



## Review

## Mucolipins: Intracellular TRPML1-3 channels

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## ABSTRACT

**The mucolipin family of Transient Receptor Potential (TRPML) proteins is predicted to encode ion channels expressed in intracellular endosomes and lysosomes. Loss-of-function mutations of human *TRPML1* cause type IV mucopolidosis (ML4), a childhood neurodegenerative disease. Meanwhile, gain-of-function mutations in the mouse *TRPML3* result in the *varitint-waddler (Va)* phenotype with hearing and pigmentation defects. The broad spectrum phenotypes of ML4 and *Va* appear to result from certain aspects of endosomal/lysosomal dysfunction. Lysosomes, traditionally believed to be the terminal “recycling center” for biological “garbage”, are now known to play indispensable roles in intracellular signal transduction and membrane trafficking. Studies employing animal models and cell lines in which TRPML genes have been genetically disrupted or depleted have uncovered roles of TRPMLs in multiple cellular functions including membrane trafficking, signal transduction, and organellar ion homeostasis. Physiological assays of mammalian cell lines in which TRPMLs are heterologously overexpressed have revealed the channel properties of TRPMLs in mediating cation ( $\text{Ca}^{2+}/\text{Fe}^{2+}$ ) efflux from endosomes and lysosomes in response to unidentified cellular cues. This review aims to summarize these recent advances in the TRPML field and to correlate the channel properties of endolysosomal TRPMLs with their biological functions. We will also discuss the potential cellular mechanisms by which TRPML deficiency leads to neurodegeneration. © 2010 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.**

### 1. Introduction

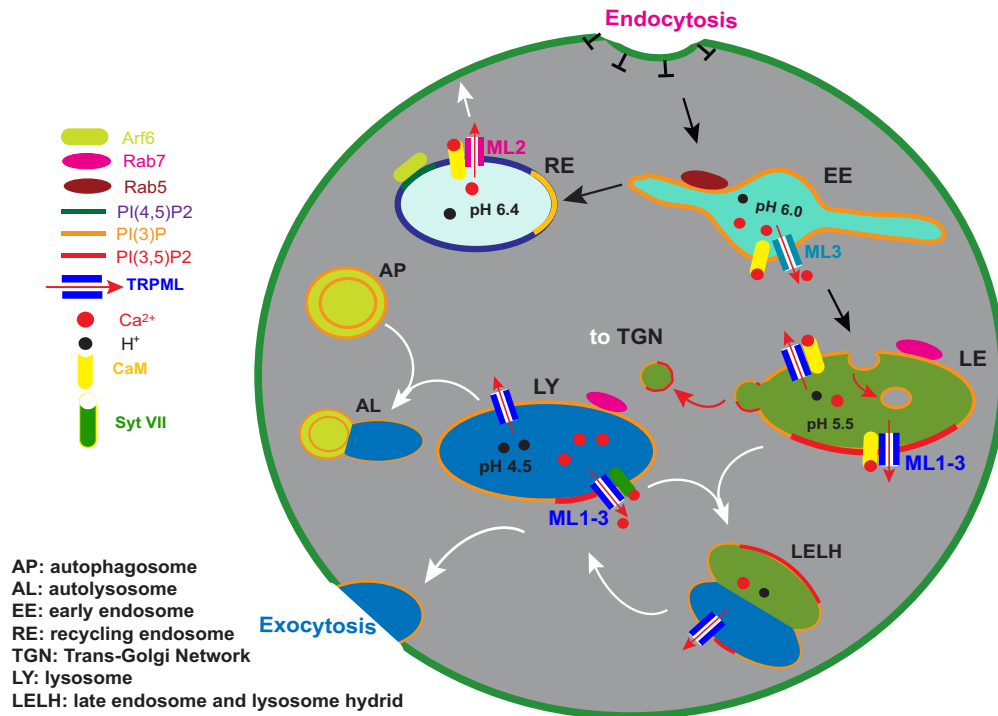
The endolysosome system in the cell is comprised of early endosomes (EEs), recycling endosomes (REs), late endosomes (LEs), and lysosomes (LYs) and is essential for a variety of cellular functions including membrane trafficking, protein transport, autophagy, and signal transduction (reviewed in Refs. [1,2]). EEs are derived from the plasma membrane upon endocytosis and are involved in intracellular trafficking of components that are sorted into recycling endosome or late endosome pathways (Fig. 1). Lysosomes are membrane-enclosed compartments that are derived from LEs and filled with hydrolytic enzymes. A hallmark feature of the endolysosome system is an acidic pH in the lumen (luminal pH is approximately 6, 5.5, and 4.5 in the EE, LE, and LY, respectively), which is established and maintained by the V-ATPase  $\text{H}^+$  pump [2]. The membrane potentials of endosomes or lysosomes are not precisely known but are presumed to be positive in the lumen (approximately in the range of +30 to +110 mV) based on measurements from other related acidic organelles [1]. Similar to other intracellular organelles, such as the endoplasmic reticulum (ER) [3], endolysosomes also provide storage for intracellular  $\text{Ca}^{2+}$  and have a luminal  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_{\text{lumen}}$ ) of approx-

imately 0.5 mM [4]. An unidentified  $\text{H}^+-\text{Ca}^{2+}$  exchanger is thought to maintain the intraendolysosomal  $\text{Ca}^{2+}$  gradient at the expense of the  $\text{H}^+$  gradient [1,2,5]. The release of  $\text{Ca}^{2+}$  from endolysosomes may play important roles in signal transduction [6,7] and membrane trafficking [2,5,8]. Although it is known that lysosomes frequently fuse with the plasma membrane and other intracellular membranes such as endosomes, autophagosomes, and phagosomes under resting conditions or upon cellular stimulation, the mechanisms underlying fusion events are not well established. While Soluble NSF Attachment Protein Receptors (SNAREs) are essential structural components involved in intracellular trafficking (e.g., membrane fusion and fission) [9], both small GTPase Rabs [10] and phosphoinositides (PIPs) [11] function as important regulatory players. In addition, the level of juxtaorganellar  $\text{Ca}^{2+}$  also plays a central role in regulating membrane trafficking [12,13]. However, the ion channels responsible for intralysosomal  $\text{Ca}^{2+}$  release have still remained elusive. Candidate endolysosomal  $\text{Ca}^{2+}$  channels include several members of the *transient receptor potential* (TRP) protein family (i.e., TRPMLs, TRPV2, and TRPM2) and the recently identified two-pore  $\text{Ca}^{2+}$  channels (TPCs) [1,6,7].

Emerging evidence suggests that the mucolipin subfamily of TRP proteins (TRPMLs) consists of endolysosomal cation channels. TRP channels are a large family of cation channels with diverse physiological functions particularly in sensory signaling [14–16]. Similar to all other TRPs, TRPMLs are six transmembrane

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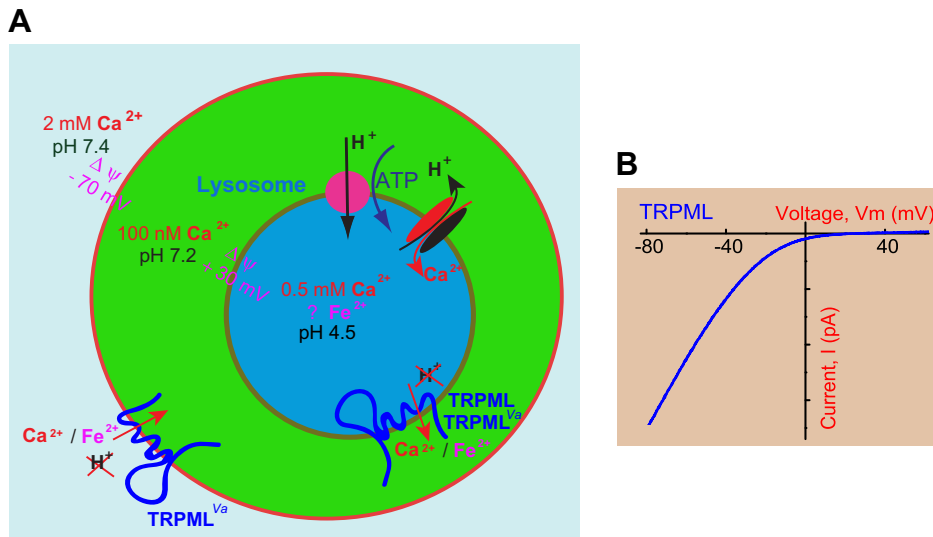
**Fig. 1.** TRPMLs in the endocytic pathway. Intracellular compartments undergo cargo-dependent maturation (indicated by black arrows), membrane fusion (white arrows), and fission/budding (red arrows). The molecular identities of intracellular compartments are defined by specific recruitment of small G proteins (Rab and Arf GTPases) and the composition of phosphoinositides (PIPs). Endolysosomes are Ca<sup>2+</sup> stores with luminal Ca<sup>2+</sup> concentration estimated to be approximately 0.5 mM. The pH of each organelle is indicated. In endolysosomes, TRPML-mediated intra-endosomal Ca<sup>2+</sup> release may activate Ca<sup>2+</sup> sensor proteins such as Synaptotagmin (Syt) and calmodulin (CaM) to trigger homotypic and heterotypic fusion. TRPML3 is present in early endosomes (EEs; pH 6.0; PI(3)P; Rab5), which are derived from the primary endocytic vesicles after endocytosis. In addition to the late endocytic pathway, contents in the EE can also be sorted into recycling endosomes (RE; pH 6.4), which are subsequently recycled back to the plasma membrane. TRPML2 is present in Arf6 – positive REs. The channel activity of TRPML2 (in RE) may regulate the activation of small GTPase Arf6, an important regulator of the recycling pathway. EEs can undergo maturation (membrane trafficking) to become late endosomes (LEs; pH 5.5; PI(3)P + PI(3,5)P2; Rab7). Membrane proteins enter the degradation pathway following membrane invagination to form multi-vesicular bodies (MVB) in LEs. LEs can fuse with LYs (LYs; pH 4.5; PI(3)P + PI(3,5)P2; Rab7) to form LE-LY hybrids. LYs can then be reformed from LE-LY hybrids. Other than fusion with LEs, LYs can also undergo fusion with autophagosomes (APs) to form autolysosomes (ALs) or with the plasma membrane during lysosomal exocytosis. TRPML1-3 channels are predominantly localized in LEs and LYs. Activation of TRPML channels by unidentified cellular cues may induce intralysosomal Ca<sup>2+</sup> release. LEs, LYs, or hybrids of LEs and LYs will then undergo CaM- or Syt-dependent membrane fusion or fission/budding. Retrograde transport vesicles, derived from EEs, LEs, or LYs upon membrane fission transport lipids and proteins in a retrograde direction to the trans-Golgi Network (TGN).

(6TM)-spanning proteins that consist of cytosolic N- and C-termini (see Fig. 2 for membrane topology). In mammals, there are three TRPML proteins (TRPML1-3, also called MCOLN1-3). Loss-of-function mutations in the human *TRPML1* gene cause type IV mucopolisoidosis (ML4), a devastating neurodegenerative disease that causes mental retardation and retinal degeneration [17–19]. In addition, some patients also develop iron deficient anemia [18]. Gain-of-function mutations of the mouse *TRPML3* gene result in the *varitint-waddler* (*Va*) phenotype that is characterized by deafness, circling behavior, and pigmentation defects [20]. The broad spectrum of phenotypes for both ML4 and *Va* might result from certain aspects of endosomal/lysosomal dysfunction. *TRPML1*<sup>-/-</sup> cells from ML4 patients are characterized by the accumulation of enlarged endosomal/lysosomal compartments (vacuoles) in which lipids and other biomaterials accumulate, suggesting defective lysosomal biogenesis and trafficking [21,22]. Nevertheless, the mechanisms of TRPMLs that are required for normal lysosomal functions are not known largely due to the lack of reliable physiological assays. Conventional assays used for assessing plasma membrane channels, such as the whole-cell patch-clamp technique, are not suitable for studying TRPMLs due to the inaccessible nature of the lysosome. Fortunately, this technical barrier has recently been overcome in a new series of studies using an activating mutation strategy [23–26]. In addition, a modified patch-clamp technique

has been developed that allows a direct recording on lysosomal membranes [27,28]. This review aims to summarize recent advances in the field and to correlate the channel properties of endolysosomal TRPMLs with their currently established biological functions.

## 2. TRPML tissue distribution and subcellular localization

In order to gain insight into the biological functions of TRPMLs, it is important to know the relative expression levels of each individual isoform in specific tissues. Unfortunately, most of the available TRPML antibodies are inadequate for this purpose and therefore published data have measured TRPML mRNA expression levels using nucleotide-based techniques such as real-time polymerase chain reaction (PCR), northern blot, and in situ hybridization. In mammals, TRPML1 is expressed in every tissue with the highest levels of expression located in the brain, kidney, spleen, liver, and heart [19] (Table 1). Consistent with its ubiquitous expression pattern, the enlarged vacuole phenotype that is associated with the loss of TRPML1 is observed in all cell types of ML4 patients and TRPML1 knockout mice [17,29]. In contrast to TRPML1, the expression of TRPML2 and TRPML3 is more restricted (Table 1). Mouse TRPML2 has two alternatively spliced variants [30]. The longer variant of TRPML2 (TRPML2lv) has an additional 28 amino



**Fig. 2.** TRPMLs in the lysosome. (A) While the ionic compositions of the extracellular space and cytosol have been well established, ion concentrations in the lumen of lysosomes are not clear. The luminal pH is between 4 and 5 and is established by a V-type ATPase. The  $[Ca^{2+}]_{Lys}$  is approximately 0.5 mM and maintained by an unidentified  $H^+$ - $Ca^{2+}$  exchanger. The resting membrane potential ( $\Delta\Psi$ ) of the cell is approximately  $-70$  mV (cytoplasmic-side negative). Based on studies of phagosomes, the membrane potential across the lysosomal membrane is estimated to be approximately  $+30$  mV (luminal-side positive). TRPMLs are permeable to  $Fe^{2+}$  (except for TRPML3) and  $Ca^{2+}$ ; none of the TRPML isoforms are permeable to  $H^+$ . Although wild-type TRPMLs are predominantly expressed in the lysosome, TRPML<sup>Va</sup> proteins are present at the plasma membrane. (B) Current–voltage ( $I$ - $V$ ) relationship of TRPML channels. All TRPMLs exhibit a strong inward rectification.

acids at its N-terminus compared to the shorter variant (TRPML2sv) [30]. Real-time PCR analysis revealed that TRPML2sv has a higher expression level than TRPML2lv in most tissues and is the dominant variant expressed in mouse thymus, spleen, and kidney [30]. RT-PCR revealed that TRPML3 mRNA is detectable in the thymus, lung, kidney, spleen, and eye [30,31]; in situ hybridization revealed expression of TRPML3 in epithelial cells of the inner ear, including strial and hair cells [26]. Using antibodies raised against TRPML3, immunoreactivities were detected in hair cells [20,26] and melanocytes of the skin [25]. TRPML3 protein was also detected in several epithelial cell lines including HEK293, HeLa, and the retinal pigmented epithelial cell line ARPE19 [32–34]. Interestingly, the expression level of TRPML2, but not TRPML3, is altered in a tissue-specific manner in TRPML1<sup>-/-</sup> cells [30]. It is likely that the three isoforms of mammalian TRPMLs have unique tissue-specific functions in addition to their shared cellular functions. In contrast to mammals, *Drosophila melanogaster* and *Caenorhabditis elegans* only express one TRPML protein [35,36].

TRPMLs primarily localize to a population of membrane-bonded vesicles along the endocytosis and exocytosis pathways. Overexpression studies using TRPML1 tagged or fused with reporter genes, such as EGFP or mCherry, have revealed that TRPML1 primarily localizes to the lysosome-associated membrane protein (Lamp-1) or Rab7-positive late endosomal and lysosomal (LEL) compartments [27,32,33,37–42]. TRPML1 localization to the LEL has been beautifully confirmed by immunostaining and gradient fractionation studies on endogenous proteins [32,34]. Lysosomal localization of TRPML1 protein is likely mediated by Clathrin adaptor AP2-dependent internalization from the plasma membrane and/or AP1/AP3-dependent trafficking from the trans-Golgi Network (TGN) by direct interaction with two individual di-leucine motifs that exist near the N- and C-termini of TRPML1 [39,41,42].

Similar to TRPML1, TRPML2 and TRPML3 that were tagged and overexpressed also accumulate in LEL compartments and co-localize with Lamp-1 and Rab7 [32,33,40,43,44]. Immunolocalization studies have also shown that TRPML2 localizes to the vesicles along the long, tubular structures within cells in addition to the LEL [43]. These long, tubular structures correspond to the recycling endosomes of the GTPase ADP-ribosylation factor-6 (Arf-6) associ-

ated pathway [43]. By using cellular fractionation and primary antibodies raised against TRPML3, Kim et al. provided evidence that TRPML3 localizes to LELs, EEs, and the plasma membrane [32]. The plasma membrane localization of TRPML3 is consistent with the substantial whole-cell currents that can be recorded from TRPML3-expressing HEK293 cells [23–26].

### 3. Channel properties of TRPMLs

TRPML1 and TRPML2 are primarily localized to the LEL, making it difficult to characterize their channel properties. In contrast, overexpressed, wild-type TRPML3 is present at the plasma membrane and has been characterized using the whole-cell patch-clamp technique [24,25]. These studies have shown that TRPML3 is an inwardly rectifying,  $Ca^{2+}$ -permeable cation channel (thoroughly reviewed in Ref. [31]). TRPML3-mediated current ( $I_{TRPML3}$ ) is inhibited by an acidic extracellular (analogous to the luminal side) pH with  $H^+$  presumably binding to multiple histidine residues within the TM1–TM2 loop [45]. Interestingly,  $I_{TRPML3}$  is potentiated by the removal and subsequent re-addition of extracellular  $Na^+$  [24,45]. The physiological implications of these two observations remain to be established. Mutation of mouse TRPML3 (A419P) causes the *varitint-waddler* (*Va*) phenotype, which is characterized by deafness, circling behavior, and pigmentation defects [20]. Much larger currents were seen for TRPML3<sup>A419P</sup> (TRPML3<sup>Va</sup>) compared to wild-type TRPML3 under basal/non-stimulated conditions [25], consistent with the high open probability of  $I_{TRPML3-Va}$  [25,26].  $I_{TRPML3-Va}$  resembles  $I_{TRPML3}$  in the basic channel pore properties including current–voltage ( $I$ - $V$ ), single channel conductance, and ion selectivity [24–26]. However, since  $I_{TRPML3-Va}$  cannot be further activated by  $Na^+$  manipulation [24], the *Va* mutation is likely to disrupt channel gating by locking the channel in an open state [31]. Therefore, the reported *Va*-induced small alteration of  $Ca^{2+}$  permeability [45] could be secondary to the effect on gating. This would be in accordance with the gating-permeation coupling phenomenon observed in other TRPs [46].

The *Va* activating mutation approach has been used to effectively characterize the pore properties of TRPML1 and TRPML2 in multiple studies [23,25,27,30]. Introduction of *Va*-like mutations

**Table 1**  
The mammalian TRPML channels.

	TRPML1 (MCOLN1)	TRPML2 (MCOLN2)	TRPML3 (MCOLN3)
Tissue expression	Ubiquitously	Thymus, liver, kidney, heart, spleen	Cochlea, thymus, kidney, lung, eye, spleen, skin (melanocytes), somato-sensory neurons
Subcellular localization	Late endosome and lysosome (LEL)	Recycling endosome, LEL	Plasma membrane, early endosome, LEL
Ion selectivity	Na <sup>+</sup> , K <sup>+</sup> , Ca <sup>2+</sup> , Mn <sup>2+</sup> , Fe <sup>2+</sup> , Mg <sup>2+</sup> , Zn <sup>2+</sup> , etc.	Na <sup>+</sup> , K <sup>+</sup> , Ca <sup>2+</sup> , Fe <sup>2+</sup>	Na <sup>+</sup> , K <sup>+</sup> , Ca <sup>2+</sup> , Mg <sup>2+</sup>
Current–voltage relationship (I–V)	Strong inwardly rectifying	Strong inwardly rectifying	Strong inwardly rectifying
Single channel conductance in pS	76 (from –140 to –100 mV) and 11 (from –80 to –40 mV)	Not described	~50
Activation mechanisms	Voltage; NAADP (?); PKA (?); extracellular or luminal low pH	Voltage; extracellular or luminal low pH	Voltage; Na <sup>+</sup> removal followed by re-addition
Blockers and inhibitors	Verapamil; Gd <sup>3+</sup> ; La <sup>3+</sup>	Not described	Verapamil; Gd <sup>3+</sup> ; extracellular or luminal low pH
Interacting proteins	TRPML2; TRPML3; Hsc70; Hsp40; ALG-2	TRPML1; TRPML3	TRPML1; TRPML2
Cellular functions	Membrane trafficking in late endocytic pathways; autophagy; lysosomal biogenesis; lysosomal iron release; lysosomal exocytosis; possible LEL pH homeostasis	Membrane trafficking in late endocytic pathways and Arf6-regulated recycling pathway	Membrane trafficking along endolysosomal pathways; autophagy; possible hair cell function
Human disease	Mucopolysaccharidosis type IV (ML4; MIM #252 650; autosomal recessive lysosomal storage neurodegenerative disease with progressive psychomotor retardation, retina degeneration, iron-deficiency, achlorhydria, and mental retardation); ML4 cells show enlarged endolysosomes, lipid accumulation, autophagic dysfunction, mitochondrial dysfunction, possible acidification defect, and lipofuscin accumulation in the brain	Not described	Not described
Genetic models	(1) TRPML1 knockout mice: inclusion bodies, enlarged vacuoles, psychomotor defect, retinal degeneration, death at age approximately 8 months (2) Cup-5 null <i>C. elegans</i> : enlarged vacuoles, excess apoptotic cells, and embryonic lethality (3) <i>Drosophila trpml</i> mutant: defective autophagy, impaired synaptic transmission, accumulation of apoptotic cells, oxidative stress, lipofuscin accumulation, mitochondrial dysfunction, motor defects, and massive neurodegeneration	Not described	Varitint-waddler mouse ( <i>Va</i> ) with A419P mutation in the 5th TM domain; <i>Va(j)</i> mouse with an additional mutation at I362T; heterozygous and homozygous mice exhibit pigmentation defects in the skin, circling behavior with degeneration of vestibular apparatus, and low fertility
References	[18,19,22,23,25–28,30,35,39,47,52,61,77–80]	[23,25,30,41,43]	[20,23–26,30,32,33,45,81]

in the analogous positions of TRPML1 (V432P) and TRPML2 (A396P) allowed for the characterization of the pore properties of TRPML1 and TRPML2 using whole-cell recordings [25,27,28,30]. We initially reported that TRPML2sv-*Va* was functional but TRPML2lv-*Va* was not [25,28]. However, a single mutation in our original mouse TRPML2lv-*Va* clone might have actually been a cloning artifact (as opposed to a polymorphism as originally thought), since correction of this mutation resulted in a measurable inwardly rectifying whole-cell current [30] (Wang and Xu, unpublished data; personal communication with Cuajunco and Grimm). Similar to  $I_{TRPML3}$ , both  $I_{TRPML1-Va}$  and  $I_{TRPML2-Va}$  are inwardly rectifying and Ca<sup>2+</sup>-permeable cationic currents. Using a lysosome patch-clamp technique, Dong et al. were able to characterize wild-type TRPML1 [27] and TRPML2 (Dong et al., unpublished results) in their native membranes (LEL). Lysosomal  $I_{TRPML1}$  and  $I_{TRPML2}$  exhibited almost identical pore properties (I–V, kinetics, and voltage-dependence) as their respective *Va* mutant channels, supporting the hypothesis that the activating mutation (*Va*) is a useful approach for characterizing the pore properties of TRPML1 and TRPML2. Nevertheless, whole lysosome  $I_{TRPML1-Va}$  is at least 10 times larger than whole lysosome  $I_{TRPML1}$  [27], suggesting that *Va* is also a gain-of-function mutation in LEL. Previous studies have resulted in very controversial results on the channel properties of TRPML1 (summarized in Ref. [21]). However, the reported currents from these studies contrast dramatically with  $I_{TRPML1}$  and  $I_{TRPML1-Va}$  data described above. For example, whole-cell patch-clamp recordings of overexpressed TRPML1 were reported to yield outwardly rectifying monovalent, cation-selective [37] or H<sup>+</sup>-selective [47] channels.  $I_{TRPML}$  and  $I_{TRPML-Va}$ , however, are in-

wardly rectifying and permeable to Ca<sup>2+</sup>, Na<sup>+</sup>, K<sup>+</sup>, and Fe<sup>2+</sup>/Mn<sup>2+</sup>, but not H<sup>+</sup> [25,27,28]. Although differences may exist for ion channel studies using different heterologous expression systems, it is unlikely that different laboratories cannot reach a consensus on the basic pore properties of one single channel protein. In the view of the authors of this article, the inwardly rectifying currents (under physiological conditions) are likely to represent the true electrophysiological functions of TRPMLs. Consistent with the overexpression studies, the endogenous TRPML-like current in LEL is also strongly inwardly rectifying (Dong et al., unpublished results).

Although the pore properties of TRPMLs have been well characterized, the mechanisms of how TRPMLs are gated/activated under physiological conditions are not known. Since the *Va* mutation appears to dramatically promote the open state of TRPML channels [25,27,28], studies of  $I_{TRPML-Va}$  may not yield useful information regarding TRPML regulation.  $I_{TRPML1-Va}$  and  $I_{TRPML2-Va}$  are both potentiated by low pH, however the effect is quite modest [25,28]. In addition, H<sup>+</sup> may have increased the single channel conductance but most likely did not increase the open probability of the channels. Therefore, the physiological implication of this pH modulation remains to be established. Heterologously-expressed wild-type TRPML3 is present in the plasma membrane [24,25,45], which allows for the study of channel activation mechanisms. Wild-type TRPML1 and TRPML2 might also be able to traffic to the plasma membrane under certain conditions or in specific cell types, though these situations have not been reported. Another possible method for investigating TRPML gating is to analyze lysosome recordings. Using this method, we are currently studying



how overexpressed and endogenous TRPMLs are regulated by luminal and cytosolic factors that are known to affect lysosomal functions (Dong et al., unpublished findings). For TRPML3, Kim et al. was able to investigate the activation mechanisms of  $I_{TRPML3}$  using whole-cell recordings and reported that  $I_{TRPML3}$  is dramatically enhanced by  $Na^+$  manipulation (removal followed by re-addition) [24]. However, it remains to be established how TRPML3 is activated by mechanisms that are physiologically more relevant.

#### 4. TRPML in membrane trafficking

Human ML4 mutations cause the abnormal accumulation of sphingolipids, phospholipids, acidic mucopolysaccharides, and cholesterol in swollen/enlarged LEL-like vacuoles in all cell types [22,48]. Consistent with this observation, abnormal lipid accumulation and enlarged LELs are also seen in cells from TRPML1 knockout mice [29], TRPML<sup>-/-</sup> (*cup-5*) *C. elegans* [36], and TRPML<sup>-/-</sup> *Drosophila* [35]. Electron microscopy (EM) studies suggest that enlarged LELs in TRPML<sup>-/-</sup> cells are likely to be the late endosome-lysosome hybrid organelles from which lysosomes are reformed under normal conditions [49]. Since the hydrolase activity that is involved in lipid processing is relatively normal [22] (but see Ref. [47]), the defect observed in TRPML<sup>-/-</sup> cells is most likely related to the sorting and trafficking processes of the endocytic pathway. Analyses of trafficking kinetics suggest that the defect is in the late endocytic pathways only [22]. Specifically, the exit of lipids from the LEL to the TGN is defective in ML4 cells [22,39]. In support of these observations, shRNA-mediated knockdown of TRPML1 expression in mouse macrophages results in a similar defect in retrograde lipid transport [40]. Taken together, TRPML1 is likely to be required for the formation of transport vesicles from the LEL compartment to the TGN and for the reformation of lysosomes from the late endosome-lysosome hybrid organelles [36,40,49]. This hypothesis is supported by the observation that TRPML1 expression is dramatically increased following induction of lysosome biogenesis [50]. Since membrane fission and/or stabilization of transport vesicles are known to be dependent on luminal  $Ca^{2+}$  [2,5,51] and/or intraluminal  $Ca^{2+}$  release [5,13], it is likely that the  $Ca^{2+}$  permeability of TRPML1 is required for the membrane fission from LEL compartments or late endosome-lysosome hybrids and the biogenesis of both retrograde transport vesicles and lysosomes (Fig. 1). TRPML1 may also be required for the reformation of lysosomes from autolysosomes [35,52].

Other trafficking defects are also observed in TRPML<sup>-/-</sup> cells. ML4 fibroblasts have been reported to exhibit a delay in the delivery of platelet-derived growth factor receptor (PDGFR) to lysosomes [52]. In mouse macrophages where TRPML1 protein level has been reduced by shRNA, the transport of endocytosed molecules to lysosomes is delayed [40]. Autophagosomes have also been shown to accumulate in ML4 cells. This could be due to either increased autophagosome formation or delayed fusion of autophagosomes with lysosomes to form autolysosomes (AL) (Fig. 1) [52]. ML4 fibroblasts also show a defect in ionomycin-induced lysosomal exocytosis [53]. However, this effect could be a secondary observation, since ionomycin ( $Ca^{2+}$  ionophore) presumably acts directly on the  $Ca^{2+}$  sensor synaptotagmin VII (SytVII) thereby bypassing upstream TRPML activation. Nevertheless, experiments showing that HEK293 cells transfected with a gain-of-function mutant TRPML1 exhibit enhanced lysosomal exocytosis provide additional evidence that TRPML1 plays a role in this process [28]. The gain-of-function mutation is likely to have mimicked an unidentified cellular stimulation to induce intralysosomal  $Ca^{2+}$  release since these experiments were performed using a  $Ca^{2+}$ -free extracellular solution to exclude the involvement of the gain-of-function TRPML1 channels at the plasma membrane. Taken together,

we hypothesize that TRPML1 plays a role in multiple membrane fusion processes related to late endosomes and lysosomes (Fig. 1). Membrane fusion in the endocytic pathway is dependent on the release of luminal  $Ca^{2+}$  that occurs as a post-docking event [54]. Using  $Ca^{2+}$  chelators in cell-free assays, Pryor et al. showed that luminal  $Ca^{2+}$  is required for both lysosome reformation and heterotypic fusion of late endosomes and lysosomes [13]. Release of luminal  $Ca^{2+}$  is thought to promote the post-docking events through the activation of Calmodulin (CaM) [13]. In consideration of these data, it is possible that TRPML1 may be tightly regulated to release  $Ca^{2+}$  as a mechanism of facilitating the endocytic process in a correct spatiotemporal method. TRPML1, therefore, may play a dual role in membrane fusion and fission in the late endocytic pathways (late endosomes, lysosomes, autophagosomes, and the plasma membrane).

In comparison to TRPML1, the role of TRPML2 and TRPML3 in regulating membrane trafficking is less well understood. TRPML2 and TRPML3 may function indirectly through the formation of heteromultimers with TRPML1, since both heterologously-expressed [41] and endogenous TRPMLs [34] can physically associate with each other (Table 1). However, the extent of co-localization appears to be limited [34]. Therefore, it is more likely that TRPML2 and TRPML3 functions are primarily cell type specific. In LEL, it is reasonable to hypothesize that TRPML2/3 play a similar role in membrane trafficking as TRPML1. TRPML2 has been reported to traffic via the Arf6-associated recycling pathway in HeLa cells [43]. While overexpression of TRPML2 promotes efficient activation of Arf6, inactivation of TRPML2 by a dominant-negative approach decreases recycling of CD59 proteins to the plasma membrane [43]. Thus, TRPML2 may interact and cross-talk with other trafficking regulators to control cargo sorting. The localization of TRPML3 is more dynamic with expression in multiple intracellular compartments. The diverse expression of TRPML3 may correlate with its role in membrane trafficking [32,33]. Overexpression of TRPML3 causes enlarged early-endosome-like structures and defective delivery and degradation of EGF and EGFR that is consistent with its expression in EEs [32,33]. Since heterologously-expressed TRPMLs display basal activity in the LEL [27], it is possible that overexpression of TRPML3 promotes endosomal membrane fusion. Interestingly, knockdown of TRPML3 appears to enhance endocytosis and degradation [33], a result that is inconsistent with the role of TRPML1 in degradation and trafficking in the late endocytic pathways [22,39,40]. It is possible that this observed effect is due to a function of TRPML3 in early endocytic pathways. The exact step where TRPML3 functions in the early endocytic pathway is still not clear. While Kim et al. showed that TRPML3 overexpression decreases constitutive and regulated endocytosis in HEK and HeLa cells [32], Martina et al. found that internalization of EGFR is not affected but that its delivery from the plasma membrane to the lysosome is impaired in human epithelial cells [33]. It is possible that these observed differences are due to the use of different cell types. Finally, TRPML3 may also be involved in the regulation of autophagy since both overexpression and knockdown of TRPML3 affect autophagy [32,33].

It is yet unclear how TRPMLs coordinate with the other key regulators of vesicle trafficking such as GTPases (Rab and Arf) and phosphoinositides since the endogenous activators of TRPMLs have not been identified. Constitutive activation of Rab5 causes enlarged early endosomes by stimulating homotypic fusion [10]. This is reminiscent of the enlarged endosomes seen in cells with TRPML3 overexpression. It is possible that Rab5 may recruit effectors to activate TRPMLs. Alternatively, the activity of TRPMLs may regulate the activity of Rabs, as shown in the case of TRPML2 and Arf6 GTPase [43]. Similarly, alterations of endolysosomal phosphoinositide (PIP) levels (PI(3)P and PI(3,5)P2) also results in enlarged endolysosomes and defective endosome-to-TGN retrograde traf-

ficking [11], a situation that is similar to the enlarged LEL and abnormal late endocytic trafficking phenotype seen in ML4 cells. It is possible that TRPMLs are regulated directly by PIPs as shown for many other plasma membrane TRPs [55]. Alternatively, TRPMLs may regulate key enzymes involved in PIP production or hydrolysis. Taken together, TRPMLs may cross-talk with these proteins and/or lipids to regulate endolysosomal traffic. Since membrane trafficking is an active process, we expect that TRPMLs would be frequently activated in the intact cell, unlike the situation in a lysosome recording configuration in which there is not substantial channel activity. It is worth noting that like most other TRPs, overexpressed TRPMLs tend to exhibit basal currents. However, as TRPMLs do not exhibit significant voltage-dependent inactivation, even a small TRPML current may lead to substantial  $\text{Ca}^{2+}$  release. Therefore, it is necessary for TRPMLs to be tightly regulated in the lysosome. It is likely that TRPMLs are only transiently activated in the intact cell. TRPML-mediated channel activation may be able to be captured as “spontaneous” lysosomal  $\text{Ca}^{2+}$  release events using high-resolution, live imaging techniques in the near future. Different  $\text{Ca}^{2+}$  sensors might be recruited in a compartment-specific manner downstream of  $\text{Ca}^{2+}$  release. While CaM has been reported to be functional in late endosomes [13,54,56], SytVII might convert the signal into  $\text{Ca}^{2+}$ -dependent fusion with the plasma membrane [57]. Recently, Vergarajaregui et al. reported that ALG-2, a penta-EF-hand protein, physically associated with TRPML1 but not TRPML2 or TRPML3 [58]. ALG-2 might also serve as a  $\text{Ca}^{2+}$  sensor to couple TRPML1 activation to endolysosomal fusion/fission events. Interestingly, the interaction of TRPML1 and ALG-2 is strongly dependent on  $\text{Ca}^{2+}$ , suggesting that this interaction is physiologically significant. Future research may indicate whether ALG-2 also plays a role in the modulation of TRPML channel activity in the LEL.

## 5. TRPMLs in signal transduction

Accumulating evidence suggests that a modest  $\text{Ca}^{2+}$  release from lysosomes plays an indispensable role in the transduction of extracellular signals by inducing a large and rapid release of  $\text{Ca}^{2+}$  from the ER through the inositol 1,4,5-trisphosphate receptor ( $\text{IP}_3\text{R}$ ) or ryanodine receptor (RyR) [7,59]. However, the mechanisms by which  $\text{Ca}^{2+}$  is released from lysosomes has remained elusive. There are two significant questions that remain unanswered. First, it is not known which intracellular messenger is generated upon extracellular stimulation. Nicotinic acid adenine dinucleotide phosphate (NAADP) has been an attractive candidate that is believed to induce  $\text{Ca}^{2+}$  release from lysosomes [7,59]. Second, identification of the lysosome  $\text{Ca}^{2+}$  channels that are activated by NAADP and other lysosome-acting cytosolic factors remains elusive. TRPMLs could potentially be the lysosomal  $\text{Ca}^{2+}$  release channels based on their subcellular location and  $\text{Ca}^{2+}$  permeability. Zhang et al. recently showed that a NAADP-sensitive current could be blocked by an anti-TRPML1 antibody or reduced by TRPML1-specific siRNA using reconstituted lipid bilayers from biochemically purified lysosomal membranes [60,61]. One caveat of these studies is that the NAADP-activated current was  $\text{Cs}^+$  permeable and possessed a linear I–V, two properties not shared by  $I_{\text{TRPML1}}$ . Nevertheless, ET-1, a known NAADP stimulator in coronary arterial myocytes [60], was found to induce a local  $\text{Ca}^{2+}$  release from lysosomes followed by a global  $\text{Ca}^{2+}$  rise [62]. This response was significantly reduced after pre-treatment with TRPML1-specific siRNA [60]. It is possible that TRPML1 may form heteromultimers with other candidate NAADP receptors such as TPCs [7]. Future work will be required to clarify the role of TRPML1 in lysosomal  $\text{Ca}^{2+}$  signaling that is mediated by NAADP or other lysosome-acting intracellular messengers.

## 6. TRPMLs in endolysosomal $\text{H}^+$ homeostasis

Lysosomal  $\text{H}^+$  homeostasis is known to play an essential role in multiple lysosome-mediated functions. First, a change of luminal pH may dramatically affect the functions of acidic hydrolytic enzymes in the lysosome and impair the degradative functions occurring in the lysosome [5]. Second, similar to  $\text{Ca}^{2+}$ , luminal  $\text{H}^+$  is known to affect membrane fusion/fission via unknown mechanisms [2,5]. Recently, Miedel et al. developed an alternative model to explain ML4 pathogenesis based on the evidence that lysosomal pH in ML4 cells or cells treated with TRPML1-specific siRNA is lower than in normal cells [47,63]. This new model is referred to as the “metabolic model” as opposed to the aforementioned “biogenesis” or “trafficking” model. The central idea of the “metabolic model” hypothesizes that a decreased lysosomal pH may interfere with the degradation of internalized lipids since  $\text{H}^+$  ions are important for sorting processes and proper function of lysosomal acid hydrolases. If this model is proven to be correct, enzyme therapies would be an effective method to treat ML4 [63].

Despite data supporting this newly proposed model, there are several observations that contradict this hypothesis. Firstly, it is not immediately clear what causes lysosomal hyperacidification in the absence of TRPML1 given that  $I_{\text{TRPML}}$  and  $I_{\text{TRPML-Va}}$  exhibit no permeability to protons [25]. However, although  $I_{\text{TRPML3}}$  is  $\text{H}^+$ -impermeable and is indeed suppressed by low pH, overexpression of TRPML3 results in the alkalization of endolysosomes [33]. Therefore, it is likely that  $\text{H}^+$  homeostasis is regulated by intralysosomal  $\text{Ca}^{2+}$  release possibly through an unidentified  $\text{Ca}^{2+}$ – $\text{H}^+$  exchanger. Leucine zipper/EF hand-containing transmembrane-1 (Letm1), the recently identified mitochondrial  $\text{Ca}^{2+}$ – $\text{H}^+$  exchanger, can function in a bidirectional manner [64]. Similarly, TRPML-mediated juxtaorganellar  $[\text{Ca}^{2+}]_{\text{cyt}}$  elevation may induce  $\text{H}^+$  efflux through a lysosomal counterpart of Letm1. It remains to be determined whether lysosomal hyperacidification associated with loss of TRPML1 is caused by such indirect mechanisms. Secondly, reduction of lysosomal pH in ML4 cells is still controversial [39] since hypoacidification and no change of lysosomal pH have also been observed in ML4 cells [22,65]. Impaired  $\text{H}^+$  homeostasis may affect the activity of a  $\text{Ca}^{2+}$ – $\text{H}^+$  exchanger and/or luminal  $\text{Ca}^{2+}$  buffering and could lead to impaired  $\text{Ca}^{2+}$  homeostasis. However, lysosomal  $\text{Ca}^{2+}$  content appears to be normal in ML4 cells [66]. Finally, based on the metabolic model, it is argued that the trafficking defect in ML4 cells may be due to the secondary effect of gradual and chronic lipid accumulation [63]. However, since aberrant lipid trafficking in ML4 cells was rescued by acute overexpression of wild-type TRPML1 [39], the secondary effect or feedback inhibition is likely to be present also in TRPML1 siRNA-treated cells.

## 7. TRPMLs in lysosomal iron release

In most mammalian cells iron is acquired through the endocytosis of  $\text{Fe}^{3+}$ -bound transferrin receptors. Iron may also enter the endocytic pathways via autophagic ingestion of iron-binding proteins, for example, cytochrome C [67]. After reduction by an endolysosomal ferrireductase,  $\text{Fe}^{2+}$  is released from the endosome or lysosome into the cytoplasm via the  $\text{Fe}^{2+}$  permeable channels or transporter [67]. As mentioned above,  $I_{\text{TRPML1}}$  is permeable to both  $\text{Ca}^{2+}$  and  $\text{Fe}^{2+}/\text{Mn}^{2+}$ . Since TRPML1 is localized to the LEL, it has become a strong candidate as an endolysosomal  $\text{Fe}^{2+}$  release channel [27]. In support of these data, some ML4 patients were reportedly associated with iron-deficiency and/or anemia [18].

Dong et al. has shown that TRPML1 can function as a  $\text{Fe}^{2+}$  permeable channel in the LEL based on patch-clamping LEL membranes [27]. Using iron imaging and staining methods, it was

shown that ML4 fibroblasts exhibit a reduction of cytosolic  $\text{Fe}^{2+}$  levels and an increase of intralysosomal  $\text{Fe}^{2+}$  levels when compared to control cells [27]. The authors proposed that  $\text{Fe}^{2+}$  release from endosomes and lysosomes is specifically blocked due to a loss of function of TRPML1. Intralysosomal iron overload caused by a TRPML1-deficiency may lead to serious physiological consequences and could contribute to the neurodegeneration seen in ML4 patients (to be discussed below).  $\text{Fe}^{2+}$  permeability is not a general feature of all TRPML isoforms. TRPML2, but not TRPML3, is also permeable to  $\text{Fe}^{2+}$  (Dong et al., 2008), suggesting a cell type specific function for TRPML2. For example, TRPML2 is expressed on B and T lymphocytes [40,44] and iron metabolism is important for lymphocyte maturation and function [68]. As impaired function of parietal cells and achlorhydria are seen in all ML4 patients [18], TRPML1 and TRPML2 might contribute to the systemic iron metabolism in mechanisms independent of  $\text{Fe}^{2+}$  permeability.

The fact that TRPML1 plays dual roles in iron metabolism and lysosomal  $\text{Ca}^{2+}$  signaling may seem counterintuitive. However, since most TRPs are non-selective for cations, it is not surprising that many of them exhibit dual permeability to ions such as  $\text{Na}^+$  and  $\text{Ca}^{2+}$ , each of which is involved with a distinct cellular output. When TRPs in somato-sensory neurons are activated by sensory cues such as temperature,  $\text{Na}^+$  influx-mediated membrane depolarization is the major signal that mediates greater than 90% of the inward current [69]. Membrane depolarization leads to the propagation of an action potential along the axon that carries the information to the spinal cord and brain. Sensory activation of TRPs also results in small amounts of  $\text{Ca}^{2+}$  influx, that is sufficient to cause sensory adaption and/or peptide release from sensory neurons [15,69]. Although TRPML1 is permeable to both  $\text{Fe}^{2+}$  and  $\text{Ca}^{2+}$ , TRPML1-deficiency results in impaired  $\text{Fe}^{2+}$  homeostasis [27] but normal  $\text{Ca}^{2+}$  homeostasis [66]. The explanation for this difference may be the fact that the lysosomal  $\text{Ca}^{2+}$  gradient is dynamically maintained by the activity of the putative  $\text{H}^+$ - $\text{Ca}^{2+}$  exchanger while lysosomal  $\text{Fe}^{2+}$  is mainly controlled by the release pathway. In order to separate the effects of  $\text{Ca}^{2+}$  versus  $\text{Fe}^{2+}$ , TRPML1 mutations that cause a selective disruption of individual permeability may prove insightful.

## 8. TRPMLs in neurodegeneration

The primary symptom of ML4 patients and TRPML1 KO mice is neurological despite the fact that TRPML-deficiency causes a clear cellular phenotype in all cell types [17,70]. Massive neuronal cell death and axonal degeneration are also seen in the brains of TRPML<sup>-/-</sup> flies [35]. Although glial cell death can also be detected in the fly brain, successful rescue of cell death by neuron-specific expression of TRPML suggests that non-neuronal cell death is secondary to neuronal death [35]. Neuronal cell death increases as an animal ages possibly because of an accumulation of toxic factors [35,70]. One potential factor is lipofuscin, which specifically accumulates in the brains of TRPML1 KO mice and TRPML<sup>-/-</sup> flies [35,70]. Lipofuscin accumulates primarily in the lysosomes of TRPML<sup>-/-</sup> cells [35,70] and its production is likely to be a direct result of TRPML-deficiency. Indeed, the  $\text{Ca}^{2+}$  and  $\text{Fe}^{2+}$  permeability of TRPML1 is sufficient to explain lipofuscin production in TRPML<sup>-/-</sup> cells. First, impaired  $\text{Ca}^{2+}$  release may result in trafficking defects and the delayed exit of lipids from the LEL compartment. This delayed exit suggests that TRPML2, TRPML3, or other lysosomal  $\text{Ca}^{2+}$  channels may compensate for the deficiency. Alternatively, less efficient trafficking pathways may exist in the cell. Regardless, this observed trafficking defect may still be tolerated by most ML4 cells since the primary symptom of ML4 patients is neurological defects despite the trafficking defect and enlarged LEL phenotype observed in all cell types [17]. Second, lysosomal  $\text{Fe}^{2+}$  overload associated

with loss of TRPML1 may convert the accumulated lipids (due to a trafficking defect) into non-degradable lipofuscin under oxidative conditions through increase production of reactive hydroxyl radicals ( $\text{OH}^{\cdot}$ ; Fenton reaction) [71]. In proliferating cells, lysosomal lipofuscin may not be toxic since it can be diluted during cell division [71]. However, in non-proliferative postmitotic cells such as neurons, accumulation of lipofuscin can result in cell death and neurodegeneration. It is hypothesized that correcting either the  $\text{Ca}^{2+}$  or  $\text{Fe}^{2+}$  defect may attenuate the symptoms of ML4 [67]. Therefore, lysosome-targeting iron chelators may be a useful therapy for ML4 [67].

Lipofuscin accumulation is often associated with disrupted autophagy, a lysosome-mediated degradation process that is impaired in many neurodegenerative diseases [72]. Lipofuscin is a non-degradable factor that may cause significant impairment of normal functions of lysosomes when in excess [71]. There are at least three basic functions of lysosomes: digestive and autophagic functions, signal transduction, and membrane trafficking. All of these functions can be inhibited by lipofuscin production. Indeed, impairment of autophagy has been reported in TRPML<sup>-/-</sup> cells [35,52,73] and TRPML1 interacts directly with the autophagic machineries [73]. In addition, overexpression of TRPMLs also results in an inhibition of autophagy [32,33]. Thus TRPMLs may have a direct role in autophagy that is independent of lipofuscin accumulation. Similarly, loss-of-function of TRPML1 affects membrane trafficking in a direct and indirect (lipofuscin-dependent) manner. Impaired autophagy appears to be a general feature of many lysosomal storage diseases [21]. Mitochondrial fragmentation is commonly seen in ML4 and other storage diseases since autophagy is required for recycling of damaged mitochondria [21,66]. Impaired mitochondrial function in turn causes oxidative stress [74]. During oxidative stress, TRPML1-deficiency causes intralysosomal  $\text{Fe}^{2+}$  overload and an increase in the production of free radicals and lipofuscin. Notably, intralysosomal  $\text{Fe}^{2+}$  concentration increases significantly during autophagic ingestion of mitochondria, which are known to contain abundant iron-binding proteins [71]. Therefore, one question to ask is how lipofuscin accumulation causes neurodegeneration. Since lipofuscin accumulation causes a significant impairment of membrane trafficking and autophagy, the subsequent effects of impaired autophagy, mitochondria fragmentation, oxidative stress, intralysosomal overload, and continued lipofuscin production may constitute a vicious cycle that leads to neuronal cell death and axonal degeneration. From a therapeutic point of view, breaking the cycle may protect neurons from degradation. For example, reducing oxidative stress by overexpressing HSP70 chaperone protein in neurons was able to break the cycle and attenuate neurodegeneration [35]. Along this line, correcting either the  $\text{Ca}^{2+}$  or  $\text{Fe}^{2+}$  defect might attenuate neurodegeneration and the neurological symptoms of ML4.

## 9. TRPML in other physiological functions

TRPMLs may also indirectly contribute to neurodegeneration. Recently, Venkatachalam et al. found that overexpression of *Drosophila* TRPML in non-neuronal cells was able to significantly reduce neuronal cell death [35]. Further analysis suggested that *Drosophila* TRPML is required for clearance of apoptotic neurons by glial and immune cells. It is not known whether mammalian TRPMLs have a similar function. However, considering the expression of all three TRPML proteins in lymphoid organs, it is possible that there is a general function for all TRPML members in immune cells. Since the reduction of oxidative stress in non-neuronal cells is ineffective in attenuating neurodegeneration [35], the mechanism by which TRPMLs are required for the functions of immune



cells is probably different from those used in neurons. It is worth nothing that while there are three mammalian TRPML isoforms, *C. elegans* and *Drosophila* only express one single TRPML protein. *C. elegans* and *Drosophila* TRPML mutants appear to exhibit more severe symptoms than ML4 patients and TRPML1 KO mice [18,29,35,36]. Therefore, TRPML2 and TRPML3 may also participate in the non-neuronal functions such as those occurring in immune cells. Human TRPML2 has been shown to co-localize with major histocompatibility complex class I (MHC-I) and CD59 antigen [43], whereas mouse TRPML1 has been shown to co-localize with MHC-II in macrophage cell lines [40]. Together, these data suggest that TRPMLs are possibly involved in immune cell development, differentiation, and antigen processing.

In addition to their putative involvement in neurodegeneration, TRPMLs may have other physiological functions as well. Although loss-of-function studies reveal a role of TRPMLs in neurodegeneration, their neuronal functions under physiological conditions are not clear. Neurite outgrowth is known to be dependent on lysosomal exocytosis [75], a process that involves TRPML1 activity [1,28]. In astrocytes, release of certain transmitters such as ATP is dependent on lysosomal exocytosis [76]. TRPML1 is also required for normal functions of secretory epithelial cells. For example, acid secretion from parietal cells is significantly reduced in ML4 patients [18]. Finally, although an abundance of data describe the intracellular functions of TRPMLs, the potential role of TRPMLs at the plasma membrane cannot be excluded. In specialized cells and/or under certain conditions, TRPMLs may be present and functional in the plasma membrane. This is especially true for TRPML3, which exhibits whole-cell currents when heterologously expressed in a variety of cell lines. The availability of TRPML2 and TRPML3 KO studies will help facilitate our understanding of their physiological functions.

## 10. Summary, perspectives, and future directions

TRPMLs channels have been found to be localized to intracellular vesicles and interact with a variety of vesicular proteins. The basic channel pore properties of TRPMLs have been characterized, however the gating regulation is currently not known. Although functional studies suggest that TRPMLs play active roles in membrane fusion and fission, signal transduction, and vesicular homeostasis, the exact mechanisms of these functions have not been elucidated. In the future, we hope to see the advancement of our knowledge of TRPMLs in the following areas:

1. Identification of TRPML regulators and activators, which will help further characterize the roles of TRPMLs in signal transduction and/or membrane trafficking.
2. Development of specific antibodies to detect endogenous TRPMLs. With these research tools, a full knowledge of TRPML subcellular localization and tissue-expression pattern can be obtained.
3. With the development of TRPML2 and TRPML3 KO mice, more cell type specific functions will be revealed for TRPML2 and TRPML3.
4. The use of real-time live imaging methods to capture TRPML-mediated juxtaorganellar  $\text{Ca}^{2+}$  transients. These seemingly “spontaneous” events may correlate with membrane fusion events that can be monitored with fluorescence imaging approaches.
5. Ion imaging methods will be applied to accurately measure luminal ion concentrations during both basal and stimulated states. This will provide basic information about the electric properties of endolysosomes and vesicles to better understand how TRPMLs function in these compartments.

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