis and oxidative stress responses, regulation of the lysosomal biogenesis by FXR-CREB and PPARα transcription factors previously implicated in the oxidative stress responses has been shown (La Spada, 2012; Lee et al., 2014; Seok et al., 2014; Ghosh et al., 2015).

Taken together, this evidence strongly suggests that the lysosomes play an important role in the cellular defense against oxidative stress not only by destroying damaged organelles, but also by taking up and removing toxic metal from the cytoplasm. Below we summarize the available data and the current concepts involving these processes, as well as highlight recent discoveries on intracellular Zn^{2+}-buffering mediated by Zn^{2+} transport proteins associated with the lysosomes.

The Lysosomal Zn^{2+} Sink

Zn^{2+} accumulation into the lysosomes via the endocytic/autophagic pathway is a logical and predictable consequence of the endocytic and autophagic activities, albeit has not been extensively pursued until recently. Lysosomal degradation of Zn^{2+}-rich organelles and Zn^{2+}-bound proteins in the acidic environment releases Zn^{2+}. Indeed, elevated free vesicular (lysosomal) Zn^{2+} has been shown shortly after the first Zn^{2+}-sensitive fluorescent dyes had become available (Gee et al., 2002).

A buildup of Zn^{2+} in the lysosomes of cells exposed to oxidative stress or high Zn^{2+} levels to induce Zn^{2+} uptake across the plasma membrane has been shown in various cell culture models using the high affinity dye, Fluozin-3 (Hwang et al., 2008; Chung et al., 2009; Lee et al., 2009; Eichelsdoerfer et al., 2010; Kukic et al., 2013; Cuajungco et al., 2014). Such buildup has led to lysosomal permeabilization followed by the release of the lysosomal digestive enzymes and cell death by the autophagic scenario. Due to the previous evidence of Zn^{2+} buildup in brains affected by stroke and other neurodegenerative diseases (Koh et

al., 1996; Lees et al., 1998; Suh et al., 2000), these findings were interpreted as an explanation for cell death in these diseases. According to this model, an excess cytoplasmic Zn^{2+} entering the cells at increased rate through the plasma membrane (Sensi et al., 1997) or due to liberation of Zn^{2+} from the cytoplasmic Zn^{2+}-binding proteins (Cuajungco and Lees, 1998; Frederickson et al., 2002; Lee et al., 2003), which floods the cytosol and subsequently, the lysosomes and mitochondria (Sensi et al., 2002; Hwang et al., 2008) (Fig. 1). Such a Zn^{2+} overload then triggers cell death (Cuajungco and Lees, 1997; Choi and Koh, 1998; Frederickson et al., 2004). Interestingly, a significant increase in cerebral Zn^{2+} levels of Mucolipin-1 knockout (*Mcoln1*$^{-/-}$) mice, a model for Mucolipidosis type IV (MLIV), has been reported (Eichelsdoerfer et al., 2010; Kukic et al., 2013; Cuajungco et al., 2014). It was surmised that lysosomal Zn^{2+} overload could potentially contribute to MLIV pathology, as well as cause progressive neuronal and retinal cell degeneration (Eichelsdoerfer et al., 2010). MLIV is a human lysosomal storage disease caused by a loss-of-function mutation or deletion in the Mucolipin-1 (TRPML1) ion channel (Bargal et al., 2000; Bassi et al., 2000; Sun et al., 2000). TRPML1 confers non-selective permeability to calcium (Ca^{2+}), Zn^{2+}, ferrous iron (Fe^{2+}), and manganese ions (Mn^{2+}) (Grimm et al., 2007; Dong et al., 2008; Dong et al., 2009; Dong et al., 2010), suggesting that TRPML1 may function in metal homeostasis. Indeed, Zn^{2+} mishandling by the lysosomes in MLIV-affected cells has been revealed consistently (Eichelsdoerfer et al., 2010; Kukic et al., 2013; Cuajungco et al., 2014), in addition to a previous report that Fe^{2+} overload may also be a contributing factor in disease etiology (Dong et al., 2008). It is interesting to note that lysosomal permeabilization and the release of Cathepsin B have been shown in an *in vitro* model of MLIV (Colletti et al., 2012), which potentially correlates with lysosomal Zn^{2+} accumulation in MLIV cells as a pathological trigger. Meanwhile, abnormal iron homeostasis has been implicated in MLIV disease because many MLIV patients suffer from anemia (Altarescu et al., 2002); however, it was reported recently that a decrease in cerebral ferric iron (Fe^{3+}) load but not total iron levels is a common feature of ten-day old *Mcoln1*$^{-/-}$ knockout mice (Grishchuk et al., 2015). The change in Fe^{3+} levels was correlated with abnormal myelination of the brains of *Mcoln1*$^{-/-}$ knockout mice relative to wild type controls (Grishchuk et al., 2015). Notwithstanding, anemia in MLIV patients is likely due to a decrease in iron uptake associated with gastrointestinal problems manifested by the disease.

Neuronal and retinal abnormalities are a hallmark of lysosomal storage diseases in which a very large fraction is associated with degenerative phenotypes and developmental delays. Both tissues are known for high Zn^{2+} requirements. Zn^{2+} is co-released with glutamate to modulate neuronal transduction (Howell et al., 1984) and very large changes in retinal Zn^{2+} content have been shown to accompany the light-dark cycle (Redenti and Chappell, 2005; Lengyel et al., 2007; Redenti et al., 2007). Owing to its importance, several reports have shown that depletion of Zn^{2+} induces degeneration of retinal pigment epithelium (Hyun et al., 2000; Hyun et al., 2001), while oxidant-induced Zn^{2+} overload may also contribute to retinal cell death (Yoon et al., 2000; Yoo et al., 2004; Chung et al., 2009). Despite these observations, the role of the lysosomes in retinal Zn^{2+} handling has not been pursued, and in general, there is a dearth of knowledge on the lysosomal function in the retina with respect to lysosomal biogenesis and exocytosis.

The first evidence of Zn^{2+} transporters localized to the lysosomal membrane came from mammary gland in which involution at the end of lactation appears to be associated with translocation of the transporter ZnT2 to the lysosomal membrane and a buildup of Zn^{2+} in the lysosomes. This is followed by the destabilization of the lysosomal membrane and cell death. In contrast to the neuronal tissue, lysosomal Zn^{2+} buildup in the mammary gland appears to be dynamically regulated component of a developmental program. For a complete account of Zn^{2+} handling and transporters in the involuting mammary gland, we refer to a recent comprehensive review on this topic by Kelleher and colleagues (McCormick et al., 2014). A confocal analysis of recombinant protein and short interfering RNA (siRNA)-mediated analysis showed that while recombinant ZnT2 and ZnT4 are routed to the lysosomes in HeLa cells, the native ZnT2 does not contribute to the lysosomal Zn^{2+} uptake in these cells. Instead, this seems to be a function of ZnT4 in HeLa cells. This is consistent with the previously shown exclusive localization of ZnT2 in the mammary gland tissue and a wider expression of ZnT4 (McCormick and Kelleher, 2012). The lysosomal localization of ZnT4 has been confirmed by the recently published lysosomal proteomic analysis (Chapel et al., 2013).

While the uptake of Zn^{2+} from the cytoplasm into the lysosomes and the resulting buildup and toxicity fits well into the idea of Zn^{2+}-mediated cell death under degenerative conditions and during the mammary gland involution, such mechanisms may play a cytoprotective role in other tissues. However, this protective process requires elimination of the lysosomal Zn^{2+} at the end of the Zn^{2+} spike. Lysosomal exocytosis seems to have emerged as such a defense mechanism (Kukic et al., 2014). Originally proposed as a means of plasma membrane repair (Reddy et al., 2001), lysosomal exocytosis is now being recognized as a key cellular detoxification and stress repair pathway. Lysosomal exocytosis is driven by the lysosomal fusion with the plasma membrane, mediated by VAMP7 and synaptotagmin 7, and by the cytoplasmic calcium ion (Ca^{2+}) spike (Logan et al., 2006). The latter was posited to depend on the endolysosomal Ca^{2+} release via TRPML1 and TRPML3 ion channels (Kiselyov et al., 2012; Samie et al., 2013; Miao et al., 2015), although its dependence on Ca^{2+} entry across the plasma membrane has been shown as well (Peña et al., 2015). As previously mentioned, lysosomal exocytosis of storage bodies and unfolded proteins was suggested to underlie the improvement of cellular phenotypes of several diseases (Medina et al., 2011). In these experiments, the *in vitro* disease models were transiently transfected with TFEB cDNA to cause overexpression of this transcription factor and stimulation of the lysosomal exocytosis. The resulting improvement was attributed to the increased lysosomal exocytosis and increased extraction of toxic material. The role of lysosomal exocytosis in the removal of excess (lysosomal) Zn^{2+} from cells has been recently shown using siRNA-mediated knockdown of VAMP7 and synaptotagmin 7, which resulted in the buildup of Zn^{2+} and oxidative stress in Zn^{2+}-treated cells (Kukic et al., 2014).

Based on the data summarized in this chapter, a concept of the lysosomal Zn^{2+} sink was proposed. We postulated that Zn^{2+} uptake into the lysosomes via the lysosomal Zn^{2+} transporters quickly lowers the cytoplasmic Zn^{2+} levels and gives the cells time to induce expression, modify, and target ionic buffering through various efflux transporters and chelating proteins (Fig. 1). Such high capacity dynamic system may serve as a first line of defense mechanism against the cytoplasmic Zn^{2+} spike – lysosomes occupy 3% to 5% of the

cellular volume according to some estimates (Draye et al., 1988). Lysosomes are thus able to respond to a wide range of signals and are dynamically regulated by the signaling loop of circuits concerning cell stress (e.g. oxidative stress, lysosomal damage, and starvation). An increase in the cytoplasmic Zn^{2+} clearance may serve a cytoprotective role, while such clearance may be decreased when cell death is a desirable outcome. As shown in a recent publication (Kukic et al., 2014) by one of us (KK), the rate of lysosomal exocytosis is a factor defining its cytoprotective role. While some molecules involved in the lysosomal exocytosis of Zn^{2+} are fairly well understood, some are still poorly understood or just beginning to shed light on their new roles. One of such molecules is discussed in the next chapter.

TMEM163 and zinc accumulation

Transmembrane-163 (TMEM163) protein, also known as synaptic vesicle 31 (SV31), was first identified in rat brain synaptosomes using proteomics (Burre et al., 2007). The *TMEM163* gene is conserved across many vertebrate species (chimpanzee, Rhesus monkey, cow, dog, chicken, mouse, rat, zebrafish, and frog) and has over 100 orthologues. TMEM163 protein expression is detected in certain glutamatergic and γ-aminobutyric acid (GABA)-ergic neuronal populations (Burre et al., 2007; Barth et al., 2011). Its presence in synaptic-like micro-vesicles, large dense core vesicles, endosomes and lysosomes (Burre et al., 2007; Barth et al., 2011) overlaps with the enrichment of zinc in pre-synaptic vesicles of these neuronal populations (Frederickson, 1989). Furthermore, subcellular fractionation of PC12 cell lysates stably expressing the rat Tmem163 also showed that this protein is detected in the plasma membrane, endoplasmic reticulum (ER), Golgi, mitochondria, and peroxisomes (Barth et al., 2011).

An interaction between TMEM163 and TRPML1 was recently shown using genetic (yeast two-hybrid) and biochemical (co-immunoprecipitation) assays (Cuajungco et al., 2014). The interaction between the two proteins appears to influence intracellular zinc homeostasis, at least in a heterologous expression system using cultured cells (Cuajungco et al., 2014; Silva and Cuajungco, 2015). Moreover, the expression level of TMEM163 is down-regulated in MLIV patient fibroblasts (Cuajungco et al., 2014). It is not clear, however, how such a reduction in TMEM163 protein potentially contributes to the disease phenotype. Nevertheless, the tissue mRNA expression pattern of *TMEM163* gene coincides well with that of *MCOLN1* (TRPML1) gene (Grimm et al., 2010; Cuajungco et al., 2014). Specifically, higher relative *TMEM163* transcripts are observed in the brain, lung, and testis, but notable levels are seen also in the pancreas, kidney, thymus, ovary, and intestines (Fig. 2). Confocal microscopy of heterologously expressed human TMEM163 shows plasma membrane (PM) and lysosomal localization (Fig. 3). A partial co-localization with TRPML1 is observed, suggesting that both proteins may have specific cellular function that is independent of each other (Fig. 3).

TRPML1 has been reported to have a di-Leucine motif [D/E]XXXL[L/I] or lysosomal targeting sequence (LTS) at the N-terminus, while another di-leucine motif situated at the C-terminus serves as an internalization signal for adaptor protein 2 (Vergarajauregui and Puertollano, 2006). Human TMEM163 (or rodent Tmem163) has a putative di-Leucine

motif [D/E]XXXL[L/I] or lysosomal targeting sequence (LTS) with amino acid residues EDRGLL at its N-terminus position 65–70 (Fig. 4) (Cuajungco et al., 2014). The LTS motif is also present on certain Zrt- and Irt-like proteins (ZIP, also known as SLC39A) and ZnT proteins. For example, ETRALL is found on ZIP1 (144–149), DDDSLL (72–77) on ZnT4, and DAAHLL on ZnT2 (103–108) or ZnT3 (105–110). In general, proteins containing a di-Leucine motif (Kozik et al., 2010) are targeted by adaptor proteins for membrane trafficking to and from the plasma membrane, tubular endosomes or endosomal-lysosomal compartment, or Golgi network (Hirst et al., 2011).

The rodent Tmem163 protein is predicted to have six transmembrane domains with long N-terminus and short C-terminus regions predicted to be cytoplasmic (Burre et al., 2007; Barth et al., 2011). A closer inspection of human TMEM163's protein sequence (Fig. 4) shows an apparent topological similarity with the ZnT proteins, whereby TMEM163 has a predicted long N-terminus but short C-terminus, while the ZnT proteins have a predicted short N-terminus but long C-terminus region. This may be, of course, a mere coincidence; however, we cannot rule out the possibility that TMEM163 may belong to the SLC39A or SLC30A family of influx or efflux transporter proteins, respectively. Further, the tissue specific expression pattern of TMEM163 coincides well with other ZnTs (Chimienti et al., 2006; Jackson et al., 2007; Bosomworth et al., 2012), and thus contributes to transporter redundancy in many cell types (Kambe et al., 2015).

The amino acid sequence alignment of rodent Tmem163 with mouse ZnT3, *E. coli* zinc transporter (YiiP), and *R. metallidurans* cobalt–zinc–cadmium resistance protein (CzcD) shows around 20% sequence identity (Barth et al., 2011). From the same alignment, two aspartate (D) residues on the predicted second transmembrane (TM) domain were conjectured to potentially bind Zn^{2+}. It is interesting to note that Histidine (H) and/or D residues such as the HXXXD motif (where X is a non-polar amino acid) located within TM2 and TM5 helices of ZnT proteins, and HXXXH motif in TM4 and TM5 helices of ZIP proteins have been suggested to facilitate tetrahedral zinc coordination (Ohana et al., 2009; Kambe et al., 2015). Collectively, these amino acid residues have been designated as HD-DD motifs present in zinc transport proteins (Kambe et al., 2015), and site-directed mutagenesis of native HD residues within TM4 and TM5 helices of the ZnT5 protein has been shown to disrupt zinc binding and transport activity (Ohana et al., 2009). Indeed, metal-binding assays on cells heterologously expressing rodent Tmem163 showed a strong binding preference to Zn^{2+} or nickel (Ni^{2+}), but weakly binds copper (Cu^{2+}) (Barth et al., 2011). Future research needs to show if any of these H and/or D residues on human TMEM163 do bind Zn^{2+}, Ni^{2+}, or Cu^{2+}. In addition, it would be interesting to know if other parts of TMEM163 that have the H-D motif such as those located between TM3 and TM4 domains, or TM5 and TM6 domains (Fig. 4) are potential binding site for Zn^{2+}, Ni^{2+}, or Cu^{2+}.

The main function of Metallothionein (MT) proteins is to act as a zinc reservoir, and to control the distribution of Zn^{2+} to other zinc-binding proteins (Maret and Vallee, 1998). However, MT is not a long-term storage for Zn^{2+} due to its short biological half-life (Krezoski et al., 1988), which necessitates vesicular or compartmental storage of zinc. Specifically, Zn^{2+} may be stored in the neuronal synaptic vesicles, endosomes or lysosomes,

endoplasmic reticulum [ER]) and mitochondria. Transport of Zn^{2+} into or out of these compartments is mediated by ZnTs, ZIPs, and divalent cation transporter protein families (Eide, 2006; Kambe et al., 2015). To further investigate the function of Tmem163, rat PC12 cells stably expressing the protein and subsequently exposed to Zn^{2+} showed accumulation within vesicular structures and cytoplasmic compartments as evidenced by the zinc-specific dye, Fluozin-3 (Barth et al., 2011). It was then suggested that rodent Tmem163 protein could be a Zn^{2+} efflux transporter. One of us (MPC) has found that cultured SH-SY5Y human neuroblastoma cells heterologously expressing TMEM163 markedly accumulate intracellular zinc following a brief exogenous zinc chloride ($ZnCl_2$, 100 μM, 1 hour exposure). A concomitant increase in the relative *MT1A* transcript expression levels is observed in TMEM163-overexpressing SH-SY5Y neuroblastoma cells compared with controls (Fig. 5). This observation suggests that TMEM163 is a novel transporter of Zn^{2+}, and is thus critical to Zn^{2+} homeostasis in specific tissues or organs. Interestingly, it was also observed that heterologously co-expressed TMEM163 and ZnT4 produces intracellular zinc elevation in cultured cells upon exogenous $ZnCl_2$ (100 μM) exposure as evidenced by Fluozin-3 fluorescence (Cantrell et al., 2016). This finding suggests that TMEM163 and ZnT4 physically interact with each other in distinct cells that express both proteins. In line with this possibility, several ZnT proteins have been reported to interact and form heterodimers with each other (Fukunaka et al., 2009; Lasry et al., 2014; Golan et al., 2015; Zhao et al., 2016). It is worth noting that ZnT2 and ZnT4 heterodimers localize to the plasma membrane, whereas ZnT2 or ZnT4 homodimer each localize to their respective vesicular compartment (Golan et al., 2015). It is thus possible that TMEM163 and ZnT4 heterodimers confer distinct function when compared to their respective homodimers. Further work needs to be done in order to prove if TMEM163 is an influx or efflux transporter, and whether it interacts with ZnT4 or other ZnTs, as well as ZIP transporters.

In summary, the lysosomal Zn^{2+} handling appears to be a critical period that could mean survival or death upon cytoplasmic Zn^{2+} overload in many cell types, especially in neurons. The TRPML1, ZnT4, and TMEM163 proteins may be central to Zn^{2+} handling that involves the lysosomes and other membrane-bound compartments (Fig. 6). Future research to define the contributions of these proteins in cellular zinc homeostasis. There are still many gaps in our knowledge regarding the function of TMEM163, and the relevance of its interaction with TRPML1 or its putative interaction with other zinc transporters. For example, does TRPML1 mediate the subcellular trafficking of TMEM163? Does TRPML1 function cooperatively with TMEM163 in terms of releasing cations from the lysosomes into the cytosol according to previous reports suggesting that TRPML1 is a “release” channel? Finally, does the functional loss of TRPML1, which creates hyperacidic and zinc-elevated lysosomes, confers concomitant inhibition of TMEM163 function? Does TMEM163 belong to a new class of ZnT- or ZIP-like proteins, which could explain its redundant expression pattern with ZnT or ZIP in various tissues? These are just a few questions that must be answered in order to advance the field and fully understand the role of TMEM163 in normal and pathological states.

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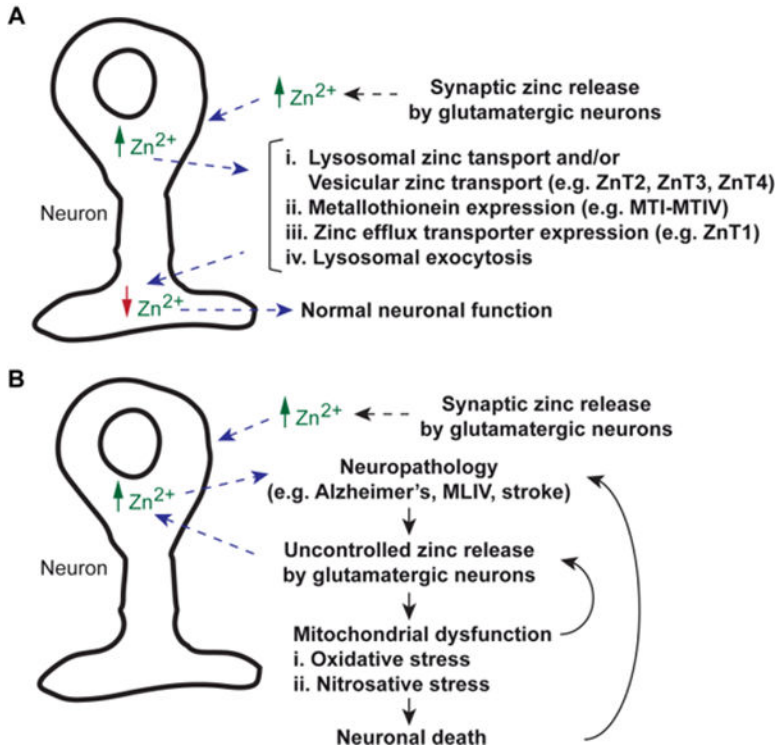

Fig. 1. Cellular zinc status

A) Normal zinc release by glutamatergic neurons results in extracellular increase of Zn^{2+}. The ions are taken up by cells, which elevate intracellular Zn^{2+} levels. The ions are taken up by lysosomes or vesicular compartments until increases in the expression levels of Metallothionein and efflux zinc transporter have occurred. Lysosomal exocytosis is also a mechanism to reduce excess intracellular Zn^{2+}. **B)** In pathological conditions caused by stroke or neurodegenerative diseases, glutamatergic neurons that release Zn^{2+} becomes a vicious cycle. Extracellular Zn^{2+} elevation perpetuates intracellular Zn^{2+} accumulation. The flood of Zn^{2+} results in oxidative and nitrosative stresses in mitochondria. Failure of lysosomes to buffer Zn^{2+} increase contributes to cellular stress, which subsequently results in cell death.

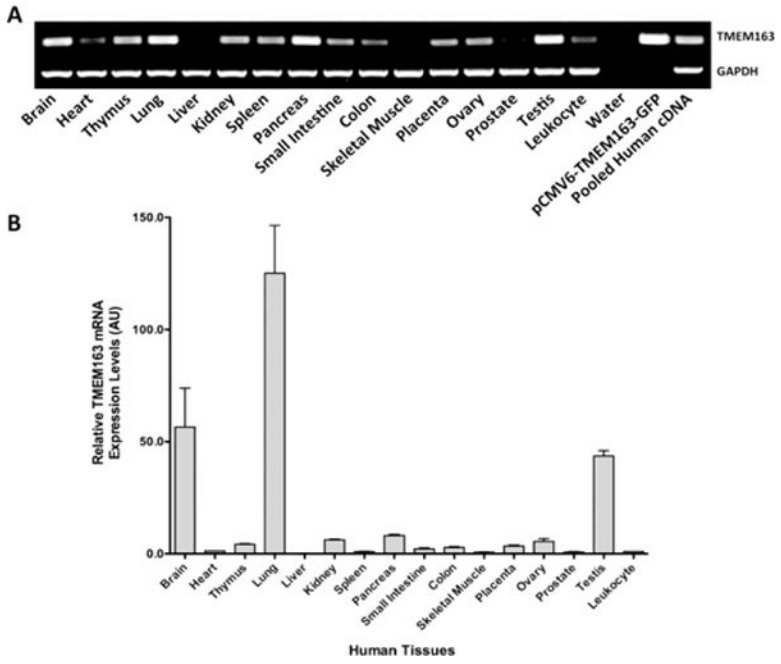

Fig. 2. Analyses of *TMEM163* gene expression in human tissues

A) Standard PCR analysis of human TMEM163 transcripts using normalized multiple tissue cDNA (MTC) panel commercially purchased from Clontech. No template control (H_2O) represented the negative control, while pCMV6-GFP-TMEM163 and non-normalized pooled cDNA were used as positive controls. The housekeeping gene, GAPDH, was used as an internal loading control. **B**) Real-time quantitative reverse-transcription polymerase reaction (RT-PCR) analysis of TMEM163 using the same MTC panel used in A. The samples were analyzed using the Livak method (ΔΔCq). The housekeeping gene, 18s rRNA, was used as a reference (normalizer). The leukocyte sample was used as the calibrator (value = 1), which makes the tissue mRNA levels all relative to leukocyte. Data are represented as mean ± SEM (n = 3). AU, arbitrary units; bp, basepair. Reprinted with permission from Cuajungco *et al.* (2014), *Traffic*, **15**, 1247-1265. Copyright 2014 Wiley.

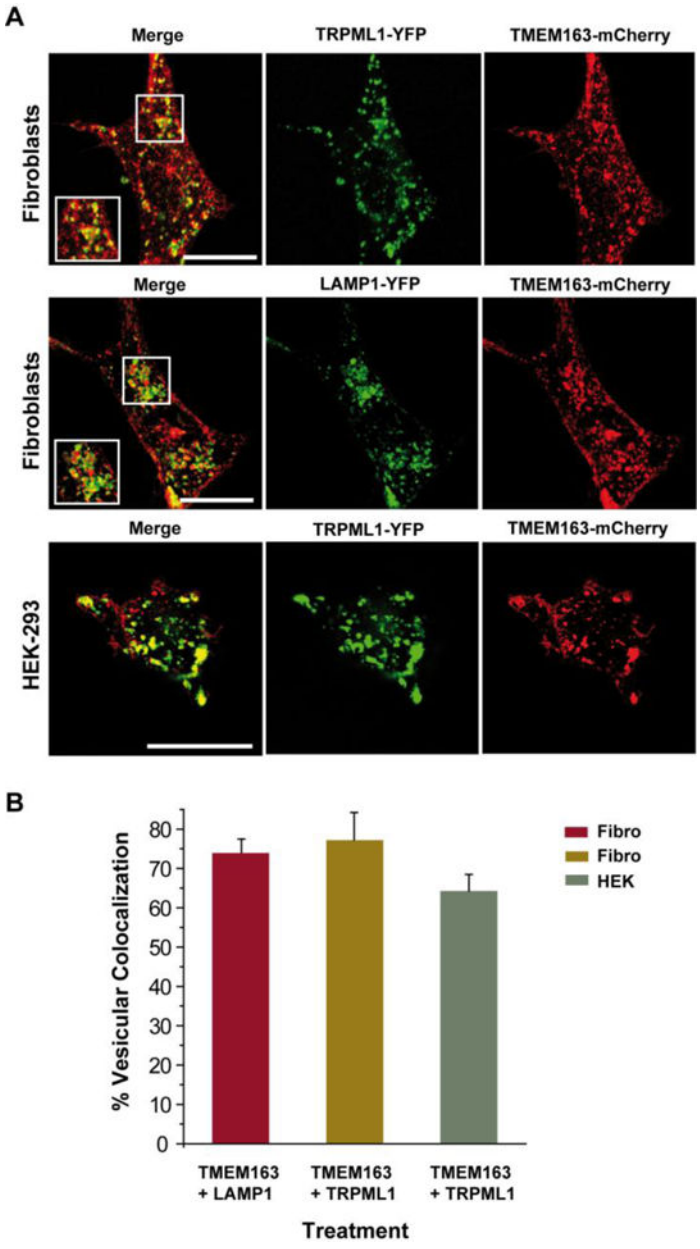

Fig. 3. Subcellular distribution of heterologously co-expressed TRPML1 and TMEM163 proteins

A) Representative laser scanning micrographs showing subcellular co-localization of TRPML1-YFP and TMEM163-mCherry upon heterologous expression in human primary fibroblast cells (*top panel*) and HEK-293 cells (*bottom panel*). TMEM163-mCherry partially co-localized with TRPML1-YFP and LAMP1-YFP (a marker for late endosomes and lysosomes). TMEM163 localized on the plasma membrane, but also exhibited a punctate distribution pattern with either TRPML1 or LAMP1. In HEK-293 cells, co-expression of TMEM163-mCherry with the TRPML1-YFP showed similar a subcellular distribution pattern to the fibroblast cells. Scale bar = 20 μm. **B**) Cell count showing the percentage of vesicular co-localization pattern between co-expressed TMEM163 plus LAMP1, and TMEM163 plus TRPML1 (for both human fibroblast and HEK-293 cells). The data showed

that 70–80% of TMEM163 co-localized with LAMP1 and TRPML1 in late endosomes and lysosomes of fibroblast cells, while 60–70% of TMEM163 co-localized with TRPML1 in late endosomes and lysosomes of HEK-293 cells (n = 50 cells). Reprinted with permission from Cuajungco *et al*. (2014), *Traffic*, **15**, 1247–1265. Copyright 2014 Wiley.

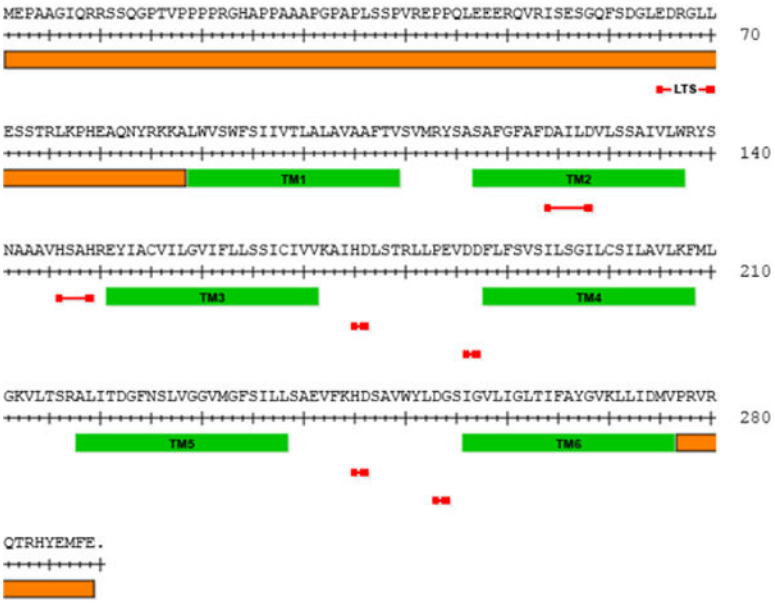

Fig. 4. Amino acid sequence map of TMEM163 protein

The map indicates H and D amino acid residues that could potentially bind zinc (red line). The predicted TM domains are shown as solid green bar, while both N- and C-termini are demarcated by a solid orange bar. The putative lysosomal targeting sequence (LTS) is indicated by a red line. The LTS contains a consensus sequence motif of [D/E]XXXL[L/I] residues where X is any amino acid.

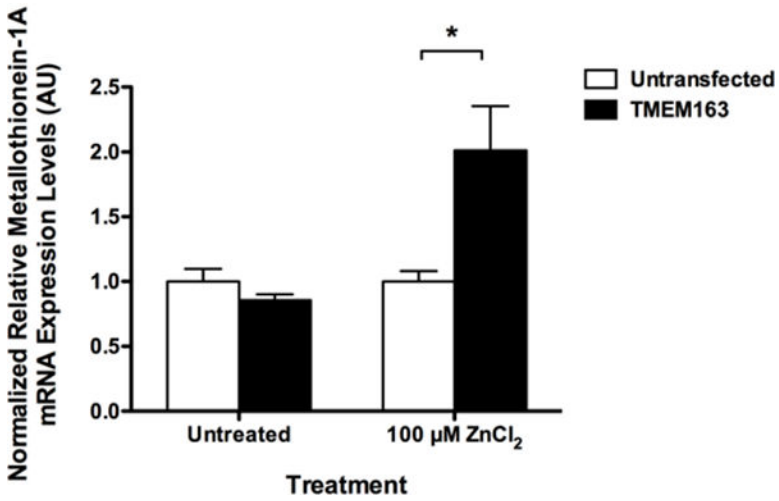

Fig. 5. Cultured SH-SY5Y neuroblastoma cells heterologously expressing human TMEM163 increases Metallothionein-1A expression levels upon exogenous zinc exposure

Real-time quantitative RT-PCR of Metallothionein-1A (*MT1A*) transcripts at 24 hours following transient ZnCl2 exposure (100 μM, 1 h) of TMEM163-expressing SH-SY5Y neuroblastoma and untransfected control cells. Significant up-regulation of MT1A transcripts is evident in the TMEM163-expressing cells exposed to zinc compared to untreated cells. This result suggests that TMEM163 mediates intracellular zinc flux upon exogenous zinc exposure. Data are represented as mean ± SEM (n = 3, Student's *t*-test, paired, two-tailed, *$p < 0.05$). AU, arbitrary units.

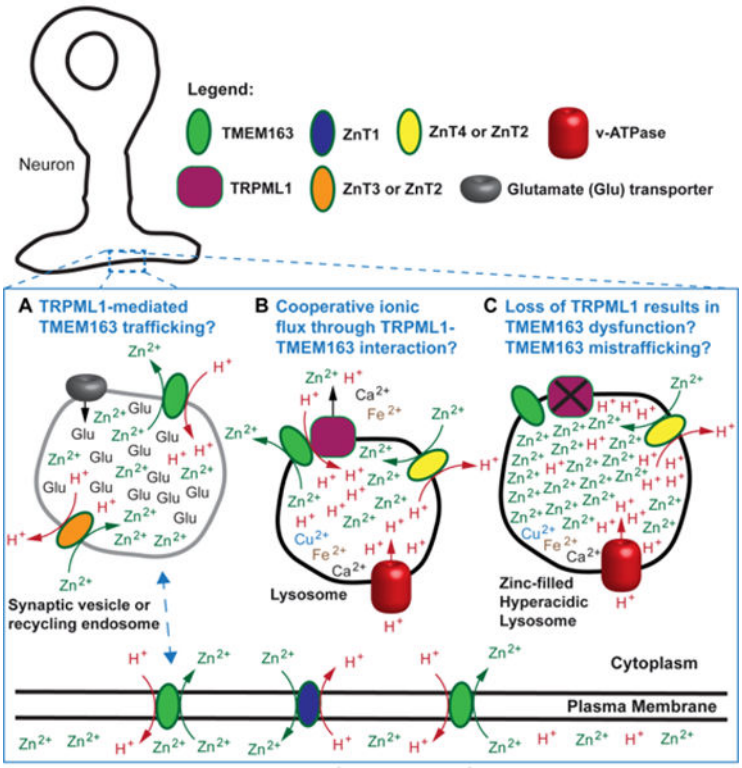

Fig. 6. Proposed cellular function of TMEM163 protein in zinc-rich cells or neurons

TMEM163 has been observed to localize in the plasma membrane, lysosomes, and vesicular compartments. **A**) The schematic model depicts that TMEM163 is a zinc transporter that is similar to the ZnT proteins in that it is a zinc (Zn^{2+})/proton (H^{+}) exchanger. It is proposed that TRPML1 may be responsible to the subcellular trafficking of TMEM163 from the plasma membrane to endocytic compartments, synaptic vesicles, or lysosomes; and vice versa. **B)** The illustration shows that TRPML1 is a release channel that controls the flux of ions (H^{+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}) within the lysosomes. The physical interaction between TMEM163 and TRPML1 is hypothesized to result in cooperative release of Zn^{2+} and possibly other cations, in order to prevent pathological buildup. **C)** The loss of TRPML1 function produces hyperacidic lysosomes that is also filled with Zn2+ through the activity of ZnT4 proteins (ZnT2 in other cell types or ZnT3 in neurons). Consequently, the loss of TMEM163 and TRPML1 interaction prevents the cooperative release and exacerbates Zn2+ accumulation.