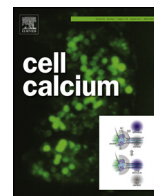




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Invited review

## The role of TRPMLs in endolysosomal trafficking and function

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## ABSTRACT

Members of the Transient Receptor Potential-Mucolipin (TRPML) constitute a family of evolutionarily conserved cation channels that function predominantly in endolysosomal vesicles. Whereas loss-of-function mutations in human TRPML1 were first identified as being causative for the lysosomal storage disease, Mucopolipidosis type IV, most mammals also express two other TRPML isoforms called TRPML2 and TRPML3. All three mammalian TRPMLs as well as TRPML related genes in other species including *Caenorhabditis elegans* and *Drosophila* exhibit overlapping functional and biophysical properties. The functions of TRPML proteins include roles in vesicular trafficking and biogenesis, maintenance of neuronal development, function, and viability, and regulation of intracellular and organellar ionic homeostasis. Biophysically, TRPML channels are non-selective cation channels exhibiting variable permeability to a host of cations including Na<sup>+</sup>, Ca<sup>2+</sup>, Fe<sup>2+</sup>, and Zn<sup>2+</sup>, and are activated by a phosphoinositide species, PI(3,5)P<sub>2</sub>, that is mostly found in endolysosomal membranes. Here, we review the functional and biophysical properties of these enigmatic cation channels, which represent the most ancient and archetypal TRP channels.

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## 1. Mucopolipidosis type IV: a clinical perspective

The major factors that drove researchers into characterizing the TRPML channels were that they belong to the TRP superfamily of channels, which have garnered a lot of interest recently [1], and that loss-of-function mutations in one of the human TRPML isoforms, TRPML1, results in an autosomal-recessive lysosomal storage disease (LSD) called Mucopolipidosis type IV (MLIV). Originally described in 1974, MLIV was identified in patients exhibiting corneal opacity and intracellular accumulation of lysosomal inclusions [2–5]. The disease causing mutations likely originated in Lithuania around the 18th/19th centuries, and although it has now been described in the general population, the vast majority of MLIV cases involve Ashkenazi Jews, whose heterozygous carrier frequency for MLIV

related mutations is ~1:100 [6–9]. Early descriptions of MLIV noted the accumulation of gangliosides and mucopolysaccharides and cytoplasmic autofluorescent puncta in the brain bearing similarities to those observed in ceroid-lipofuscinoses [5,6,10,11]. These histological alterations still remain some of the most salient and defining features of MLIV and may be used for diagnosis of MLIV [12,13]. Subsequent biochemical investigations revealed that patient cells accumulated 2–3 fold excess of GM3 and GD3 gangliosides, mucopolysaccharides, and phospholipids [14–17]. This abnormal accumulation of storage material in MLIV cells occurs due to their retention in acidic organelles, delayed metabolism, and diminished trafficking to the plasma membrane and Golgi [18–22]. Thus, the storage in MLIV is secondary to the trafficking and metabolic defects associated with those lipids. Indeed, the secondary defects in vesicular trafficking could underlie the heterogeneity of storage material in MLIV and other LSDs [23]. However, we cannot rule out whether some of the storage material also accumulates due to primary defects in the activity of lysosomal enzyme(s) due to alterations in trafficking of these enzymes to the lysosomes or alterations in the intraluminal environment of the lysosomes, which prevent the enzymes from functioning optimally.

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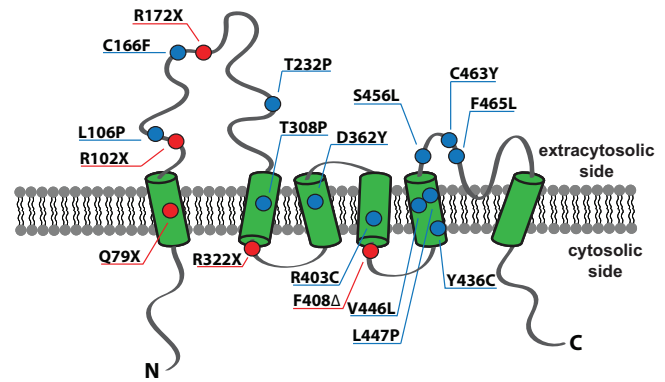
### 1.1. Clinical features of MLIV

Often misdiagnosed as cerebral palsy [24], the characteristics of MLIV include profound psychomotor disability resulting in hypotonia (and sometimes spasticity), and severe unrelenting cognitive impairment, which becomes evident by ~1–2 years of age when patients fail to meet the normal developmental milestones [6,12,25–28]. Both gross and fine motor skills are severely compromised [26,28]. As they get older, patients are unable to walk unassisted and are restricted to wheelchairs [26]. Although MLIV patients surviving into the 30s have been reported [27], the peak cognitive capacity of the more severely affected patients rarely progresses beyond that typical of 2–3 year old children. Most patients do not demonstrate significant expressive lingual ability [26,27]. However, over the subsequent decades of life the course of MLIV is heterogeneous and slowly progressing with some symptoms showing negligible deterioration [26]. Thus, MLIV is a developmental disease with a slowly degenerative component. Indeed, head MRIs have indicated a characteristically thin corpus callosum [28,29], which does not worsen with age – likely indicating developmental defects in axonal growth and/or myelination. However, postmortem analyses reveal that MLIV brains exhibit significant neuronal loss in the cortex and basal ganglia, and reactive astrogliosis in the brain stem [30]. The involvement of the basal ganglia in MLIV patients may underlie the deficits in cognition as well as motor control.

One exception to the overall slow time-course of MLIV progression is the rapidly progressive retinal degeneration. Even if some of the less severely affected patients do not display clear psychomotor disabilities, the ocular disturbances may still be present [31,32]. Along with bilateral corneal opacity and optic atrophy, the retinal degeneration results in complete blindness by the end of the first decade of life [25–28,33,34]. Patients have also been reported to exhibit other ocular disturbances including pain, strabismus, bouts of excessive tearing and conjunctivitis, and occasional photophobia [26,35]. Altered electroretinograms (ERGs) in MLIV patients indicate photoreceptor involvement [36]. Although other sensory modalities such as hearing are normal in MLIV patients [26], anecdotal evidence from parents suggests an elevated threshold for peripheral pain possibly suggesting diminished neuronal excitability. As opposed to some other LSDs like Gaucher's disease and mucopolysaccharidoses, skeletal abnormalities are absent in MLIV [26,37]. Other characteristic features of MLIV, which set this disease apart from other LSDs, include constitutive achlorhydria resulting in iron deficiency and markedly elevated serum gastrin levels – used as a diagnostic tool to detect MLIV [28,37,38]. The achlorhydria is likely a consequence of the large lysosomal inclusions in gastric parietal cells [39].

### 1.2. Identification of mutations in *MCOLN1* as causative for MLIV

Early attempts to identify the MLIV causing mutations suggested the presence of a single mutated gene on a 1 cM region of chromosome 19p [40,41]. Within a year, three independent studies identified mutations in *MCOLN1*, which encodes Muco1ipin-1, as the genetic lesion underlying MLIV [8,42,43]. The predominant Ashkenazi Jewish haplotype (~72% of the alleles) was found to be a mutation in the splice acceptor site of the 3rd intron on *MCOLN1* resulting in aberrant splicing and skipping of exons 4 and 5 [8,42–44]. In this haplotype, a frameshift mutation results in premature termination [42]. Another Ashkenazi haplotype (albeit relatively minor, ~23% of the alleles) was identified as a ~6 kb deletion spanning the first 6–7 exons of *MCOLN1* [8,42–44]. These two mutations account for ~95% of the MLIV mutations in the MLIV population [8,40,42]. Other mutations included nonsense and



**Fig. 1.** Topology of TRPML1 and MLIV-causing mutations that lead to codon change. TRPML1 is predicted to consist of six transmembrane domains (green barrels). Reported MLIV-causing mutations cause changes to the amino acid residues located in both loops and transmembrane regions. Missense mutations causing amino acid substitution are indicated in blue circles. Mutations causing premature stop codon (X) or in-frame deletion ( $\Delta$ ) are indicated in red circles. N and C denote amino-terminus and carboxyl terminus, respectively.

missense mutations and an in-frame deletion in *MCOLN1* (Fig. 1), some of which result in relatively milder phenotypes [8,9,44,45].

## 2. TRPMLs: lysosomal cation channels

Analysis of the protein encoded by the *MCOLN1* gene immediately revealed significant homology to the TRP superfamily of cation channels [8,42,43,46]. Owing to the homology to TRP channels, mucolipin-1 was also named TRPML1, and was classified into the TRP superfamily of cation channels [1,47]. Most mammals express two additional TRPML isoforms called TRPML2 and TRPML3 [47]. In contrast, zebrafish express 5 *trpml* related genes [48]. A single *trpml* gene is encoded in each of the genomes of yeast (called *ycv1/trpy*) [1,49,50], *Dictostelium* [51], *Caenorhabditis elegans* (called *cup-5*) [52], and *Drosophila* [53]. A detailed description of the *trpml* genes in these model organisms is given in a subsequent section of this review.

### 2.1. Subcellular localization of mammalian TRPMLs

When overexpressed in cultured cells, TRPML1 is largely a late-endosomal/lysosomal (LE/lys) transmembrane protein [21,54–56]. One of the MLIV related variants (TRPML1<sup>T232P</sup>) is retained in the ER [54] indicating that diminished localization of TRPML1 to the lysosome can result in milder MLIV-like phenotypes. TRPML1 has two di-leucine motifs (on the N- and C-terminal cytoplasmic domains) that regulate the trafficking of TRPML1 to LE/lys [21,55,56]. Deletion of these di-leucine motifs causes the accumulation of TRPML1 in the plasma membrane [21,55,56]. Conversely, abrogation of clathrin-mediated endocytosis by the expression of a dominant-negative variant of dynamin also results in the accumulation of TRPML1 in the plasma membrane [56]. Although these findings strongly suggest that TRPML1 is trafficked to the lysosome via the cell surface, TRPML1 can also be delivered to the lysosome directly from the *trans*-Golgi without intermediate delivery to the plasma membrane [55,57].

TRPMLs can also be trafficked from endolysosomes to the plasma membrane in a regulated and activity dependent manner. Constitutively active TRPML1 mutants, which mimicked the varitint-waddler (*Va*) mutation of mouse TRPML3 [58–61], exhibit pronounced plasma membrane localization, which is likely a consequence of lysosomal exocytosis owing to the elevated  $\text{Ca}^{2+}$  efflux via TRPML1 [62]. Increasing the activity of the mechanistic target of rapamycin complex-1 (MTORC1) activity in *Drosophila* cells

also leads to elevated plasma membrane localization of fly TRPML [63]. This observation could have multiple explanations including MTORC1 decreasing TRPML endocytosis from the plasma membrane or driving TRPML exocytosis potentially by elevating TRPML channel activity.

TRPML2 and TRPML3 localize to intracellular vesicles and long-tubular structures [56,64,65]. Similar to TRPML1, TRPML2 has been observed in the LE/lys compartment [56,64], and traffics via the Arf6-associated pathway and colocalizes with the MHC class I and GPI-anchored proteins [65]. In contrast, overexpressed TRPML3 localizes to multiple subcellular compartments including the early and late-endosomes [66,67], endoplasmic reticulum [56], plasma membrane, and autophagosomes [67]. These findings indicate that TRPML3 is very dynamic in the cell, and regulates multiple steps in the endolysosomal pathway and autophagy. Moreover, the three mammalian TRPMLs are capable of forming heteromultimers [56,68–70]. Once coupled into heteromultimers with either TRPML1 or TRPML2, the localization of TRPML3 is largely determined by either TRPML1 or TRPML2 indicating hierarchical control of TRPML subcellular distributions [56]. Moreover, the biophysical properties and regulation of the heteromultimers may be somewhat distinct from the homomultimers, which could contribute to the functional diversity of these proteins [69]. Nevertheless, despite the potential for the mammalian TRPMLs to physically interact, these interactions may have limited overall biological significance because whereas TRPML1 is ubiquitously expressed, at least TRPML2 is expressed predominantly in the lymphoid organs and kidneys [71]. Interestingly, the hierarchical control of TRPML2 by TRPML1 in these organs may also extend to transcriptional regulation because TRPML2 transcription correlates with the presence and/or activity of TRPML1 [71].

## 2.2. Biophysical properties of TRPML channels

Early studies relying on the constitutive activity of a small proportion of the overexpressed TRPML1 present on plasma membrane revealed non-selective cation conductance with permeability to  $\text{Ca}^{2+}$  [72–75]. The observed currents were outwardly rectifying and inhibited by low pH on the extracellular side. These features were also observed for TRPML1 reconstituted in planar lipid bilayers [76]. The outwardly rectifying TRPML1 current was inhibited by activation of protein kinase A and phosphorylation of two C-terminal serine residues S557 and S559 was shown to be critical for this regulation [77]. However, the biophysical properties described in the early studies are inconsistent with those reported in later ones, which were carried out employing rather different methods, including: (1) the use of mutant constructs that mimicked the varitint-waddler (Va) mutation of mouse TRPML3 and expressed on plasma membrane with high constitutive activities [58–61]; (2) induction of wild type TRPML2 and TRPML3 activity on plasma membrane by removal of extracellular  $\text{Na}^+$  [58,78–80]; and (3) direct whole-lysosome recording of TRPML currents from enlarged LE/lys [50,61]. Under these conditions, all TRPML channels show inward rectification and the current mediated by either TRPML1 or TRPML2 is enhanced by the low pH that mimics the luminal environment of lysosomes [59,61]. On the other hand, the activity of TRPML3 is inhibited by low pH with a sigmoidal current–pH relationship and  $\text{IC}_{50}$  of  $\sim$ pH 6.4 for current mediated by either  $\text{Na}^+$  or  $\text{Ca}^{2+}$  [59,78]. The proton-mediated inhibition appears to involve three histidines (H252, H273, and H283) in the first extracytosolic loop of TRPML3 [78]. However, for the TRPML3<sup>Va</sup> mutant (A419P), low extracytosolic pH was shown to either have no effect [78] or be inhibitory [59]. The reason for the discrepancies between the earlier and more recent studies remains obscure. Although variations in experimental conditions could account for some of the observed differences, only inward,

but not outward, rectification is reported in all recent studies from different groups (see [72,74,81,82]). Therefore, the nature of the outwardly rectifying currents in earlier studies remains mysterious.

Interestingly, the extracytosolic pH dependence of TRPML1<sup>Va</sup> (V432P) was found to be biphasic, showing potentiation between pH 7.5 and 4.5 and then inhibition at lower pH [81]. The optimal pH for maximal TRPML1 activity was pH 4.5, which is close to the mean pH value of the lysosomal lumen of mammalian cells. Also interesting is that *Drosophila* TRPML has a similar biphasic dependence on extracytosolic pH, but the optimal pH for maximal activation was shifted to pH 5.2 [81], consistent with the mean luminal pH of insect lysosomes [83].

The luminal side of the lysosomes contains mostly  $\text{Na}^+$ , with relatively low amounts of  $\text{Ca}^{2+}$  and  $\text{K}^+$  ions [61]. In addition, the  $\text{Ca}^{2+}$  selectivity of TRPML3 is  $\sim$ 350 times that of  $\text{K}^+$  [78]. As non-selective cation channels, TRPML channels are equally permeable to  $\text{Na}^+$  and  $\text{K}^+$  [81]. The inward rectification property and the relatively positive potential in the luminal side of the lysosome [84] suggest that TRPML channels mainly mediate  $\text{Na}^+$  efflux from these organelles.

TRPML1 and TRPML2, as well as the fly TRPML, conduct  $\text{Fe}^{2+}$  and other divalent cations [61,81]. However, TRPML3 appears to be relatively  $\text{Fe}^{2+}$ -impermeable [61]. Although the divalent cation permeability of TRPML1 has been shown to be important for cellular  $\text{Fe}^{2+}$  and  $\text{Zn}^{2+}$  metabolism [61,85], it is not known whether TRPML1 is directly permeable to  $\text{Zn}^{2+}$  and TRPML1 may be affecting  $\text{Zn}^{2+}$  metabolism indirectly without actually being permeable to the cation.

All the TRPML channels tested so far are activated by phosphatidylinositol 3,5-bisphosphate ( $\text{PI}(3,5)\text{P}_2$ ) – a phosphoinositide enriched in endolysosomes [61,81]. The findings that TRPMLs are activated by  $\text{PI}(3,5)\text{P}_2$  are consistent with the notion that TRPMLs mainly function in endolysosomes. Further supporting this idea is that TRPML1 and *Drosophila* TRPML are inhibited by phosphatidylinositol 4, 5-bisphosphate ( $\text{PI}(4,5)\text{P}_2$ ) [81,86], the main  $\text{PIP}_2$  species in plasma membrane. Thus, although TRPML channels are trafficked to plasma membrane, they may not be functional there because of the high  $\text{PI}(4,5)\text{P}_2$  levels.

A number of small-molecule TRPML3 agonists have been identified by high throughput screening [87]. Many of them also act at TRPML2 [80], but only a few, for instance SF-22 and SF-51, also stimulate TRPML1 [50,82,88]. Both compounds have now been optimized and renamed MK6-83 (for SF-22) and ML-SA1 (for SF-51). These compounds have allowed detection of endogenous TRPML-like currents in fibroblasts and macrophages [50,88,89]. Recently, it was shown that ML-SA1 is not an agonist for *Drosophila* TRPML, but rather an allosteric activator that acts specifically on open TRPML channels [90]. In contrast, SF-22 has no effect on the fly channel [90].

The commonly used cation channel and/or  $\text{Ca}^{2+}$  channel blockers, 2APB (200  $\mu\text{M}$ ), SKF 96365 (50  $\mu\text{M}$ ), amiloride (100  $\mu\text{M}$ ), ruthenium red (10  $\mu\text{M}$ ), or nifedipine (100  $\mu\text{M}$ ) did not inhibit TRPML1 or TRPML3, while verapamil blocked TRPML3 only at 1 mM [59]. Recently, novel TRPML1 inhibitors, ML-SI1-3, have been described and shown to inhibit particle uptake and lysosomal exocytosis in bone marrow-derived macrophages [91], demonstrating their utility in assessing the functions of native TRPML1 channels.

## 3. TRPMLs in animal models

### 3.1. Invertebrate TRPMLs

The only TRP channel gene in the budding yeast, *Saccharomyces cerevisiae*, is *Yvc1* (also referred to as TRPY [1]) – a channel that mediates release of  $\text{Ca}^{2+}$  from the vacuole when yeast cells



are exposed to a hyperosmotic shock [49]. Multiple features of yeast Yvc1/TRPY indicate functional homology with TRPMLs. First, Yvc1/TRPY shows significant homology at a sequence level to other invertebrate and vertebrate TRPML channels (identity/similarity: with *C. elegans* CUP-5 = 16/40%; with *Drosophila* TRPML = 15/42%; with human TRPML1 = 16/40%; with human TRPML2 = 16/41%; with human TRPML3 = 16/38%). Second, similar to vertebrate TRPML1 and *Drosophila* TRPML, Yvc1/TRPY is a PI(3,5)P<sub>2</sub> activated Ca<sup>2+</sup> release channel expressed in the membrane of the vacuole [50,81]. The activation of TRPML1 by PI(3,5)P<sub>2</sub> has been shown to depend on a cluster of basic amino acid residues on the N-terminus prior to the 1st transmembrane domain of the protein (amino acids residues 55–62: **KFRAGGRK**) [50]. A similar polybasic domain is found in the cytosolic N-terminus of Yvc1/TRPY (amino acid residues 84–91: **KDKANKRK**). It would be interesting to determine whether this domain mediates the activation of Yvc1/TRPY by PI(3,5)P<sub>2</sub>. Third, expression of TRPML1 in yeast cells lacking Yvc1/TRPY partially restored the hyperosmotic shock dependent vacuolar Ca<sup>2+</sup> release [50]. Owing to these similarities of yeast Yvc1/TRPY to TRPMLs, it is likely TRPMLs represent the most ancient and archetypical TRP channels. *Dictostelium* TRPML was reported to be required for lysosomal Ca<sup>2+</sup> uptake, and TRPML-deficient *Dictostelium* cells were characterized by reduced lysosomal Ca<sup>2+</sup> levels, and enhanced lysosomal exocytosis [51], which is in sharp contrast to several observations made with mammalian and other invertebrate TRPMLs.

*C. elegans* express a single TRPML homolog called Coelomocyte Uptake Defective-5 (CUP-5) [52]. Coelomocytes are scavenger cells that line the worm body cavity and are responsible for uptake of the fluid from the body cavity and lysosomal degradation of the proteins dissolved therein. The uptake and degradation of the body cavity fluids and dissolved solutes is defective in all the CUP mutants [52]. As is the case for mammalian TRPMLs, CUP-5 was found to be a LE/lys protein and loss of the protein led to the accumulation of large lysosome-like organelles [52,92]. In addition, the *cup-5* null worms are characterized by embryonic lethality and elevated accumulation of apoptotic cells [92,93]. Interestingly, these phenotypes are rescued by the exogenous expression of human TRPML1 or TRPML3 indicating functional redundancy between these isoforms [92,93].

*Drosophila* also express a single TRPML isoform, which is a LE/lys transmembrane protein [53,63]. Whereas the neuronal phenotypes associated with the *cup-5* hypomorphs have yet to be described, *trpml* deficient *Drosophila* are characterized by several neuronal phenotypes, many of which bear remarkable congruence with the established neurological features of MLIV. The plethora of phenotypes observed following loss of *Drosophila trpml* include elevated accumulation of acidic organelles and autophagic vacuoles in neurons and other tissues, severely compromised autophagic flux leading to age-dependent accumulation of ubiquitinated intermediates, diminished glutamatergic synaptic transmission, elevated lethality during the pupal period, severe locomotor defects during larval and adult phases, and progressive neurodegeneration in the CNS and retina [53,63]. The cell death in the *Drosophila* nervous system is incredibly precipitous, and is caused by two simultaneously occurring defects. First, defective autophagy, mitochondrial dysfunction, and elevated levels of reactive oxygen species result in cell autonomous neuronal apoptosis [53]. Second, a concomitant defect in the phagocytic clearance of these cell corpses results in a secondary “neuroinflammation”, which results in non-cell autonomous decline in neuronal viability and ultimately underlies the rapid onset of the phenotypes [53]. Thus, the expression of wild-type TRPML in the *trpml* mutant phagocytic cells or elevating the phagocytic clearance of the apoptotic cells significantly delays the onset of the phenotypes despite the continued cell autonomous demise of neurons [53].

Interestingly, the pupal lethality associated with the loss of *Drosophila trpml* was only partially suppressed by the expression of human TRPML1, which shares 38% amino acid sequence identity with *Drosophila* TRPML (~50% suppression with human TRPML1 compared to 100% rescue following expression of *Drosophila* TRPML) [53,63,81]. We anticipate that there could be multiple explanations for this observation. First, the optimal pH of *Drosophila* TRPML is ~0.6 pH units higher than that for human TRPML1 [81], which is consistent with the slightly higher pH of endolysosomes in *Drosophila* compared to mammals (~5.1 in *Drosophila* lysosomes vs. ~4.5 in mammalian lysosomes) [83,94]. Therefore, *Drosophila* lysosomal pH might be optimized for *Drosophila* TRPML but not for human TRPML1 preventing full activation of the human channel in *Drosophila* cells. Second, *Drosophila* TRPML also shares features of mammalian TRPML2 and TRPML3 [81] indicating that expression of these other isoforms or coexpression of the three isoforms in *Drosophila* cell might be necessary for the complete rescue of the *Drosophila trpml* mutant phenotypes. Third, human TRPML1 might have a necessary subunit that is missing in *Drosophila* cells. For instance, human two-pore channels, which are lysosomal channels that share functional similarities with TRPML1 [89,95–97] and may be necessary for the optimal functioning of TRPML1, are not expressed in *Drosophila* [98].

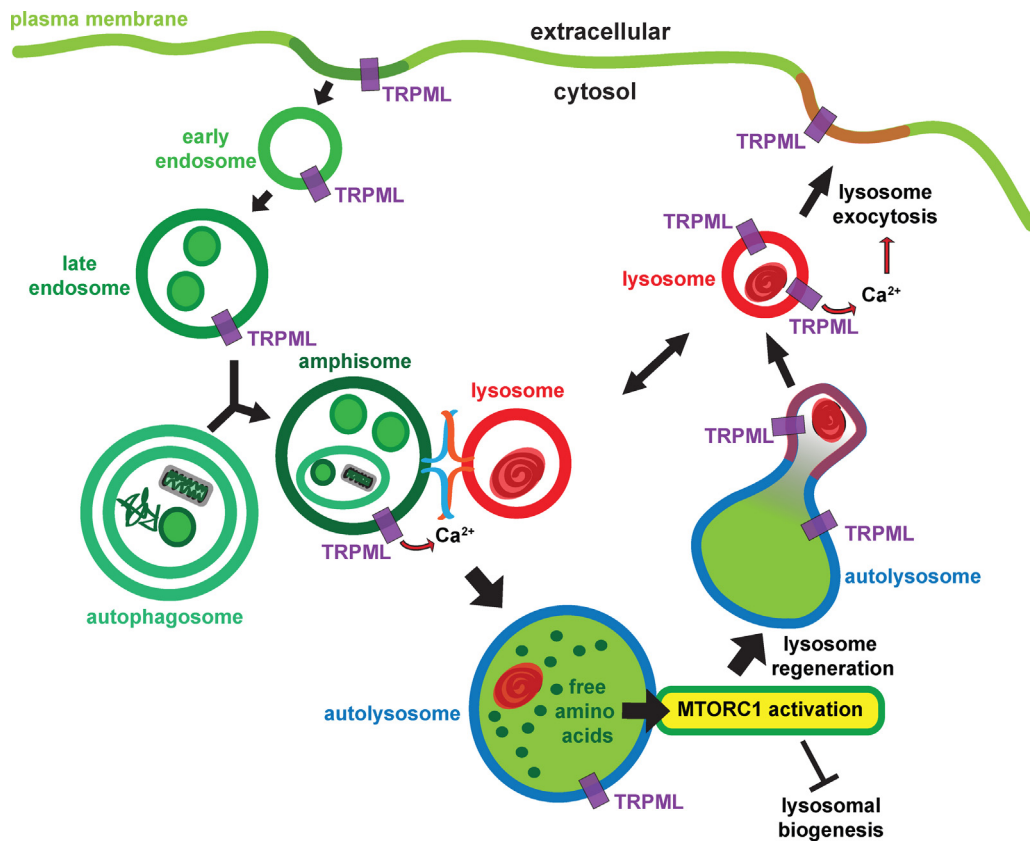
### 3.2. Vertebrate TRPMLs

The zebrafish genome encodes five TRPML genes (two TRPML1 orthologs, one TRPML2 ortholog, and two TRPML3 orthologs) [48]. Similar to mammalian TRPML1, the two TRPML1 orthologs in zebrafish are localized to LE/lys [48]. However, mutational analyses of these genes have not yet been described. Two independently generated TRPML1 knockouts in mice have been described [99,100]. One of the lines faithfully recapitulates many of the MLIV phenotypes including accumulation of lysosome-like inclusions in the nervous system and elsewhere, progressive neurological deficits, and retinal degeneration [99]. Further evaluation of the neuropathological deficits of the MLIV mice revealed elevated ganglioside accumulation in the CNS, presence of p62 puncta characteristic of diminished autophagic flux, reduced myelination, and the presence of activated glial cells in the nervous system indicative of neuroinflammation [101]. These findings indicate that both the mouse and *Drosophila* MLIV models exhibit elevated neuroinflammation indicating that this may be an evolutionarily conserved outcome of loss of TRPML activity in the nervous system. Both mouse models of MLIV also exhibit achlorhydria and elevated serum gastrin levels [99,100].

## 4. Biological functions of TRPMLs

### 4.1. Role of TRPMLs in vesicular trafficking

Both homotypic and heterotypic fusion of endosomal vesicles requires Ca<sup>2+</sup> [102]. In *Drosophila*, the Ca<sup>2+</sup> required for the fusion of late-endosomes and amphisomes (hybrid organelles formed by LE-autophagosome fusion) with lysosomes is provided by TRPML [63,103] (Fig. 2). These data are consistent with the findings that loss of *Drosophila* TRPML or mammalian TRPML1 results in the elevation of LE/lys Ca<sup>2+</sup> levels [63,104]. Loss of *Drosophila* TRPML results in the formation of “fusion-clamped” amphisomes and lysosomes, which resemble synaptic vesicles docked and primed on the presynaptic membrane, but are unable to undergo fusion resulting in diminished degradation of endocytosed and autophagic material [63,103]. *cup-5* deficient worms also exhibit diminished endolysosomal degradation of cell surface receptors [105] and loss of TRPML1 in mammalian cells results in delayed fusion of



**Fig. 2.** Biological functions of TRPMLs in different cellular compartments. TRPML localizes to endolysosomes and facilitates  $\text{Ca}^{2+}$ -dependent heterotypic fusion between amphisomes and lysosomes resulting in lysosomal degradation of endocytosed and autophagic material, which provides free amino acids for activation of the MTORC1. MTORC1 activation reduces lysosomal biogenesis via TFEB and promotes lysosome regeneration from autolysosomes. TRPML present on lysosome membrane is also responsible for triggering  $\text{Ca}^{2+}$ -dependent lysosomal exocytosis, which in turn delivers TRPML to the plasma membrane.

autophagic vacuoles with lysosomes [106]. Both mammalian cells and fly neurons lacking TRPMLs accumulate autophagic vacuoles, polyubiquitinated proteins, and p62 – features that are characteristic of diminished autophagic flux [53,106,107]. These studies are consistent with early findings that the delivery of endocytosed material such as lipids is delayed to the lysosomes in MLIV cells [18–21]. However, this issue is somewhat controversial in mammalian cells because other studies have found that the delivery of endocytosed material to lysosomes is not significantly delayed or impaired following acute knockdown of TRPML1 in mammalian cells [108]. These apparently conflicting findings could be explained on the basis of alterations in kinetics as opposed to a complete block of lysosomal delivery in the TRPML1 deficient cells, whereby different experimental conditions and time-courses result in variable findings [21].

The reformation of lysosomes from hybrid organelles formed after the fusion of late-endosomes with lysosomes is also a  $\text{Ca}^{2+}$ -dependent process [102,109]. Consistent with a role of TRPMLs in regulating lysosomal reformation, loss of CUP-5 results in a failure in lysosomal reformation and progressive defects in lysosomal transport in *C. elegans* [93,110,111]. However, detailed ultrastructural studies using electron microscopy, which is capable of distinguishing between lysosomes, MVBs, amphisomes, and autophagosomes, did not reveal a defect in lysosomal biogenesis/reformation in *Drosophila trpml* mutants [63]. Rather, the levels of electron dense lysosomes are ~10-fold elevated in the *trpml* deficient cells compared to wild-type controls [63] – a phenotype not consistent with diminished lysosomal reformation.

Although the biological functions of TRPML2/3 are incompletely understood, TRPML2 promotes the activation of the Arf6 pathway,

and regulates the sorting of GPI-anchored proteins [65]. TRPML3, which is a non-selective cation channel that localizes to both early- and late-endosomes and is inactivated by low pH, has been shown to be involved in the regulation of endosomal  $\text{Ca}^{2+}$  homeostasis, luminal acidification, and fusion/trafficking of endosomal vesicles [58,66,67,112,113]. Knockdown of TRPML2 and TRPML3 also results in the accumulation of lysosome-like vesicles, indicating significant overlap in the functions of mammalian TRPMLs-3 in regulating vesicular trafficking [68].

Similar defects in membrane and vesicular trafficking could also be occurring in other LSDs, especially since the function of TRPML proteins can be altered in these diseases. For instance, in Niemann–Pick disease cells, sphingomyelins are shown to abnormally accumulate in the lysosomes [88]. Interestingly, sphingomyelins inhibit the activity of TRPML1 leading to subsequent lysosomal storage, which can be corrected by exogenously activating TRPML1 using specific agonists [88]. These data suggest that the vesicular trafficking defects in Niemann–Pick disease and MLIV may show considerable overlap given that a decrease in TRPML1 activity underlies both diseases. It would also be useful to determine whether additional LSDs are characterized by decreased TRPML1 activity as a causal event for the vesicular storage because TRPML1 agonists could be beneficial in these diseases.

#### 4.2. Role of TRPMLs in LE/lys and autophagosome biogenesis

The absence of TRPML genes is characterized by profound accumulation of LE/lys, as well as constitutive autophagy and elevated de novo autophagosome biogenesis [53,63,105–107]. Indeed, the accumulation of acidic organelles such as LE/lys is the

quintessential feature of all LSDs. We recently asked: *what might be the biochemical signaling pathways that underlie the accumulation of lysosomal inclusions in LSDs?* Following lysosomal degradation, the amino acids derived from proteolysis are fed into anabolic pathways. Lysosomal free amino acids activate the Mechanistic Target of Rapamycin (MTOR) kinase containing complex, MTORC1 (Fig. 2), which couples the levels of free amino acids to protein translation and growth [114–116]. When MTORC1 activity is elevated in the presence of abundant cellular free amino acids, the kinase complex negatively regulates both autophagosome and LE/lys biogenesis [117]. In contrast, during periods of cellular amino acid starvation, MTORC1 activity drops resulting in elevated autophagosome and LE/lys biogenesis [117]. As a consequence, increased lysosomal protein degradation ensues resulting in amino acid replenishment, reactivation of MTORC1, and cessation of autophagosome biogenesis [117,118].

We recently found that diminished delivery of endocytosed and autophagic material to lysosomes in *Drosophila* cells lacking TRPML results in lower MTORC1 activity [63,103]. Therefore, lysosomal dysfunction results in lower free amino acid levels and subthreshold MTORC1 activation. Interestingly, restoration of MTORC1 activity completely suppressed the accumulation of LE/lys in the TRPML deficient *Drosophila* cells [63]. These data indicate that despite the continued absence of TRPML, the accumulation of acidic vacuoles is reversed simply by the reactivation of MTORC1 [63,103]. Although the role of MTORC1 downstream of CUP-5 has not been examined in worms, starvation due to inefficient lysosomal degradation of endocytosed embryonic yolk protein (degradation of yolk protein provides amino acids and lipids required for survival of embryos) and a ~10-fold decrease in ATP levels has been proposed to partially underlie the lethality observed in *cup-5* mutants and restoration of ATP levels partially suppresses the mutant phenotypes [105]. Because elevation of cellular ATP levels should also result in increased MTORC1 activity due to suppression of AMPK activity – a negative regulator of MTORC1 activity [119], the pronounced biogenesis of autophagosomes and LE/lys and other consequence of amino acid and ATP starvation in the absence of TRPMLs is likely a consequence of diminished signaling through the MTORC1 pathway.

#### 4.3. Role of TRPMLs in lysosomal exocytosis and vesicular delivery to the plasma membrane

Early studies of the properties of TRPML1 found that it drives the exocytosis of lysosomes [72,73,120]. Indeed, TRPML1 variants carrying mutations that elevate the channel activity induce constitutive lysosomal exocytosis leading to pronounced plasma membrane localization of the channels [62]. TRPML1 has also been shown to promote the formation of tubular extensions from the cell surface that are a consequence of LE/lys exocytosis via a process requiring the serine-lipase domain present in an extracytosolic domain [121]. In mouse macrophages, TRPML1 is required for the exit of endocytosed macromolecules from the lysosomes and the delivery of major histocompatibility complex II to the plasma membrane [22]. Moreover, overexpression of transcription factor EB (TFEB), a master regulator of endolysosomal biogenesis that promotes TRPML1 transcription [122], promotes lysosomal exocytosis by upregulating the expression of TRPML1 [123,124] – yet again confirming the critical function of TRPML1 in endolysosomal exocytosis. Recently, TRPML1 was shown to be required for focal exocytosis of LE/lys to the site of phagosome formation at the plasma membrane and thereby plays a significant role in phagocytosis [91]. Therefore, loss or inhibition of TRPML1 results in a decrease in the phagocytic uptake of senescent and apoptotic cell corpses [91]. A defect in the phagocytic uptake of apoptotic neurons was also found in *Drosophila* lacking *trpml* and this defect underlies

the precipitous onset of neurodegeneration in the *Drosophila* MLIV model [53].

#### 4.4. Role of TRPMLs in mitochondrial function and cell viability

The concept of a “lysosome–mitochondrial” axis indicates close functional crosstalk between these two organelles [125,126]. Indeed, unbiased protein interaction studies have found that TRPML1 physically interacts with several mitochondrial proteins [127]. Therefore, lysosomal dysfunction in cells lacking TRPML1 lead to concomitant aberrations in mitochondrial function, morphology, Ca<sup>2+</sup> buffering capacity, and caspase-mediated cell death [128]. Knockdown of endogenous TRPML2 and TRPML3 also result in mitochondrial abnormalities [68]. *Drosophila* adults lacking TRPML show pronounced mitochondrial dysfunction, accumulation of mitochondria with dissipated electrochemical membrane potentials, diminished autophagic-clearance of damaged mitochondria, and increased reactive-oxygen species [53]. Together, these cytological insults result in neuronal cell loss in the brains of *Drosophila* adults lacking TRPML [53]. Recently, loss of TRPML1 in mammalian cells was also shown to result in loss of mitochondrial membrane potential and the buildup of reactive oxygen species [129].

Whereas the loss of TRPMLs results in mitochondrial dysfunction and eventual cell death, excessive activity of TRPMLs also results in cell death, presumably by inducing Ca<sup>2+</sup> excitotoxicity. The varitint-waddler mice (bearing the *Va* mutation (A419P) in TRPML3) are characterized by deafness, vestibular alterations, and diminished pigmentation [130]. Several groups found that the *Va* mutation in murine TRPML3 leads to a gain of function phenotype whereby excessive Ca<sup>2+</sup> flux through TRPML3 results in Ca<sup>2+</sup> cytotoxicity, cell death, and auditory hair cell dysfunction [58–60,78,112,131]. Similarly, introduction of the *Va* mutation in TRPML2 also results in elevated plasma membrane localization, Ca<sup>2+</sup> overload, and subsequent demise of the cells [79]. Introduction of the *Va* mutation in conserved residues of TRPML1 or *Drosophila* TRPML also results in a constitutively active channel [61,62,81]. These studies demonstrate that TRPML channel activity in cells needs to be tightly regulated and uncontrolled cation flux through these channels induces cellular toxicity.

Cell death in the absence of TRPMLs can also take place via mitochondria-independent mechanisms. For instance, acute knockdown of TRPML1 also results in the loss of lysosomal integrity and the release of the lysosomal protease cathepsin B into the cytosol [132]. The cytoplasmic cathepsin B contributes to the apoptosis observed in the absence of TRPML1 [132]. In this regard, decreasing the levels of LE/lys by activating MTORC1, which should also decrease the levels of cathepsin B in cell, might prove beneficial in decreasing the toxicity associated with the loss of TRPML1.

#### 4.5. Role of TRPMLs in the regulation of lysosomal Fe<sup>2+</sup> and Zn<sup>2+</sup>

Lysosomes and mitochondria serve as the major cellular reserve of Fe<sup>2+</sup> and Zn<sup>2+</sup> – cations whose homeostatic regulation by TRPMLs is critical for the survival of cells [104]. Interestingly, TRPML1 serves as an endolysosomal Fe<sup>2+</sup> channel and loss of TRPML1 results in Fe<sup>2+</sup> accumulation in these acidic organelles [61]. The MLIV-associated point mutations affect Fe<sup>2+</sup> flux via TRPML1 to varying extent, and this variability correlates with disease severity associated with those mutations [61]. *Drosophila* TRPML is also capable of fluxing Fe<sup>2+</sup> (but not Fe<sup>3+</sup>) [81] indicating evolutionary conservation of Fe<sup>2+</sup> fluxing capacity of TRPML channels. Loss of the TRPML1 also results in Zn<sup>2+</sup> accumulation in acidic organelles [133]. Although the permeability of TRPML1 to Zn<sup>2+</sup> has not been directly measured, these data might indicate a role for TRPML1 in regulating Zn<sup>2+</sup> efflux from LE/lys. Besides mediating Zn<sup>2+</sup> efflux directly, TRPML1



could be affecting lysosomal Zn<sup>2+</sup> levels by mediating the exocytosis of Zn<sup>2+</sup> laden lysosomes, trafficking of Zn<sup>2+</sup> transporters between various subcellular locations, or the levels of Zn<sup>2+</sup> binding proteins.

#### 4.6. Central role for TRPMLs in LSDs

LSDs such as Niemann–Pick are also characterized by significantly reduced activity of TRPML1 [88]. In addition, overexpression of TFEB decreases the storage of acidic vacuoles in LSDs by activating TRPML1-dependent lysosomal exocytosis [122–124,134]. Therefore, TRPML channels may have roles in multiple LSDs and activating these channels could be beneficial in a wide array of storage diseases.

#### 4.7. A controversial role of TRPMLs in the regulation of endolysosomal pH

The impact of TRPMLs on endosomal pH has been a matter of debate and scrutiny. Early studies reported that lysosomal pH is elevated by one pH unit in MLIV cells [135]. However, subsequent studies suggested that TRPML1 might be a lysosomal H<sup>+</sup> channel responsible for the release of H<sup>+</sup> from the lysosomal lumen, thus a lower luminal pH in the absence of TRPML1 [74,75]. Lower lysosomal pH following the loss of TRPML channel activity has also been reported by other studies [53,108]. Moreover, slightly elevating the lysosomal pH using Nigericin or Chloroquine has been shown to be beneficial in MLIV cells [75]. However, another study found that Nigericin/Chloroquine treatment led to very limited attenuation of the lysosomal storage observed in MLIV cells [136]. In addition, other studies have reported that TRPML1 is not permeable to H<sup>+</sup> [59,61]. In summary, the exact effect of the loss of TRPML on H<sup>+</sup> flux and endolysosomal pH, if any, is still incompletely understood.

### 5. Physical and genetic interactors of TRPMLs

#### 5.1. Physical interactors of TRPMLs

A large-scale screen identified several candidate proteins that interact with TRPML1 [127]. Briefly, some of the interesting interactors of TRPML1 in this screen involved the cytoskeleton modifying small G-proteins such as Rac2 and Cdc42 (indicating a potential role of TRPML1 in cytoskeleton regulation), lysosomal enzymes such as Cathepsin B, members of the solute carrier family of transporters, the ER Ca<sup>2+</sup> release channel IP<sub>3</sub>-receptor as well as the Ca<sup>2+</sup> transporting ATPase (SERCA2), markers of the ER-Golgi intermediate compartments (ERGIC) and Yif1, which regulate ER to Golgi trafficking of proteins (these interactions may mediate the exit of TRPML1 and associated proteins from the ER), and peroxisome-associated Pex16 [127]. Interestingly, both approaches identified several mitochondrial proteins as TRPML1-interactors that among others include NADH dehydrogenase, ATP synthase subunits, and subunits of the mitochondrial voltage-dependent ion channel (VDAC, also known as Porin) [127].

Another screen found that TRPML1 physically associates with members of the lysosome-associated protein transmembrane (LAPTM) family of lysosomal transporters [137]. Knockdown of LAPTMs in mammalian cells led to the accumulation of lysosomal inclusions morphologically similar to those observed in MLIV cells [137]. These data strongly implicate the loss of interactions between TRPML1 and LAPTMs in the pathophysiology of MLIV. TRPML1 also interacts with the EF-hand containing protein, ALG-2, in a Ca<sup>2+</sup> dependent manner and this interaction impacts endolysosomal vesicle trafficking [138].

#### 5.2. Genetic interactors of TRPMLs

In *C. elegans*, loss of CUP-5 was found to be associated with elevated accumulation of a lysosomal ABC transporter, MRP-4, which is responsible for the transport of lipids into the lysosomal lumen [139]. Loss of function MRP-4 mutations suppressed the lysosomal degradation defects and the embryonic lethality observed in the CUP-5 deficient animals suggesting that the lysosomal accumulation of MRP-4 and the lipophilic molecules transported by the protein contribute to the toxicity associated with the loss of *cup-5* [139]. In *Drosophila*, the toxicity associated with the loss of *trpml* is suppressed by the expression of molecular chaperones such as heat shock proteins and is enhanced by the expression of polyglutamine-stretch containing proteins [53]. These findings indicate that the toxicity associated with the loss of *trpml* might be related to the accumulation of macromolecular aggregates and potentially and unfolded protein response. In humans, gene expression profiles revealed that transcription of several hundred genes, many of which are involved in lysosomal function and hydrolytic activity, endolysosomal trafficking and vesicle fusion, solute transport, organellar biogenesis, and lipid metabolism, are altered in MLIV fibroblasts compared to controls [140].

### 6. Concluding remarks

Over the last decade and a half, several in-roads have been made into understanding the biological functions and biophysical properties of TRPMLs in mammalian cells and animal models. We anticipate that in the future a host of biological functions of the TRPML channels unrelated to MLIV will also be described. However, we still face several challenges. For instance, identifying the precise nature of the neuronal dysfunction in vertebrate models of MLIV and human patients is essential before we come up with viable concepts for therapy. Another interesting question is whether lipids such as PI(3,5)P<sub>2</sub> that activate TRPMLs are dynamically regulated in response to physiological stimuli that drive endolysosomal Ca<sup>2+</sup> release. Also, whether or not manipulation of TRPML function can ameliorate other LSDs remains to be categorically established. Nevertheless, the future likely holds exciting new findings, which will undoubtedly drive our understanding of these unique channels and lysosomal physiology to new heights.

#### Conflict of interest

The authors declare no conflicts of interest.

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