4 TRPML Subfamily of Endolysosomal Channels
Concepts and Methods

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4.1 INTRODUCTION

The mucolipin subgroup of the transient receptor potential superfamily of cation channels (TRPMLs) are evolutionarily conserved non-selective cation channels that function in endolysosomal membranes, and play key roles in the regulation of endocytosis, autophagy, and intracellular trafficking. Mammalian genomes encode three TRPML paralogs – TRPML1, TRPML2, and TRPML3 – that differ in tissue

distribution and exhibit subtle, yet significant, differences in subcellular localization and molecular function. In humans, *MCOLN1*, which encodes TRPML1, is ubiquitously expressed, and loss-of-function mutations in this gene cause a paediatric-onset lysosomal storage disease called mucolipidosis type IV (MLIV) (Bargal et al., 2000; Bassi et al., 2000; Sun et al., 2000).

Owing to the significance of TRPMLs in human health and disease, much has been learned about the function of endosomal proteins in a variety of biological contexts. The effort to understand TRPML has been facilitated by the proliferation of many tools and techniques to study these channels. For example, robust models of MLIV exist in several genetically tractable organisms, and reliable protocols that leverage small molecules that modulate channel activity have been identified. These developments have aided researchers in elucidating the function of TRPMLs, which has contributed to the redefinition of endolysosomes as complex and dynamic organelles that serve myriad functions that extend well beyond the notion of these vesicles being the sites of macromolecular degradation. Additional studies have focused on the development of therapies that mitigate the pathology associated with MLIV and other related lysosomal diseases.

4.2 TRPMLS IN HEALTH AND DISEASE

Loss-of-function mutations in *MCOLN1*, which is located on chromosome 19p, lead to MLIV (Bargal et al., 2000; Bassi et al., 2000; Sun et al., 2000). The incidence of this disease is estimated to be 1 in 40,000 live births, but within the Ashkenazi Jewish population, the heterozygous carrier frequency is closer to 1:100, which points to a strong founder effect (Venkatachalam et al., 2015; Zeevi et al., 2007). The disease is found across other ethnicities as well, but the true frequencies in these cohorts are likely underrepresented due to misdiagnosis (Wakabayashi et al., 2011). Indeed, the manifestations of MLIV are very similar to those described in cerebral palsy. Whereas two mutations account for ~95% of Ashkenazi MLIV cases and lead to complete loss of gene expression, over 20 other mutations have been identified in total (Venkatachalam et al., 2015; Wakabayashi et al., 2011; Zeevi et al., 2007). Amongst the latter cohort are missense mutations that lead to intermediate phenotypes. As such, genotype–phenotype correlation is reasonably strong for MLIV.

Although appearing normal at birth, patients with MLIV present with a wide range of clinical symptoms early in post-natal development (Venkatachalam et al., 2015; Wakabayashi et al., 2011; Zeevi et al., 2007) (Figure 4.1). Severe psychomotor delay, characterized by intellectual disability and difficulty developing a variety of motor skills, is a prominent feature of MLIV. By adolescence and adulthood, most patients are unable to ambulate independently and suffer from cognitive impairment. Patients with MLIV also exhibit ophthalmological abnormalities including corneal opacity, which is considered a hallmark of the disease. The visual dysfunction apparent in MLIV is usually progressive and culminates in fulminant blindness. Although corneal clouding contributes to the loss of vision, the eventual onset of blindness is a consequence of progressive retinal degeneration. Degeneration in the central nervous system is not extensive, with patients manifesting neurological phenotypes early due to neurodevelopmental deficits, which is followed by a protracted

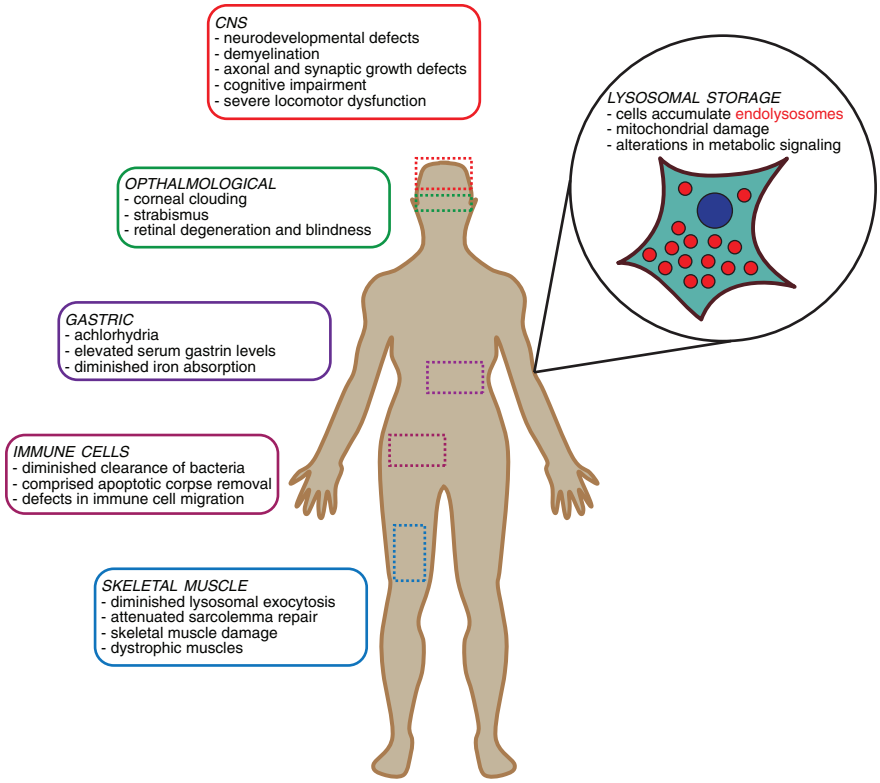

FIGURE 4.1 Symptoms observed in MLIV patients. MLIV symptoms are categorized on the left on the basis of affected organ systems. Note that although the skeletal muscle and immune defects have been described in animal models, these alterations have not been directly examined in human patients. Nevertheless, the remarkable conservation of phenotypes across species suggests the existence of these defects in MLIV patients. The inset on the right depicts accumulation of endolysosomal vesicles (red) in virtually every cell of the patient. Other cellular phenotypes are also indicated.

phase of slow decline in central nervous system function. Intriguingly, neurological regression and seizures – defining features of the neural ceroid lipofuscinoses (NCL) cohort of lysosomal diseases (Bennett and Rakheja, 2013; Boustany, 2013) – have not been described in MLIV. Although the absence of seizures in diseases with lysosomal dysfunction is not unique to MLIV (Pastores and Maegawa, 2013), it remains to be determined whether the absence of seizures in MLIV reflects a potential role for TRPML1 in the onset of seizures in NCL patients. Another unusual feature of MLIV is the appearance of gastrointestinal ailments, which occur predominantly due to achlorhydria resulting from diminished secretion of gastric acid by parietal cells (Altarescu et al., 2002). Achlorhydria prevents adequate iron absorption in the gut, leading to iron deficiency and attendant anaemia. Patients also exhibit elevated levels of the hormone gastrin in the plasma, which has been used as a diagnostic criterion for MLIV.

Compared to *MCOLN1*, *MCOLN2* and *MCOLN3* exhibit relatively restricted tissue expression (Castiglioni et al., 2011; Cuajungco et al., 2016; Miao et al., 2015; Sun et al., 2015). Although human diseases with mutations in *MCOLN2* and *MCOLN3* have not been defined, naturally occurring gain-of-function mutations in murine *Mcoln3* cause the pathological varitint-waddler (Va) phenotype (Di Palma et al., 2002). The Va-associated mutation, A419P, has been shown to lock TRPML3 in the open state, resulting in constitutive channel activity and resistance to regulatory inhibition (Grimm et al., 2007; Kim et al., 2007; Nagata et al., 2008; Xu et al., 2007). Mice bearing this mutation are hypopigmented, deaf, and exhibit vestibular abnormalities that result in stereotypical wandering behaviour (Atiba-Davies and Noben-Trauth, 2007). The neurosensory phenotype results from degeneration of the sensory hair cells (Van Aken et al., 2008; Castiglioni et al., 2011; Nagata et al., 2008). The Va mutation in TRPML3 inspired the creation of analogous mutations in other TRPML proteins (Feng et al., 2014; Lev et al., 2010; Xu et al., 2007). These mutated channels are also constitutively active and have aided in the study of TRPML function.

TRPMLs also contribute to the pathophysiology of diseases that are not directly related to MLIV. Alzheimer's disease-linked mutations in presenilin-1 lead to elevated Ca^{2+} release from endolysosomes via TRPML1 (Lee et al., 2015). Mitigating TRPML1 hyperactivation under these circumstances alleviates disease phenotypes. However, the role for TRPML1 in neurodegenerative diseases is likely more complex than initially envisioned since other studies have found that a decrease in TRPML1 activity is causally associated with Aβ1–42-dependent degeneration in Alzheimer's disease (Zhang et al., 2017b, 2017a). These findings are consistent with other reports that activation of TRPML1 is involved in the clearance of toxic Aβ aggregates in models of HIV infection (Bae et al., 2014). Furthermore, mutations in *VAC14* or *FIG4* – genes encoding proteins regulating levels of the endogenous TRPML agonist, phosphatidylinositol 3,5-bisphosphate – lead to Yunis-Varón syndrome and neurodegenerative motor neuron disease with features that mimic lysosomal storage (Lines et al., 2017; Zou et al., 2015). In these cases, reactivation of TRPML1 by synthetic agonists has been shown to ameliorate disease phenotypes. Notably, the importance of TRPMLs in health extends beyond the nervous system since the contribution of these channels to immune cell function highlights their involvement in preventing infections and/or immunodeficiency (Bretou et al., 2017; Dayam et al., 2015; Miao et al., 2015; Sun et al., 2015; Wong et al., 2017; Zhong et al., 2017).

4.3 BIOPHYSICAL AND MOLECULAR CHARACTERIZATION OF TRPMLS

TRPMLs are non-selective cation channels that exhibit inwardly rectifying currents (Dong et al., 2008; Feng et al., 2014; Grimm et al., 2007; Kim et al., 2007; Lev et al., 2010; Nagata et al., 2008; Xu et al., 2007). Mammalian TRPML1 and TRPML2 and *Drosophila* TRPML have all been found to be potentiated by low pH on the luminal side, which indicates that their activity increases as the vesicular environment acidifies during endolysosomal maturation (Dong et al., 2008; Feng et al., 2014; Lev et al., 2010). TRPML1 is optimally active at pH 4.5, which is also the pH of

the mammalian lysosome. In contrast to the other isoforms, TRPML3 is inhibited by low pH, which is consistent with the activity of the channel in early endosomes before the lumen of the vesicles is acidified (Kim et al., 2008).

TRPMLs are activated by the endosomal phosphoinositide – phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) (Dong et al., 2010; Feng et al., 2014). Interestingly, phosphatidylinositol 4, 5-bisphosphate ($PI(4,5)P_2$), which is abundant in the plasma membrane, inhibits TRPMLs indicating that these channels are inactivated at the plasma membrane. Being non-selective cation channels, TRPMLs are permeable to monovalent cations such as Na^+ as well as divalent cations such as Ca^{2+} (Dong et al., 2008; Feng et al., 2014; Grimm et al., 2007; Kim et al., 2007; Lev et al., 2010; Nagata et al., 2008; Xu et al., 2007). Further validating the versatility of these channels, TRPML1 also permeates Fe^{2+} and Zn^{2+} with important consequences to cellular metabolism and gene transcription (Cuajungco and Kiselyov, 2017; Cuajungco et al., 2014; Dong et al., 2008; Eichelsdoerfer et al., 2010; Feng et al., 2014; Grimm et al., 2007; Kim et al., 2007; Kukic et al., 2013; Lev et al., 2010; Nagata et al., 2008; Xu et al., 2007).

As with other TRP channels, TRPMLs function as tetramers that can be either homo- or heteromultimeric (Curcio-Morelli et al., 2010; Venkatachalam and Montell, 2007; Venkatachalam et al., 2006; Zeevi et al., 2010). Förster resonance energy transfer (FRET) and immunoprecipitation assays have demonstrated that TRPML1 can form homomultimers or heteromultimers with either TRPML2 or TRPML3. Given that particular combinations of TRPMLs may possess different biophysical properties, the ability of TRPMLs to form heteromultimers may expand the functional diversity of these channels. Another shared feature is that each TRPML monomer is comprised of six membrane-spanning helices with N- and C-terminal domains residing in the cytosol (Venkatachalam and Montell, 2007). Recently, the first cryo-electron microscopy structures of TRPML proteins were described (Chen et al., 2017; Hirschi et al., 2017; Schmiege et al., 2017; Zhang et al., 2017c; Zhou et al., 2017). These studies correlate functional observations describing various regulatory mechanisms – such as the influence that $PI(3,5)P_2$ and pH have on channel activity – with structural information at the atomic level. Owing to the contributions of these studies, it is now understood that the TRPML1 agonist, ML-SA1, binds to a hydrophobic cavity that is *not* equivalent to the activating site on TRPV1 (Schmiege et al., 2017). The native ligand and inhibitor, $PI(3,5)P_2$ and $PI(4,5)P_2$, respectively, bind to the N-terminus of TRPMLs, and an intermediate domain couples ligand-binding to pore opening or closing (Chen et al., 2017; Hirschi et al., 2017). The intermediate domain is proposed to act as a 'gating pulley' in transferring the signal from the phosphoinositide to the channel pore (Hirschi et al., 2017). Additional identified features include the residues in the selectivity filter of TRPML1 that confer pH sensitivity (Chen et al., 2017), and a 'gating rod' on TRPML3 that connects to the pore loop and mediates the inhibition of this paralog by low pH (Zhou et al., 2017).

4.4 TISSUE AND SUBCELLULAR DISTRIBUTION OF TRPMLS

The lack of effective antibodies against TRPMLs, which is exacerbated by the low expression levels of these proteins, has hindered detailed examination of their tissue

and subcellular distribution. Consequently, most studies investigating the location of these channels across tissues rely on mRNA quantification. These studies have revealed that *MCOLN1* is expressed in all tissues with relative enrichment in the brain, heart, kidney, liver, and spleen (Sun et al., 2000). Ubiquitous expression is conserved in evolution, as *Drosophila trpml* is also expressed at low levels in multiple tissues (Venkatachalam et al., 2008). In contrast to *MCOLN1*, expression of mammalian *MCOLN2* appears to be restricted to kidney and lymphoid tissues (Cuajungco et al., 2016; Samie et al., 2009; Sun et al., 2015). Indeed, the presence of TRPML2 in lymphoid organs informs a potential role in the function of immunity (Sun et al., 2015). In addition, there are long and short splice variants of TRPML2 – TRPML2lv and TRPML2sv, respectively – with the short variant exhibiting higher expression than the long variant (Samie et al., 2009). Interestingly, it appears that the expression of mucolipins is coordinated in certain contexts. For instance, expression of *Mcoln2*, but not *Mcoln3*, is diminished in mice lacking *Mcoln1*, which points to the existence of hierarchical control mechanisms that coordinate mucolipin gene expression (Samie et al., 2009). As is the case for *MCOLN2*, *MCOLN3* is expressed in the kidney and lymphoid organs. In addition, *MCOLN3* is expressed in the eyes, skin (melanocytes), and cochlea (hair cells). The pathological consequences of the aforementioned Va phenotype, including pigmentation abnormalities and deafness, are well-aligned with the tissue distribution of TRPML3 (Van Aken et al., 2008; Atiba-Davies and Noben-Trauth, 2007; Castiglioni et al., 2011; Miao et al., 2015; Nagata et al., 2008).

TRPMLs are endolysosomal membrane proteins. Fluorescently tagged TRPML1 is predominantly localized to late-endosomes and lysosomes as evidenced by colocalization with Rab7, lysosomal-associated membrane protein 1 (Lamp1), endolysosomal lipid LBPA, and LysoTracker (Manzoni et al., 2004; Thompson et al., 2007; Venkatachalam et al., 2006; Vergarajauregui and Puertollano, 2006). The TRPML1 ortholog in *Drosophila* also localizes to the membranes of late-endosomes and lysosomes (Venkatachalam et al., 2008; Wong et al., 2012). Human TRPML1 bears two di-leucine motifs that mediate the localization of the protein from the plasma membrane to endosomes (Venkatachalam et al., 2006; Vergarajauregui and Puertollano, 2006). Since expression of dominant-negative dynamin was sufficient to mislocalize TRPML1 to the cell surface, we envision that localization of TRPML1 to endolysosomal membrane requires internalization from the plasma membrane (Venkatachalam et al., 2006). However, TRPML1 can also be delivered to endocytic compartments directly from the *trans*-Golgi via a pathway mediated by adapter protein complex-1 (AP-1) (Vergarajauregui and Puertollano, 2006). The existence of multiple routes of endolysosomal delivery likely serves as a failsafe mechanism to ensure that TRPML1 reaches the endocytic compartment. Loss of *MCOLN1* or its orthologs in different species results in accumulation of endolysosomes, which is a defining feature of lysosomal storage diseases such as MLIV (Figure 4.1).

Given the physical interactions between TRPML1 and TRPML2, it stands to reason that the latter protein also localizes to late-endosomes (Venkatachalam et al., 2006). In addition, TRPML2 has been detected in long tubulovesicular compartments associated with GTPase ADP-ribosylation factor-6 (Arf-6) (Karacsonyi et al., 2007; Radhakrishna and Donaldson, 1997). Indeed, activation of Arf-6 has

been shown to cause accumulation of TRPML2 in tubulovesicular structures where it colocalizes with major histocompatibility complex I and glycosylphosphatidylinositol-anchored proteins (Karacsonyi et al., 2007). Interactions with TRPML1 drive TRPML3 to the endolysosomal membrane (Venkatachalam et al., 2006). When overexpressed alone in cell culture models, murine TRPML3-YFP was found localized to the endoplasmic reticulum (ER) (Venkatachalam et al., 2006). Only upon coexpression with TRPML1 or TRPML2, which possess endolysosomal targeting motifs, was TRPML3 delivered to the vesicles, yet again pointing to a hierarchical relationship between the channels. TRPML3 does appear to exhibit the greatest diversity in subcellular compartments. In addition to localizing to endolysosomal compartments, TRPML3 has also been shown to reside in the plasma membrane, early endosomes, and autophagosomal membranes (Kim et al., 2009; Martina et al., 2009; Miao et al., 2015).

4.5 ANIMAL MODELS OF MLIV

Evolutionarily, TRPMLs are highly conserved proteins with homologs identified in diverse lineages. Vertebrates encode multiple paralogs of TRPML – the mammalian and zebrafish genomes are marked by the expression of three and five TRPML encoding genes, respectively (Benini et al., 2013; Li et al., 2017). Two independently generated mouse models with *Mcoln1* deletions have been described (Chandra et al., 2011; Venugopal et al., 2007). Both models faithfully recapitulate various aspects of MLIV including neurodevelopmental and psychomotor defects, ophthalmological abnormalities and retinal degeneration, achlorhydria and elevated serum gastrin levels, and of course, diminished endolysosomal Ca^{2+} release. The neurological phenotypes of the *Mcoln1*$^{-/-}$ mice include diminished strength, shorter gait, and eventual paralysis of the hindlimbs. Remarkably, these mice also demonstrated MLIV phenotypes on the cellular level including ubiquitous presence of endolysosomal inclusions. Zebrafish express two orthologs of human TRPML1, and their deletions elicit many of the features of MLIV including retinal and neuromuscular defects (Benini et al., 2013; Li et al., 2017).

The commonly studied invertebrates, *Drosophila melanogaster* and *Caenorhabditis elegans*, possess single TRPML encoding genes – *trpml* and *coelomocyte uptake defective-5* (*cup-5*), respectively (Fares and Greenwald, 2001; Venkatachalam et al., 2008). The worm *cup-5* mutants exhibit maternal-effect embryonic lethality and endolysosomal accumulation. Flies lacking *trpml* exhibit defects in completion of autophagy with a concomitant build-up of endolysosomes in a wide range of tissues, high rates of pupal lethality, age-dependent neurodegeneration, and locomotor impairment. The MLIV flies also exhibit defects in glutamatergic synapse development and neurotransmission (Venkatachalam et al., 2008; Wong et al., 2015). Interestingly, the neurological phenotypes in the MLIV flies are a result of a complex interplay between neurons and phagocytic cells (Venkatachalam et al., 2008). First, cell autonomous endolysosomal defects in neurons led to accumulation of damaged mitochondria and diminished cell viability. A secondary, non-cell autonomous effect in phagocytic cells such as glia was triggered by the dying neurons and led to neuroinflammation. Remarkably, reintroduction of wild-type *trpml* in

only the phagocytic cells was sufficient to significantly delay the locomotor defects and attendant lethality. These findings led to the intriguing proposal that bone marrow transplantation to introduce functional phagocytic cells such as microglia in patients lacking TRPML1 could delay the onset of MLIV – a concept that was successfully validated in a mouse model of MLIV (Walker and Montell, 2016).

In the yeast *Saccharomyces cerevisiae*, *yvc1* encodes a protein that possesses several striking functional similarities to TRPMLs at the level of protein sequence (~40% similarity with mammalian TRPMLs) (Denis and Cyert, 2002; Dong et al., 2010). Furthermore, in a manner similar to TRPML1, YVC1 is activated by $PI(3,5)P_2$ leading to the release of Ca^{2+} from the yeast vacuole. In yeast lacking *yvc1*, expression of human TRPML1 led to a partial suppression of phenotypes (Dong et al., 2010). Conservation of function has also been demonstrated in *C. elegans* and *Drosophila*, where expression of human TRPML1 suppresses mutant phenotypes (Feng et al., 2014; Hersh et al., 2002; Treusch et al., 2004). Although the *C. elegans* mutant phenotype was suppressed by expression of human TRPML3 (Treusch et al., 2004), we have found that the phenotypes in *trpml*-deficient *Drosophila* larvae were not suppressed by the expression of human TRPML2 or TRPML3 (unpublished observations). Nevertheless, our observations argue in favour of fundamental and conserved biological roles for TRPML1 in eukaryotic cell biology.

4.6 CELLULAR FUNCTIONS OF TRPMLS

4.6.1 Endocytosis and Autophagy

The endocytic pathway is initiated at the plasma membrane, where molecularly defined regions of the bilayer are pinched inwards to form early endosomes via a pathway orchestrated with exquisite spatiotemporal precision (Kaksonen and Roux, 2018). Although likely inactive in that compartment, the delivery of TRPML1 to endosomes also occurs at the level of the plasma membrane, (Pryor et al., 2006) (Figure 4.2). The neutral extracellular pH as well as the abundance of $PI(4,5)P_2$ in the plasma membrane synergize to prevent TRPML activation at the cell surface (Dong et al., 2010; Feng et al., 2014). As TRPML1-bearing endosomes mature, the vacuolar ATPase (V-ATPase) pumps protons into the endolysosomal lumen such that the pH of the late-endosomal vesicles settles at ~4.5–5 in mammalian cells. In addition, activity of the Pikfyve/Vac14/Fig4 complex in late-endosomes leads to the generation of $PI(3,5)P_2$ (McCartney et al., 2014). The coincidence of low pH and $PI(3,5)P_2$ is necessary for TRPML1 activation. It is therefore not surprising that deletion of genes encoding the Pikfyve/Vac14/Fig4 complex leads to phenotypes that are remarkably similar to those observed in the absence of TRPMLs in both mammals and *Drosophila* (Rusten et al., 2006; Zolov et al., 2012).

Activation of TRPML1 has profound effects on the trafficking and fate of endolysosomal vesicles. The fusion of late-endosomes or autophagosomes with lysosomes are Ca^{2+}-dependent processes that depend upon luminal Ca^{2+} released via endolysosomal channels such as TRPMLs (Pryor et al., 2000; Wong et al., 2012) (Figure 4.2). Therefore, TRPMLs participate in the heterotypic fusion of late-endosomes or amphisomes with lysosomes, which is important for the appropriate degradation of

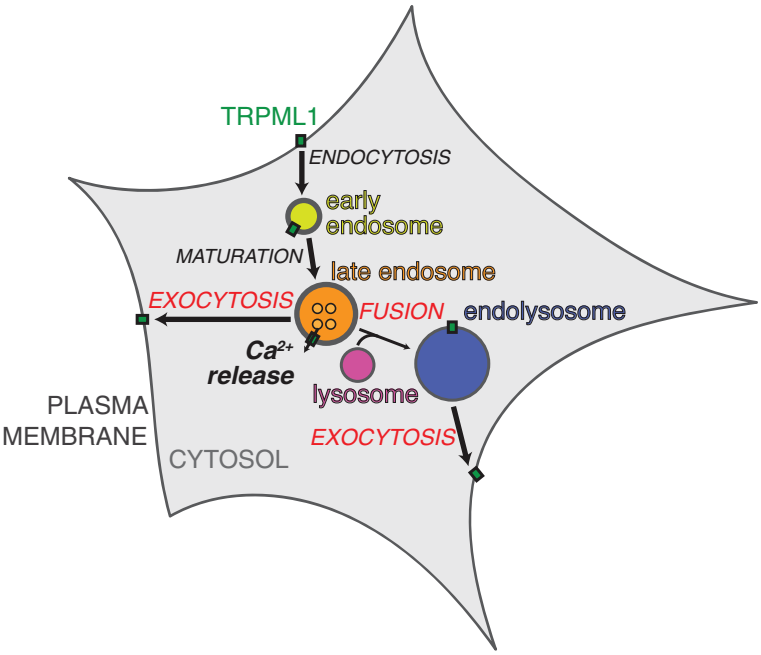

FIGURE 4.2 Schematic diagram showing the functions of TRPML1 in endolysosomal vesicular trafficking. The route of TRPML1 (green rectangle) trafficking from the plasma membrane to endolysosomal vesicles is shown. Vesicular trafficking steps described in red letters require TRPML1 activity.

the material nucleated within those vesicles. Evidence from multiple model systems suggests that Ca^{2+} efflux from the late-endosomes/amphisomes via TRPMLs promotes Ca^{2+}-dependent vesicle fusion (Schaheen et al., 2006; Vergarajauregui et al., 2008a; Wong et al., 2012). In *Drosophila*, loss of TRPML leads to attenuated late-endosome/amphisome–lysosome fusion leading to a build-up of these unfused vesicles (Venkatachalam et al., 2008; Wong et al., 2012). Similarly, in mammalian cells lacking TRPML1, fusion of vesicles in the autophagic pathway is delayed (Miedel et al., 2008; Vergarajauregui et al., 2008a). Consequently, markers of diminished endolysosomal and autophagic flux – accumulation of p62, polyubiquitinated proteins, and cellular waste destined for degradation – have been observed in TRPML-deficient human cells and model systems.

4.6.2 Regulation of Endolysosomal Exocytosis

In addition to mediating endosomal fusion, TRPMLs also regulate endolysosomal exocytosis – the process by which cells direct the traffic of vesicles to the plasma membrane for the release of endolysosomal contents into the extracellular space (Bretou et al., 2017; Cao et al., 2015; Cheng et al., 2014; Lima et al., 2012; Miao et al., 2015; Ravi et al., 2016; Sahoo et al., 2017; Samie et al., 2013; Zhong et al., 2016) (Figure 4.2). This notion is supported by evidence from studies

demonstrating that gain-of-function mutations of mammalian TRPML1 cause an elevation in lysosomal trafficking to the plasma membrane in HEK293T cells and a predictable increase in the levels of TRPML1 at the plasma membrane (Dong et al., 2009). Furthermore, lysosomal exocytosis is impaired in MLIV (LaPlante et al., 2006).

Owing to the role of TRPMLs in exocytosis, many biological processes that require endolysosomal exocytosis exhibit a functional dependence on TRPMLs. In the murine macrophage cell line, RAW 264.7, delivery of the major histocompatibility complex II to the plasma membrane is diminished following *Mcoln1* knockdown (Thompson et al., 2007). In the context of immune cell function, TRPML1-dependent endolysosomal exocytosis has additional functions. For instance, focal exocytosis following TRPML1 activation provides membrane to the growing phagocytic cup during the internalization of apoptotic cell corpses (Samie et al., 2013). Diminished phagocytosis of apoptotic cells is also observed in the nervous system of *trpml*-deficient flies, where this defect contributes to the precipitous onset of neurodegeneration (Venkatachalam et al., 2008). More recently, TRPML1-mediated vesicular exocytosis has been shown to be critical for immune cell chemotaxis via a process requiring the actin cytoskeleton and Rac GTPases (Bretou et al., 2017; Dayam et al., 2017). TRPML1-mediated endolysosomal Ca^{2+} release is required for the exocytosis of tubulovesicles for gastric acid in the parietal cells of the stomach (Sahoo et al., 2017). This cellular property of TRPML1 explains the achlorhydria that characterizes patients suffering from MLIV. The release of gastric acid-laden tubulovesicles in the parietal cell is triggered by an elevation of cytosolic cyclic AMP (cAMP) and the subsequent activation of protein kinase A (PKA) (Sahoo et al., 2017). These data indicate that cAMP and PKA are capable of triggering TRPML1-dependent endolysosomal exocytosis. Although the exact mechanism underlying TRPML1 activation by PKA remains undefined, these findings are aligned with a previous study that PKA phosphorylates TRPML1 (Vergarajauregui et al., 2008b). It also remains to be seen whether the crosstalk between cAMP–PKA–TRPML1 relates to other processes requiring TRPML1-dependent vesicle release. Conversely, it would be worth investigating whether TRPML1 plays a role in other pathways that require cAMP- and PKA-dependent exocytosis.

From a mechanistic perspective, the role of TRPML1 activation in endolysosomal exocytosis is especially intriguing in the context of situations that are marked by dramatic elevations in cytosolic Ca^{2+}. For instance, TRPML1-mediated exocytosis is required for the repair of skeletal muscle membrane (also called sarcolemma), following damage that is a consequence of normal muscle function or following acute injury (Cheng et al., 2014). Indeed, *Mcoln1*$^{-/-}$ mice exhibit muscle degeneration that is attributed to diminished sarcolemma repair. It is well known that skeletal muscle fibres exhibit dramatic elevations in cytosolic Ca^{2+} during contraction, which will only be amplified if the sarcolemma were damaged. In this framework, it stands to reason that the role of TRPML1 in endolysosomal exocytosis is not related to elevation of cytosolic Ca^{2+} *per se*. We envision that specific steps in the endolysosomal trafficking process require the TRPML1 channel in a manner that cannot be explained by global cytosolic Ca^{2+} elevations although a role for localized Ca^{2+} elevations remains a possibility.

4.6.3 Bidirectional Interactions with the Mechanistic Target of Rapamycin Complex 1 (mTORC1) Signalling

By mediating the fusion of vesicles carrying material destined for degradation, TRPML1 influences several signalling modalities in the cell. Diminished lysosomal degradation of proteins in the absence of TRPMLs results in diminished activity of mTORC1 – a master regulator of anabolism and protein synthesis (Sancak et al., 2010; Venkatachalam et al., 2013; Wong et al., 2012, 2015; Zoncu et al., 2011). Normally, lysosomal degradation of proteins and the consequent increase in the availability of free amino acids activate mTORC1, which in turn prevents the necessity for endolysosomes and autophagosomes during periods of amino acid sufficiency (Efeyan et al., 2012). However, cells lacking functional TRPMLs are characterized by attenuated endolysosomal protein degradation and the consequent paucity of free amino acids leading to low mTORC1 activity (Venkatachalam et al., 2013; Wong et al., 2012, 2015) (Figure 4.3). In *Drosophila*, the decrease in mTORC1 activation in cells lacking *trpml* can be restored by ectopic supplementation of a high protein diet or the genetic reactivation of mTORC1. In addition to the effects of TRPML activation on the bioavailability of amino acids that activate mTORC1, endolysosomal Ca^{2+} release via TRPML1 can also directly activate mTORC1 via calmodulin (Li et al., 2016; Sun et al., 2018). Thus, the activity of TRPML1 influences mTORC1 activity through multiple pathways.

Pointing to a bidirectional relationship between mTORC1 and TRPML1, the activation of mTORC1 influences TRPML1-mediated endolysosomal Ca^{2+} release. The reciprocal relationship likely reflects an intricate feedback loop since starvation activates TRPML1-mediated endolysosomal Ca^{2+} response via mTORC1 (Onyenwoke et al., 2015; Wang et al., 2015). This model is consistent with mTORC1 negatively regulating the activity of TRPML1 channels. Besides decreasing channel conductance, the activation of mTORC1 in *Drosophila* leads to elevated localization of TRPML at the plasma membrane (Wong et al., 2012). Mislocalization of TRPML in the plasma membrane would be expected to compromise the function of the channels due to the

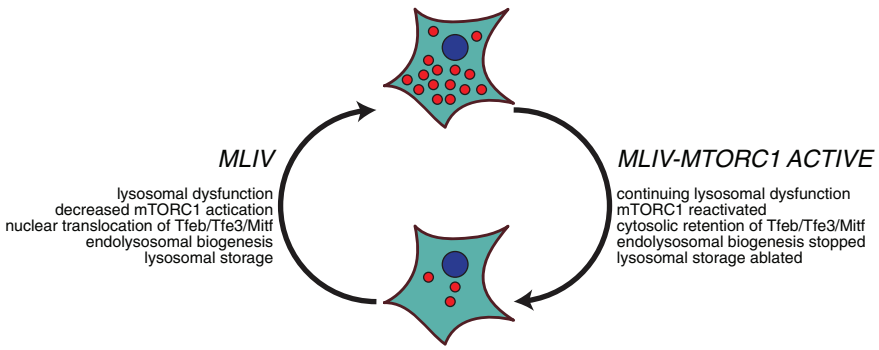

FIGURE 4.3 Schematic diagram showing the role played by mTORC1 in endolysosomal accumulation in cells lacking TRPML1. Steps on the left depict the alterations in MLIV cells that underlie lysosomal storage. Reactivation of mTORC1 in those cells mitigates lysosomal storage despite continuing lysosomal dysfunction.

fact that plasma membrane-enriched $PI(4,5)P_2$ negatively influences the activity of *Drosophila* TRPML (Feng et al., 2014). These studies also indicate that starvation-induced decrease in mTORC1 activity stimulates TRPML1, which in turn would restore mTORC1 activity via a combination of direct Ca^{2+}-dependent and indirect amino acid-dependent processes.

4.6.4 Regulation of Endolysosomal Biogenesis

Pioneering studies have demonstrated that expression of many genes encoding endolysosomal proteins is under the transcriptional control of Tfeb/Mitf/Tfe3 (Bouché et al., 2016; Martina et al., 2014; Palmieri et al., 2011; Ploper and De Robertis, 2015; Ploper et al., 2015; Sardiello et al., 2009; Settembre et al., 2011). Pointing to functional relationships between endolysosomal biogenesis and growth-inducing kinases – mTORC1 and AKT – both kinases phosphorylate and inactivate Tfeb leading to diminished endolysosomal gene expression (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012). Since *MCOLN1* is under the control of Tfeb/Mitf/Tfe3-mediated endolysosomal gene expression (Medina et al., 2011; Sardiello et al., 2009; Wang et al., 2015), low mTORC1 activity during starvation also promotes the transcriptional upregulation of *MCOLN1*. This high-level regulatory pathway likely synergizes with direct activation of TRPML1 in cells characterized by diminished mTORC1 function. The activation of TRPML1 eventually leads to the restoration of mTORC1 kinase activity and termination of the feedback loop. This model also explains the endolysosomal accumulation observed in cells characterized by diminished TRPML function – absence of TRPML function results in decreased mTORC1 activity and disinhibition of Tfeb/Mitf/Tfe3-dependent endolysosomal biogenesis. Indeed, ectopic stimulation of mTORC1 with a high protein diet or genetic manipulations mitigates the endolysosomal storage phenotype in *trpml*-deficient tissues in *Drosophila* (Wong et al., 2012, 2015). Alternatively, it has been proposed that endolysosomal Ca^{2+} released via TRPML1 activates the Ca^{2+}-responsive phosphatase calcineurin, which in turn, dephosphorylates Tfeb and promotes its translocation into the nucleus (Medina et al., 2015). The consequences of TRPML1 activity on both Tfeb inactivation via mTORC1 *and* Tfeb activation via calcineurin point to the complexities of endolysosomal biogenesis that will likely be resolved in future studies.

4.6.5 Regulation of Trace Metal Biology

Although the permeability to Ca^{2+} underpins many of the biological functions of TRPML1, the permeability to trace cations such as Fe^{2+} and Zn^{2+} also fulfil important roles (Cuajungco and Kiselyov, 2017; Cuajungco et al., 2014; Dong et al., 2008; Eichelsdoerfer et al., 2010; Feng et al., 2014; Grimm et al., 2007; Kim et al., 2007; Kukic et al., 2013; Lev et al., 2010; Nagata et al., 2008; Xu et al., 2007). Given that iron is delivered to the cell via endocytosis or autophagic degradation of iron-containing proteins, a role for TRPML1 in the regulation of Fe^{2+}-dependent processes can be envisioned (Mills et al., 2010). Indeed, impaired myelination in mice lacking *Mcoln1* is attributed to alterations in iron levels in the brain (Grishchuk et al., 2015).

In addition to Fe^{2+}, there is also evidence that TRPML influences Zn^{2+} metabolism, including the observation that Zn^{2+} also accumulates in endolysosomes upon loss of TRPML1 (Cuajungco and Kiselyov, 2017; Cuajungco et al., 2014; Dong et al., 2008; Eichelsdoerfer et al., 2010; Feng et al., 2014; Grimm et al., 2007; Kim et al., 2007; Kukic et al., 2013, 2014; Lev et al., 2010; Nagata et al., 2008; Xu et al., 2007). However, unlike with Fe^{2+}, direct measurements of Zn^{2+} conductance have not been conducted, leaving to possibility that TRPML1 may regulate Zn^{2+} transport indirectly.

4.6.6 Crosstalk Between Lysosomes and Other Organelles

Multiple lines of evidence support the notion of functional crosstalk between lysosomes and other cellular organelles, in particular the ER and mitochondria (Plotegher and Duchen, 2017; Repnik and Turk, 2010; Ronco et al., 2015). Interestingly, TRPMLs could be important participants in interorganellar communication, and loss of TRPMLs has a strikingly adverse impact on mitochondrial health, which may contribute significantly to the aetiology of MLIV (Jennings et al., 2006; Venkatachalam et al., 2008). Conversely, mammalian TRPML1 responds to oxidative stress that can originate from damaged or stressed mitochondria (Ravi et al., 2016; Zhang et al., 2016). In addition, Ca^{2+} dynamics of the lysosomes and ER exhibit functional relationships via a process requiring TRPML1 (Garrity et al., 2016; Kilpatrick et al., 2016). It would be interesting to evaluate whether phenotypes associated with MLIV and other lysosomal diseases involve aberrant crosstalk between endolysosomes and ER.

4.7 METHODS TO STUDY TRPML TRAFFICKING AND FUNCTION

The purpose of the following sections is to provide an overview of some of the tools and resources that may be used to study the localization, trafficking, and channel function of TRPMLs. As evidenced by the extensive contribution of TRPMLs to endolysosomal function, many of the protocols described below can also be utilized to study the properties of these vesicles in general.

4.7.1 Visualization of Functionally Relevant Vesicles

Human TRPML1 and its orthologs in other species localize to late-endocytic and lysosomal compartments, which by the virtue of low luminal pH can be visualized by LysoTracker – a fluorescent dye that accumulates in acidic organelles. Mammalian and invertebrate TRPML1 decorate the periphery of LysoTracker-positive vesicles (Venkatachalam et al., 2006, 2008). An important caveat that needs to be considered when using LysoTracker is that despite being useful for evaluating the abundance, shape, and size of acidic vesicles, the dye cannot be used to distinguish between late-endosomes, lysosomes, and other acidic vesicles. Furthermore, neither early endosomes nor autophagosomes can be detected using LysoTracker since these structures are not acidic.

To overcome the limitations of LysoTracker, co-labelling with specific-protein markers is also required. For instance, Rab5 and Rab7, which localize to the

early- and late-endosomes, respectively, may be used as markers. Other markers of early endosome include HRS and EEA1, and late-endosomes/lysosomes include proteins such as LAMP1 and LAMP3 (Manzoni et al., 2004; Thompson et al., 2007; Venkatachalam et al., 2006). Lysosomes can be visualized using fluorescently tagged substrates for lysosomal enzymes such as cathepsin (e.g. Magic Red cathepsin L substrate) (Johnson et al., 2016). Autophagosomes are detected by labelling with fluorescently tagged Atg proteins or LC3, whereas amphisomes, which are generated by the fusion of autophagosomes and endosomes (Tanida et al., 2008), can be identified by appropriate co-labelling. In addition to these fluorescent techniques, autophagosomes, endosomes, amphisomes, and lysosomes can be effectively discriminated by electron microscopy, and have been shown to accumulate in cells lacking TRPMLs (Wong et al., 2012). Unfortunately, the absence of effective antibodies against TRPML proteins has prevented visualization of natively expressed proteins, which can be circumvented by examining the expression of ectopic expression of tagged TRPMLs.

The sample protocol provided below can be used to visualize LysoTracker staining in cells from a variety of tissues or organisms. Figure 4.4 shows a representative image of primary fibroblasts from control and two different MLIV patients. These data demonstrate the accumulation of LysoTracker-labelled vesicles in MLIV fibroblasts.

1. Dissect tissues or prepare cells that express tagged TRPML variants.
2. While still alive, incubate the tissues/cells with LysoTracker Red (Molecular Probes: 2 mM for *Drosophila* fat bodies, although other tissues may require different concentration) added to full-growth cell culture medium for the desired amount of time (usually 30 minutes).
3. Wash samples once with phosphate-buffered saline (PBS).
4. Fix tissues/cells in 4% paraformaldehyde PBS for a minimum of 30 minutes at room temperature.

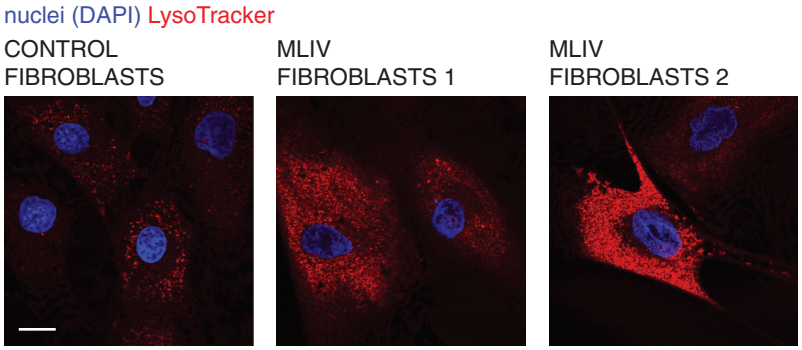

FIGURE 4.4 LysoTracker staining in control and MLIV fibroblasts. Confocal images of fibroblasts from control or MLIV patients labelled with DAPI (blue) and LysoTracker (red). Scale bar shown in the panel on the left depicts 10 μm and applies to all panels. MLIV fibroblasts 1 and 2 refer to cells from two separate MLIV patients. The cells shown here were obtained from the Coriell Institute for Medical Research.

5. Wash samples thrice with 0.1%-Triton X-100 PBS.
6. Incubate samples with primary antibodies against the tag on TRPMLs in 0.1%-Triton X-100 PBS + 5% donkey serum at 4°C overnight.
7. Wash samples thrice with 0.1%-Triton X-100 PBS.
8. Incubate samples with appropriate Alexa Fluor-conjugated secondary antibodies at room temperature for 2.5 hours.
9. Wash samples thrice with 0.1%-Triton X-100 PBS.
10. Mount samples on glass slide using VECTASHIELD with DAPI (Vector Labs).
11. Image using confocal microscope.

4.7.2 Ca^{2+} Imaging to Assay TRPML Function

The activity of mammalian and *Drosophila* TRPML channels has been evaluated using the endolysosomal-patch clamp technique (Dong et al., 2008; Feng et al., 2014). Given that TRPMLs release endolysosomal Ca^{2+}, imaging changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]$) also inform alterations in TRPML activity. In this context, TRPML activity can be evaluated either by measuring changes in bulk cytosolic $[Ca^{2+}]$ using Ca^{2+} sensors such as fura-2 or by tagging TRPML proteins with genetically encoded Ca^{2+} indicators (GECIs) such as GCaMPs (Kilpatrick et al., 2016; Shen et al., 2012; Wong et al., 2012, 2017).

Fura-2 is maximally excited at 340 nm when bound to Ca^{2+} or 380 nm when unbound, and always emits maximally at 510 nm (Paredes et al., 2008). Therefore, the ratio of fura-2 emission at 510 nm following excitation at 340 nm and 380 nm, respectively, is a function of cytosolic free $[Ca^{2+}]$. Since fura-2 measurements are inherently ratiometric, potential sources of error such as varying concentration of dye in individual cells are of limited concern.

The use of GECIs provides an alternative and potentially more sophisticated way to measure intracellular $[Ca^{2+}]$, for instance, by targeting to subcellular compartments or genetically defined cell types. The popular GECIs, GCaMPs, are comprised of circularly permutated GFP and calmodulin, with the latter binding to free Ca^{2+} and thereupon imposing an increase in the fluorescence of the former (Nakai et al., 2001). The power of GECIs is realized upon targeting to specific organelles, which allows for unprecedented spatiotemporal resolution of Ca^{2+} measurements (Mao et al., 2008; Pologruto et al., 2004). Indeed, by tagging the termini of TRPMLs with derivatives of GCaMP, Ca^{2+} in the vicinity of the channel can be detected in a cell type or tissue of interest in any genetically tractable organism. This approach allows measurement of Ca^{2+} changes in the vicinity of endolysosomes when the GCaMP is tagged to TRPML proteins (Samie et al., 2013; Wong et al., 2017). It is, however, important to bear in mind that TRPML-GCaMP can detect Ca^{2+} in the vicinity of the channel even if the actual source of the cations is not TRPML. As long as adequate free Ca^{2+} can diffuse to the GCaMP moiety, this signal will be reported by an increase in fluorescence.

Provided below are two adaptable protocols to image endolysosomal Ca^{2+} release. The first protocol describes a procedure to detect $[Ca^{2+}]$ in the bulk cytosol using fura-2 and the second protocol describes a more specialized approach that reflects

Ca^{2+} signals in the vicinity of endolysosomal membranes. We have also provided a brief primer on pharmacological approaches to manipulate TRPML activity, which is of tremendous utility when examining endolysosomal Ca^{2+} release.

4.7.3 Protocol 1: Fura-2

1. If using cultured cells, the experiment is performed on glass bottom culture dishes or cover slips. If the cells are transfected with specific constructs, Ca^{2+} imaging should not be performed <24 hours post-transfection.
2. For loading the cells or tissues with fura-2, the acetoxymethyl (AM) ester fura-2 (Invitrogen: 10 μM) should be applied for a duration ranging from 30 minutes to 1 hour. The fura-2-AM should be dissolved in a buffer containing: 125 mM NaCl, 5 mM KCl, 10 mM $MgSO_4$, 10 mM KH_2PO_4, 1 mM $CaCl_2$, 5.5 mM glucose, and 5 mM HEPES; pH 7.4. The duration of fura-2 loading might have to be determined empirically for the cells/tissues being used. During this step, it is important to keep the cells covered so as to prevent exposure to light, which may bleach the fura-2 signal. Some cell lines are inherently harder to load with fura-2, due to the presence of organic anionic transporters that extrude the dye (for example, S2 cells from *Drosophila*). In these cases, the anionic transport can be blocked by the inclusion of 5 mM probenecid in the buffer.
3. After the completion of the loading phase, remove the buffer containing fura-2-AM and replace with fresh buffer. At this point, the fura-2-AM will be de-esterified by endogenous esterases in the cytosol into fura-2, which is inherently membrane-impermeable and will remain in the cytosol. De-esterification should be complete in ~30 minutes after removal of fura-2-AM.
4. Using an appropriate Ca^{2+} imaging set-up and a wide-field fluorescence microscope, the ratio of emission intensities at 510 nm, following excitation at 340 nm and 380 nm, will provide an indication of cytosolic free [Ca^{2+}]. To convert the ratio to [Ca^{2+}], an appropriate calibration using known Ca^{2+} standards would be needed.
5. Different pharmacological agents can be used to trigger the movement of Ca^{2+} across organellar membranes and/or the plasma membrane in order to gain insights into the biological processes of interest.

4.7.4 Protocol 2: GCaMP

1. Mammalian cells or animal tissues expressing TRPML1-GCaMP5G or *Drosophila* TRPML-GCaMP5G can be used to measure [Ca^{2+}] in the endosomal periphery. If isolated from an animal, TRPML-GCaMP5-expressing cells should be first allowed to adhere to glass bottom dishes coated with concanavalin A.
2. After a few washes with full-growth cell culture medium, the cells are ready for imaging using a wide-field fluorescence imaging system.

3. GCaMP5G emission following excitation at 490 nm may be interpreted as a measure of cytosolic [Ca^{2+}].
4. After subtracting baseline, the GCaMP5G signals may be expressed as real-time fluorescence relative to intensity at the beginning of the run.

4.7.5 Pharmacological Manipulation of TRPML Function

There are several compounds that either directly or indirectly influence the activity of TRPML channels, and therefore, permit the rapid investigation of channel function in a multitude of contexts. The first TRPML inhibitor, mucolipin synthetic inhibitor 1 (ML-SI1), also known as GW-405833, was originally found to be a selective partial agonist of the endocannabinoid receptors, CB2 and CB1, having a weaker affinity for the latter (Clayton et al., 2002). In addition to its action on endocannabinoid receptors, ML-SI1 is an antagonist of TRPML1 (Samie et al., 2013; Wang et al., 2015). Since the description of ML-SI1, newer generation TRPML1 antagonists with relatively higher specificity have been developed (Kilpatrick et al., 2016; Samie et al., 2013). These compounds are particularly valuable because many conventional cation channel blockers are not effective inhibitors of TRPML1. Given that TRPML channels are activated by $PI(3,5)P_2$, inhibitors of the enzyme involved in the biogenesis of this endolysosomal phosphoinositide – Pikfyve – are indirect inhibitors of TRPML activity. In this context, YM201636 and apilimod are Pikfyve inhibitors that inhibit TRPML-mediated endolysosomal Ca^{2+} release (Dong et al., 2010; Lee et al., 2015; Wong et al., 2017). Direct TRPML agonists have also been described. First in this class was a compound named Mucolipin Synthetic Agonist 1 (ML-SA1) (Shen et al., 2012). Other drugs that act on TRPML1, as well as TRPML2 and TRPML3, have also been described (Chen et al., 2014; Grimm et al., 2010, 2012). The utility of these drugs is highlighted by the fact that they are all cell-permeable and can be added to the extracellular media to influence channel activity within cells. Alternatively, we have found that mixing these drugs into fly food is an effective approach to inhibit the activity of TRPML in *Drosophila* (unpublished observations).

4.8 CONCLUSIONS

Here, we have provided an overview of the biological functions of TRPMLs and described several methods that can be used to further study these intriguing channels. Over the last few decades, the effort to understand the molecular underpinnings of MLIV has yielded a wealth of information about the function of TRPMLs. Given the availability of numerous models and techniques to study these proteins, we expect the field continues to expand and provide fresh biologic insights. We hope this review serves as a reliable guide for those interested in contributing to research in this direction.

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