TRPML1 in Autophagy and Neuromuscular Diseases

by

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Abstract

Autophagy is a process that maintains cellular homeostasis and monitors nutrient requirements. During autophagy, cytoplasmic components are engulfed by autophagosomes. These autophagosomes fuse with lysosomes, where lysosomal hydrolases degrade engulfed material. Autophagic flux is regulated by a series of events, including autophagy initiation, autophagosome-lysosome fusion, autolysosome/lysosome degradation, and autophagic lysosome reformation. Disruption of any of them could lead to compromised autophagy and subsequent abnormalities.

X-linked myotubular myopathy (XLMTM) is a severe form of centronuclear myopathy that is caused by the loss of myotubularin 1 (MTM1), a member of the family of phosphoinositide phosphatases that dephosphorylate PI3P and PI3,5P2. MTM1-deficient cells are characterized by PI3P and PI3,5P2 accumulation and autophagic defects.

Amyotrophic lateral sclerosis (ALS) is a late-onset fatal neurodegenerative disorder characterized by the loss of both upper and lower motoneurons in the central nervous system (CNS). ALS is traditionally classified into two categories, i.e., familial ALS (fALS) and sporadic ALS (sALS). Within all familial cases of ALS, approximately 20% have point mutations in the gene encoding superoxide dismutase type 1 (SOD1), a redox enzyme. While it is believed that the disease onset occurs inside motoneurons, different cell types, such as glial cells in CNS, may control the disease progression. Microglia are resident immune cells of the CNS activated by infection, neuronal injury, and inflammation. Since glial-mediated neuroinflammation is one of the most striking hallmarks of ALS, much effort has been devoted to understanding the role of microglia in ALS progression.

Transient receptor potential cation channel 1 (TRPML1, also known as mucolipin 1) serves as an ion channel on the lysosomal membrane, activated by PI3,5P2 and reactive oxygen species (ROS). Mutations in the human TRPML1 gene cause Mucolipidosis type IV (ML-IV), a genetic disease characterized by neurodegeneration and movement disorders. TRPML1 mutant cells are characterized by abnormal autophagosome accumulation. Intriguingly, activation of TRPML1 results in autophagosome accumulation due to increased autophagosome biogenesis or impaired autophagic degradation.

In this study, we focused on understanding the role of TRPML1 in XLMTM and ALS, two neuromuscular diseases associated with muscle and motoneuron, respectively. We reported that in XLMTM, MTM1 deficiency hyperactivated TRPML1 due to PI3,5P2 elevation, and this suppressed autophagosome-lysosome fusion and myogenesis. The defective autophagosome-lysosome fusion and myogenesis in MTM1 deficient cells were corrected by TRPML1 suppression. Mechanistically, TRPML1 overactivation in MTM1 deficient cells led to a dynamin 2 increase due to the activation of transcription factor EB (TFEB), which further caused muscle defects. In ALS, we reported that $SOD1^{G93A}$ mutation activated TRPML1 in a ROS-dependent manner in microglia. Activated TRPML1 in $SOD1^{G93A}$ microglia impaired autophagic flux, potentially by inhibiting autophagosome-lysosome fusion. Activated TRPML1 also increased proinflammatory cytokine release from $SOD1^{G93A}$ microglia. Downregulating TRPML1 rescued the impaired autophagic flux and proinflammatory cytokine release in $SOD1^{G93A}$ microglia.

Taken together, our studies suggest that hyperactivation of TRPML1, either by PI3,5P2 in XLMTM muscle or by ROS in ALS microglia, disrupts autophagic flux, thereby affecting disease progression. Inhibiting TRPML1 could be an approach to mitigate the progression of the disease.

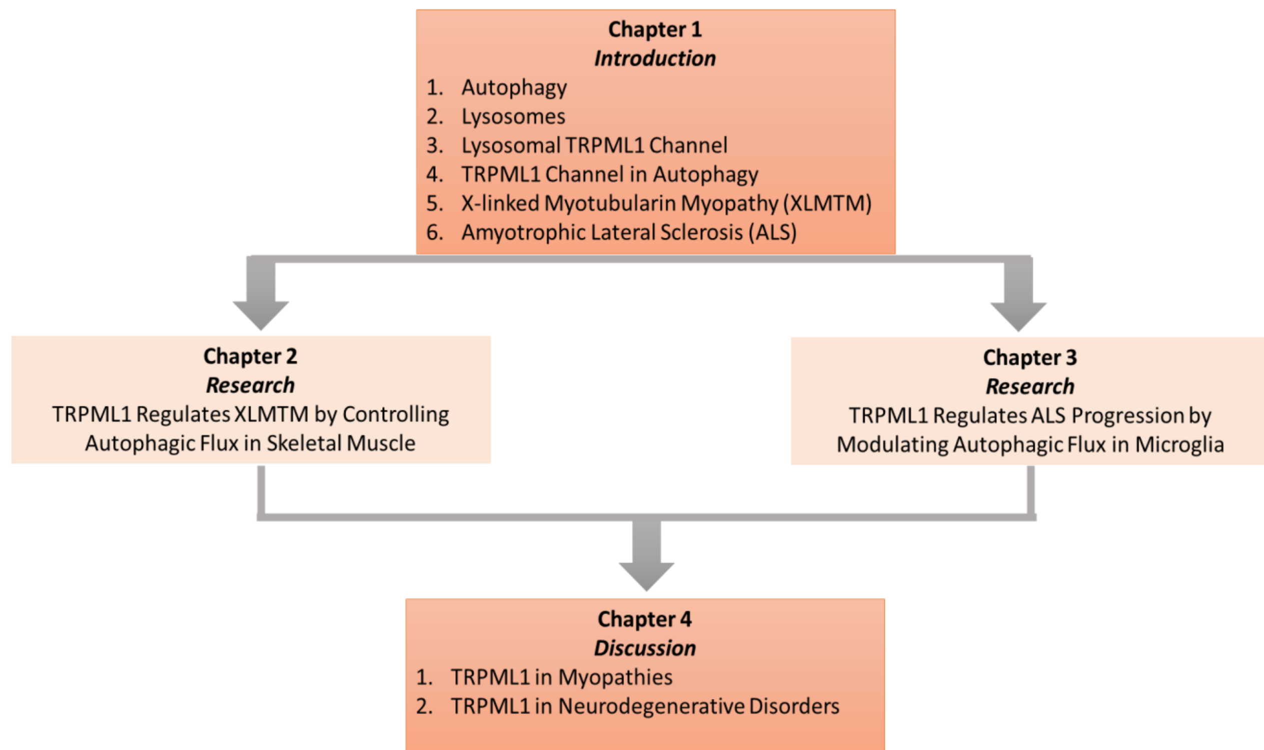
List of Abbreviations Used

ALS	Amyotrophic Lateral Sclerosis
AMPK	AMP-activated Protein Kinase
DNM2	Dynamin 2
ELISA	Enzyme-Linked Immunosorbent Assay
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
IL-1β	Interleukin 1 beta
LC3	Microtubule-associated Protein 1A/1B Light Chain 3
LPS	Lipopolysaccharide
MTM1	Myotubularin 1
MTMR	Myotubularin-Related Proteins
mTORC1	Mammalian Target of Rapamycin, Complex 1
NAC	N-Acetyl Cysteine
PI3,5P2	Phosphatidylinositol 3,5 Bisphosphate
PI3K	Phosphatidylinositol 3 Kinase
PI3P	Phosphatidylinositol 3 Phosphate
PIP	Phosphoinositide

RFP	Red Fluorescent Protein
ROS	Reactive Oxygen Specie
SNARE	Soluble N-Ethylmaleimide–sensitive Factor Attachment Protein Receptor
SOD1	Superoxide Dismutase 1
SQSTM1/P62	Sequestosome 1
STX17	Syntaxin 17
TDP43	Transactive-response DNA-binding Protein 43
TFEB	Transcription Factor EB
TNF-α	Tumour Necrosis Factor Alpha
WT	Wild Type
XLMTM	X-linked Myotubularin Myopathy

Statement

In this thesis, we used two neuromuscular disease models, i.e., XLMTM and ALS, to study the effects of TRPML1 on autophagy and its relevance to disease progression and treatment. In Chapter 1, we introduced autophagy, TRPML1, modulation of autophagy by TRPML1, XLMTM, and ALS. In Chapter 2 and 3, we studied the role of TRPML1 in XLMTM and ALS, respectively. In Chapter 4, we summarized and discussed the role of TRPML1 in myopathies and neurodegenerative disorders.



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CHAPTER 1: INTRODUCTION

1.1 Autophagy

Autophagy is a fundamental cellular process that removes unnecessary or dysfunctional components through a lysosome-dependent degradation mechanism. Under basal conditions, it helps maintain cellular homeostasis by eliminating damaged organelles and protein aggregates. In contrast, in stress such as nutrient deprivation, hypoxia, pathogen insult, and diseases, autophagy supports cell survival by providing nutrients and energy and removing potentially dangerous elements.

Autophagy is divided into several subtypes: macroautophagy, microautophagy, and chaperon-mediated autophagy (CMA). Macroautophagy is a major subtype of autophagy that is characterized by the enclosure of damaged cytoplasmic content within a double membraned vesicle, autophagosome and delivered to lysosomes for degradation. Macroautophagy, from hereon referred to as "autophagy," comprises four main steps: (1) vesicle nucleation characterized by engulfment of target material and formation of the phagophore, (2) elongation and enclosure in doubled membraned autophagosome, (3) vesicle fusion with the lysosome to form autolysosome, and (4) digestion of target material through lysosomal hydrolyses. The digested products are used as nutrients for cell proliferation and intracellular signaling, which in turn also modulate autophagy induction. (Codogno et al., 2011; Marshall & Vierstra, 2018).

The autophagy process commences with the downregulation of the mammalian target of rapamycin (mTOR), which acts as a nutrient sensor (Marshall & Vierstra, 2018). mTOR forms two multiprotein complexes, mTORC1 and mTORC2. mTORC1 is a serine/threonine protein kinase that belongs to the phosphatidylinositol kinase (PIKK) family (Noda & Ohsumi, 1998) and negatively regulates autophagy by phosphorylating unc51-like kinase 1 (ULK1), which is an integral member of autophagic protein complex for the initiation of autophagy (Hosokawa et al., 2009; Zachary & Ganley, 2017). Interestingly, ULK1 is alternatively regulated by an energy sensor, AMP-activated protein kinase (AMPK), which activates ULK1 by phosphorylation at Ser 317 and Ser 777 and promotes autophagy (J. Kim et al., 2011). ULK1 forms a complex with ATG13 (autophagy-related protein 13), ATG101, FIP200 (focal adhesion kinase family interacting protein of 200 kDa) (Hosokawa et al., 2009; Zachari & Ganley, 2017). Upon activation, the ULK1 complex is free to translocate to the autophagy initiation site to sequester the targeted material in

a phagophore and recruit a second set of autophagic-protein complex, VPS 34 complex (Herman & Emr, 1990; Mizushima, 2010). VPS34 is a phosphatidylinositol 3-kinase class III (PI3KC3) responsible for producing PI3P at the phagophore formation site (Devereaux et al., 2013). PI3P enables the recruitment of a third set of the protein complex, ATG16L1-ATG5-ATG12 (Dooley et al., 2014; Russell et al., 2013), and promote phagophore elongation and closure to form a double membraned autophagosome. ATG16L1-ATG5-ATG12 and PI3P mediate lipidation of ATG8, well known as microtubule-associated protein 1 light chain 3 (LC3) (Dooley et al., 2014; Runwal et al., 2019). LC3 further facilitates the sequestration of cytosolic material in autophagosomes through proteins containing LC3-interacting region (LIR) domain such as SQSTM1/P62 (Sequestosome 1), plays a role in sequestering cytosolic material in autophagosome (W. J. Liu et al., 2016). LC3 recruitment and lipidation to LC3-II marks the end of the first step of autophagy and the generation of mature autophagosomes containing damaged material to be recycled.

The next stage of autophagy is characterized by the formation of single-membraned autolysosomes, generated by the fusion of double-membrane autophagosomes and lysosomes. Lysosomes are present at the perinuclear region of the cell, and autophagosomes need to be transported toward lysosomes for the final degradation step to complete. Failure of this step leads to the accumulation of toxic proteins, damaged organelles, and energy depletion (Lőrincz & Juhász, 2020a); hence, the delivery and fusion of autophagosome to the lysosome are necessary for degradation in autophagy. This vesicle transport and fusion are coordinated by Soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins, small GTPases, membrane adapter proteins, and phosphoinositides (Stenmark, 2009; Wickner & Schekman, 2008). SNARE proteins are the main components of membrane fusion of intracellular vesicles, and they are present on both autophagosome and lysosome membranes to direct fusion. A well-established autophagosome-lysosome fusion process is moderated by SNARE proteins, including Syntaxin 17 (STX17) that is present on autophagosome membrane, and Vamp7 or Vamp 8, which is localized on lysosomes (Itakura et al., 2012a; Saleeb et al., 2019). STX17 localizes to LC3-positive autophagosomes under starvation (Itakura et al., 2012a) and is essential for the fusion step to proceed as it interacts with multiple other SNAREs on lysosome to drive autophagosome-lysosome fusion (Lőrincz & Juhász, 2020b; Saleeb et al., 2019; Tian et al., 2020). Interestingly, mTOR also regulates the fusion steps, disrupting STX17 and Vamp8 complex formation (H. Huang et al., 2021; Liang et al., 2023). Moreover, mature autophagosomes are labeled with

phosphoinositides, PI3P (Devereaux et al., 2013; Y. Liu et al., 2013; Nascimbeni et al., 2017), which is not only crucial for autophagosome formation but also for fusion (Behrends et al., 2010; Ogawa et al., 2011). PI3P forms a complex to tether autophagosomes to the lysosomal membrane and acts upstream of the SNARE complex. PI3P provides binding platform to small GTPase Rab7 (Cabrera et al., 2014), and adaptor proteins such as FYVE and Coiled-Coil Domain Autophagy Adaptor 1 (FYCO1) (Pankiv et al., 2010), homotypic fusion and vacuole protein sorting (HOPS) (Cabrera et al., 2014) and tectonin beta-propeller repeat containing 1 (TECPR1) (D. Chen et al., 2012) to facilitate the autophagosome-lysosome fusion. Similarly, PI3,5P2, another phosphoinositide located on the lysosomal membrane, is also associated with autophagosome-lysosome fusion (Fernandez-Mosquera et al., 2019; Rusten et al., 2007). Additionally, both PI3P and PI3,5P2 modulate the function of mTORC1 by activating VPS34 and lysosomal TRPML1 channel (Nobukuni et al., 2005; X. Sun et al., 2018). Inversely, mTORC1 also acts on VPS34 to control PIP synthesis and lysosomal reformation (Munson et al., 2015). This implies a feedback mechanism of phosphoinositides and mTORC1 to ensure proper autophagic flux. Proteolytic degradation and macromolecule release in cytosol denotes autophagy's final step. Once autophagosomes fuse with lysosomes, the lysosomal hydrolyses facilitate the active degradation of autophagosomal cargo into small molecules and ions. Lysosomes contain approximately 70 hydrolytic enzymes active at low pH (Settembre et al., 2013; Settembre & Ballabio, 2014). Acidic luminal pH is crucial for enzymes to function and perform the degradation, and this pH is achieved by lysosomal membrane v-ATPase that pumps proton across the membrane (R. Chen et al., 2020). The expression and translocation of v-ATPase are essential for lysosomal enzymes to function, and dysfunction of v-ATPase affects lysosomal acidification, leading to disruption in the clearance of autophagosomal cargo and defective autophagy (F. Chen et al., 2022; Song et al., 2020). The assembly of v-ATPase on the lysosomal membrane is regulated by signaling cascades, which control this process during different nutrient requirements. For example, mTORC1 mediates v-ATPase expression through transcription factor EB (TFEB), a significant transcription regulator of lysosome and autophagy pathway (Peña-Llopis et al., 2011), and its assembly on lysosomes through PI3K (Liberman et al., 2014). Immediately after proteolytic degradation, the macromolecules are released in the cytosol or exocytosed, and mTORC1 relocates on the lysosomal membrane to abate further autophagy (L. Yu et al., 2010a). Later autophagic steps also require abundant lysosomes to maintain autophagic flux. Lysosomes have de-novo biogenesis;

alternatively, lysosomes are formed by recycling autolysosomal membranes, the process termed autophagic lysosome reformation (ALR) (L. Yu et al., 2010b). During this process, mTORC1 activity promotes proto-lysosome tubulation from autolysosome and maturation into functional lysosomes (Yang et al., 2019; L. Yu et al., 2010a).

In conclusion, the autophagic process is systematically regulated by a series of events, including autophagy initiation, autophagosome-lysosome fusion, and lysosome degradation. Disruption of any of them could lead to compromised autophagy. The housekeeping role of autophagy in protein and organelle quality control is essential for cell growth, proliferation, and differentiation. Autophagy is especially important for postmitotic cells such as neurons and muscle cells because garbage accumulation due to dysfunctional autophagy could lead to cell death in those cells. Therefore, autophagic defects are the hallmark of neurodegenerative diseases and muscle myopathies (F. Chen et al., 2022; Colacurcio & Nixon, 2016; Raben et al., 2008; Xia et al., 2021).

1.2 Lysosomes

Lysosomes have been viewed as the degradation center of the cell, containing a large number of acidic hydrolytic enzymes. Lysosomes supplies raw material for all kind of biosynthesis in the cell, and this degradation center breaks down macromolecules to allow cell survival in adverse conditions. Therefore, lysosomes provide a platform for various nutrient sensors, including mTORC1 that integrates amino acid levels (Zoncu et al., 2011), AMPK that acts as an energy sensor, and solute carrier (SLC38A9)-Niemann-Pick C1 (NPC1) complex that functions as a lipid sensor (Castellano et al., 2017b; C. S. Zhang et al., 2017). mTORC1 is translocated onto lysosomes to inhibit autophagy and lysosomal function in nutrient-rich conditions (Rabanal-Ruiz et al., 2017; Zoncu et al., 2011), whereas AMPK acts antagonistically to mTORC1 during starvation, specifically glucose depletion, and encourages autophagy (S.-C. Lin & Grahame Hardie, 2018; C. S. Zhang et al., 2017). Similarly, in the presence of cholesterol, NPC1, a cholesterol transporter, binds to the SLC38A9, lysosomal transmembrane protein, to drive mTORC1 activation (Castellano et al., 2017a; Davis et al., 2021). Considering lysosomes mediate the degradation of proteins, carbohydrates, and lipids, more than 50 membrane proteins are equipped to transport the digested material out of lysosomes (H. Xu & Ren, 2015), and subsequently modulating the nutrient sensors on lysosomes. In addition to macromolecule transport, lysosomal

membrane ion channels also regulate luminal pH to facilitate the activity of lysosomal hydrolases (J. H. Lee et al., 2015; W. Wang et al., 2015a).

Lysosomal ion conduction not only modulates the luminal pH and transport of material but also lysosomal membrane fusion and fission, intracellular vesicle transport, and signaling (Samie & Xu, 2014). For example, Ca^{2+} concentration in the lysosome lumen is estimated to be about 0.5mM, which is about 5000 folds higher than cytosolic Ca^{2+} levels. Therefore, the lysosome is recognized as one of the significant Ca^{2+} reservoirs. In alignment with this, a vast number of Ca^{2+} channels have been identified to control Ca^{2+} efflux from lysosomes to cytosol firmly. These include TRPML1, P2X4, and Two Pore Channels (TPC1 and TPC2) (Y. Wu et al., 2021; H. Xu & Ren, 2015), with TRPML1 acting as a critical player in lysosomal Ca^{2+} conductance (X. Sun et al., 2018; Y. Wu et al., 2021). Ca^{2+} release from the lysosomes has been implicated in autophagy, cell proliferation, cell differentiation, protein expression, and disease (Christensen et al., 2002; Feng & Yang, 2016; Medina et al., 2015a).

1.3 Lysosomal TRPML1 Channel

TRPML1 belongs to the mucolipin family and is encoded by the *MCOLN1* gene (Cheng et al., 2010). It is ubiquitously expressed in all types of tissues (Cheng et al., 2010). Immunostaining and density gradient centrifugation confirmed that TRPML1 is predominantly localized on late endosomes/lysosomes (Dong et al., 2010; H. J. Kim et al., 2009a). Loss of TRPML1 results in the accumulation of lysosomal storage material that is characterized by enlarged lysosomes in ML-IV patient cells (Bassi et al., 2000; Dong et al., 2008) and elevated lysosomal luminal pH (Venkatachalam et al., 2008). The channel is permeable to Ca^{2+}, Na^{+}, K^{+}, Fe^{2+}, and Mg^{2+} (Dong et al., 2008; W. Wang et al., 2014). Physiologically, TRPML1 is potentiated by acidic luminal pH (Liberman et al., 2014; H. Xu et al., 2007) and PI3,5P2, a lysosome-specific phosphoinositide (Dong et al., 2010). Cellular stress conditions, including nutrient starvation and increased reactive oxygen species (ROS), also activate TRPML1 (W. Wang et al., 2015b; X. Zhang, Cheng, et al., 2016), implying its role in mediating cell adaptation in adverse conditions.

TRPML1-mediated Ca^{2+} efflux from lysosomes is proposedly essential for lysosomal homeostasis and signal transduction (Cheng et al., 2010; P. Huang et al., 2020; W. Wang et al., 2014). Considering lysosomal luminal Ca^{2+} concentration is believed to be 0.5mM, which is significantly higher than cytosolic Ca^{2+} levels (50-100nM), making TRPML1 a strong contender

for lysosomal Ca^{2+} release and Ca^{2+}-dependent lysosomal fusion events and autophagy (H. J. Kim et al., 2009b; Morgan et al., 2011) as discussed below.

1.4 TRPML1 Channel in Autophagy

TRPML1 has a multifaceted role in autophagic regulation, including autophagy induction, autophagosome-lysosome fusion, lysosomal degradation, and autophagic lysosome reformation (Huang et al., 2020; Xu et al., 2019; X. Zhang et al., 2016).

During nutrient starvation, TRPML1 mediates the nuclear translocation of a major transcription regulator of autophagic genes, TFEB (Medina et al., 2015b), hence initiating the transcription of genes required for autophagy and lysosomal function. In addition to TFEB translocation, recent studies have provided another avenue of TRPML1's involvement in autophagy initiation as it activates calmodulin-dependent protein kinase kinase β (CaMKKβ) and AMPK pathway to facilitate autophagosome biogenesis (Scotto Rosato et al., 2019). Similarly, TRPML1 is sensitive to increased ROS released by damaged mitochondria. TRPML1 mitigates this oxidative stress by promoting autophagy to remove damaged mitochondria (X. Zhang, Cheng, et al., 2016). In addition to the autophagy initiation, TRPML1 also modulates the later autophagic stage, autophagosome-lysosome fusion, and lysosomal degradation. Pollmanns et al. (2022) showed that activating TRPML1 by its agonist ML-SA1 increased autophagosome maturation. This finding was supported by other studies showing that TRPML1-mediated Ca^{2+} efflux activates apoptosis-linked gene 2 (ALG-2) in CaMKKβ dependent manner (Scotto Rosato et al., 2019a), which then promotes centripetal movement of lysosomes towards the perinuclear region where they fuse with autophagosomes (X. Li et al., 2016). TRPML1 controls lysosomal function as well, as it has been suggested to regulate lysosomal degradation by modulating luminal pH. Furthermore, TRPML1 is also necessary for autophagic lysosome reformation through mTORC1, it modulates mTORC1 activation and translocation on the lysosomal membrane in stress conditions (R.-J. Li et al., 2016; X. Sun et al., 2018).

Interestingly, an inhibitory effect of TRPML1 on autophagy has also been reported, more so in the context of disease conditions where cellular stress cues may activate TRPML1 unfavorably. Wang's group (Qi et al., 2021) reported that TRPML1 activation via synthetic agonists, ML-SA5 or MK6-83, blocks the interaction of STX17 and Vamp8 and leads to blockage of autophagosome-lysosome fusion. They further suggested that TRPML1-mediated autophagic

arrest leads to the accumulation of damaged mitochondria and increased ROS, inhibiting cancer cell invasion and metastasis (Xing et al., 2022). Considering that mTORC1 acts as a negative regulator of autophagy (Hosokawa et al., 2009; Zachari & Ganley, 2017), increasing mTORC1 TRPML1 has also been suggested to inhibit autophagic flux (R.-J. Li et al., 2016; X. Sun et al., 2018).

Overall, TRPML1 plays a dual role in the autophagic pathway. In some conditions, it promotes autophagy, whereas in disease or stress conditions, it is shown to be inhibiting autophagy.

1.5 X-linked Myotubular Myopathy (XLMTM)

Heckmatt, Sewry, Hodes, and Dubowitz, in 1985, identified a myotubular myopathy in 6 patients. They observed that it has an X-linked inheritance pattern with early onset muscle weakness, respiratory failure, and premature death, thus giving the disorder its name, XLMTM. Laporte (2000) and colleagues used single-strand polymorphism to provide more insight into the molecular mechanism and established the Myotubularin-1 (*MTM1*) gene to be the cause of XLMTM. At present, there is no specific treatment for XLMTM, although MTM1 gene therapy (Childers et al., 2014), inhibition of PI3 kinase class II (PI3KC2) (Sabha et al., 2016; Samsó et al., 2022), and dynamin 2 (DNM2) downregulation (Cowling et al., 2014; Tasfaout et al., 2018) are being tested as potential therapeutics.

MTM1 belongs to the family of phosphoinositide phosphatases that dephosphorylate PI3P and PI3,5P2 (Dove et al., 2009; Tronchère et al., 2004a; Tsujita et al., 2004a). The absence of MTM1 causes the accumulation of two phospholipids, PI3P and PI3,5P2 (Kutchukian et al., 2016; Sabha et al., 2016). Earlier studies suggested that MTM1 deficiency impaired EC coupling in muscle cells by disrupting calcium homeostasis (Al-Qusairi et al., 2009; Dowling et al., 2009), whereas later studies demonstrated that loss of MTM1 leads to autophagosome accumulation, mTORC1 activation and disruption in phosphoinositides metabolism that eventually plays a vital role in pathological characteristics of XLMTM (Al-Qusairi et al., 2009; Dowling et al., 2010; Fetalvero et al., 2013a, 2013b; Reifler et al., 2014).

1.5.1 Phosphoinositide Metabolism

Phosphoinositides (PIP) are important signaling molecules involved in a vast array of cellular processes, including autophagy, vesicle trafficking, exocytosis, and signal transduction

(Falkenburger et al., 2010). These lipid molecules comprise 2 fatty acids and glycerol, attached to an inositol ring that can be phosphorylated at up to 3 positions (3,4 and 5), giving rise to seven distinct pools of phosphoinositides. All phosphoinositides share a common precursor, i.e., phosphatidylinositols, on the membrane (De Craene et al., 2017). The seven phosphoinositides are widely distributed in cells, such as PI4,5P2, PI3,4,5P3, have been observed at the plasma membrane, PI4P can be located on Golgi apparatus, and PI3P and PI3,5P2 shown to be localized at endosomes, autophagosomes, and lysosomes (Burman & Ktistakis, 2010). Notably, PI4P is located on the plasma membrane and involved in the exocytosis pathway, while PI3P and PI3,5P2 are localized on autophagosomes and lysosomes, regulating the autophagic pathway (Vergne & Deretic, 2010; Wills & Hammond, 2022).

Phosphoinositide's synthesis and cellular concentration are regulated by phosphatase and kinase enzymes. On the one hand, the generation of PIPs such as PI3P, PI4P, and PI5P depends on the action of various kinases. For example, Phosphoinositide 3-kinases (PI3Ks) are kinases grouped into classes I, II, and III (Balla, 2013; De Craene et al., 2017; Falkenburger et al., 2010). PI3P is an autophagosomal phosphoinositide that is mainly synthesized by VPS34, a PI3 kinase class III (Devereaux et al., 2013) and PIK3C2B, a PI3 kinase class II (Yoshioka, 2021). On the other hand, phosphatases, including myotubularins, are responsible for the degradation of phosphoinositides by removing the phosphate group from the inositol ring. They play a huge role in regulating the levels of PIPs in cells (Laporte et al., 2003; Mochizuki et al., 2013). For example, the action of phosphatase MTM1 can dephosphorylate PI3,4P2 and PI3,5P2 to form PI3P (Liu & Bankaitis, 2010).

PIPs modulate cellular processes by directly binding to effector proteins, including ion channels present on the membrane. These membrane proteins or ion channels generally contain PIP binding domains that allow compatible PIP to regulate their function. In response to PIP binding, proteins could either have a direct effect on the membrane dynamics, for example, budding, fission, fusion, or coordinate intracellular signaling by activating or inhibiting proteins such as GTPase and Ca^{2+} sensor proteins (Hasegawa et al., 2017). To elaborate on the previous statement, PI4,5P2 serves as a recruiter or activator of protein complexes in the plasma membrane, which initiate membrane budding, exocytosis of a synaptic vesicle, endocytosis, and remodeling of actin cytoskeleton (T. F. J. Martin, 2012; Wills & Hammond, 2022). Likewise, PI3P modulates

endocytotic pathways by interacting with membrane proteins on early endosomes and autophagosomes (Cabrera et al., 2014; Dooley et al., 2014; Pankiv et al., 2010). It regulates intracellular vesicle transport through small GTPase effector proteins, which enables tethering of vesicles prior to fusion (Cabrera et al., 2014) and loading of vesicles to microtubule network for transport towards lysosomes (Pankiv et al., 2010). Another less abundant PIP, PI3,5P2, mitigates cell adaptation to stress conditions by activating various cellular pathways by the modulation of Ca^{2+} signaling and binding to target proteins such as ATG18 and Raptor (mTORC1) (Baskaran et al., 2012; Bridges et al., 2012; Hasegawa et al., 2017). For example, PI3,5P2 binds and activates TRPML1 on the lysosomal membrane (Dong et al., 2010) and TPC1-2 channels on the endosomal membrane (X. Wang et al., 2012). It also recruits V-ATPase on the lysosomal membrane in salt stress conditions (S. C. Li et al., 2014). Collectively, PIPs regulate through effector proteins and ion channels.

1.5.2 Phosphoinositides in Autophagy

Phosphoinositides are involved in all steps of autophagy, from phagophore formation to autolysosome degradation. (Di Paolo & De Camilli, 2006). PI3P synthesis has been established as one of the main events for initiation (Petiot et al., 2000). At the location of phagophore formation, PI3P is produced by PI3KC3/VPS34 and PI3KC2 (S. He et al., 2013; Palamiuc et al., 2020). PI3P synthesis is the hallmark of autophagy initiation as it is detected on the ER omegasome site, which elongates to form a phagophore (Axe et al., 2008). The PI3P platform at ER leads to the recruitment of the WIPI2 and ATG16L1-5-ATG12 complex (Hanada et al., 2007). WIP12-ATG16L1 mediates phagophore expansion by recruitment and lipidation of LC3 (Dooley et al., 2014), which is important for membrane closure autophagosome formation. Studies have shown that a substantial distribution of PI3P pool is present on the cytoplasmic and luminal leaflets of the autophagosomal membrane (Nascimbeni et al., 2017). Once autophagosome maturation completes, it fuses with lysosome for the degradation of material by lysosomal protease. Membrane adaptor protein FYCO1 can bind to PI3P and microtubules. Under basal conditions, FYCO1 enables the loading of PI3P-positive autophagosomes on microtubules to facilitate the transport of autophagosomes to the cell periphery, where they fuse with lysosomes (Pankiv et al., 2010). PI3P clearance from autophagosome in yeasts is critical for its fusion with the lysosome (Cebollero et al., 2012). Contrarily, in mammals, PI3P is required for the fusion between autophagosomes and lysosomes (Behrends et al., 2010; Ogawa et al., 2011).

In addition to PI3P, its derivative PI3,5P2 has also been reported to modulate the late stages of autophagy. PI3,5P2 is generated by the phosphorylation of PI3P by PIKfyve (Zolov et al., 2012). It is a low abundance PI, estimated to comprise 0.1% of the total cellular phosphoinositide pool, and mainly localized on late endosomes and lysosomes (Dove et al., 2009; X. Li et al., 2013). During starve conditions, AMPK phosphorylates PIKfyve and mediates its translocation on lysosome-initiate PI3,5P2 synthesis (Y. Liu et al., 2013). PI3,5P2 acts as a nutrient sensor through mTORC1 in the presence of insulin and amino acids. It is implicated in inhibiting autophagy by directly interacting with mTORC1 and mediating the localization of mTORC1 on lysosomes (N. Jin et al., 2014). Sun et al. (2018) further demonstrated that PI3,5P2 mediates mTORC1 translocation on lysosomes in starved conditions by activating the lysosomal Ca^{2+} channel TRPML1. Similar reports have shown that PI3,5P2 regulates lysosomal ion channels in the autophagy pathway, and increased levels of PI3,5P2 leads to decreased autophagosome-lysosome fusion, defective lysosomal reformation (Palamiuc et al., 2020), and accumulation of inclusion bodies (Hasegawa et al., 2016). These reports suggest that failure of PI3,5P2 degradation results in autophagic defects.

Other phosphoinositides, including PI4P and its subproducts, have been reported in various stages of autophagy. PI4P autophagosomes facilitate fusion with lysosomes (H. Wang et al., 2015) both PI4P and PI4,5P play a vital role in lysosome reformation under normal conditions (Rong et al., 2012). Interestingly, despite its role in lysosome reformation, PI4,5P2 accumulation at lysosomes inhibits autophagy in Lowe Syndrome (De Leo et al., 2016).

Collectively, phosphoinositide regulates autophagy at various steps. Their function largely depends on the conditions and cell type; they could either carry out stimulatory or inhibitory effects on the autophagic flux. Accumulation of these lipid molecules is implicated in different diseases, including Lowe syndrome, Joubert syndrome, Amyotrophic lateral sclerosis, X-linked myotubularin myopathy, and Charcot-Marie-Tooth diseases (Chow et al., 2009; Dove et al., 2009; Tronchère et al., 2004a).

1.5.3 Phosphoinositides in XLMTM

Phosphoinositides are short-lived and low-abundant phospholipids that control a vast array of cellular processes. Consequently, their cytosolic concentrations are regulated by the action of phosphatase that mediates the dephosphorylation of PIPs immediately after they serve their

purpose. Phosphatase ensures the inactivation of PIPs by removing the phosphate group (Dyson et al., 2015). Although PIPs are essential for cellular processes, their rapid removal is equally important. The mutation and loss of function of the myotubularin family of phosphoinositide phosphatase have been implicated in neuromuscular diseases, obesity, and cancer (Dyson et al., 2015; Hnia et al., 2012). For example, Charcot-Marie-Tooth neuropathy is caused by the mutation in MTMR2 and MTMR13 (Raess et al., 2017), Creutzfeldt-Jakob disease and Epilepsy involves MTMR7 mutation (Baulac et al., 2008; Sanchez-Juan et al., 2012) and X-linked myotubularin myopathy is caused by MTM1 deficiency (Jocelyn Laporte et al., 2000; Oliveira et al., 2012).

MTM1 is one of the phosphoinositide phosphatases that was discovered in relation to myopathies (Blondeau et al., 2000). It was suggested that MTM1 may regulate autophagy by binding to PIs on endosomes/lysosomes (Lorenzo et al., 2006), which was later supported by another work showing MTM1 modulates endosomal trafficking and function by regulating PI3P and PI3,5P2 levels on the vesicle(C. Cao et al., 2007). Generally, PI3P is produced by PI3KC3 determines autophagy induction (Backer, 2008; Nascimbeni et al., 2017; Petiot et al., 2000; Reifler et al., 2014). Because MTM1 interacts with PI3KC3 and because PI3P accumulation induced by MTM1 deficiency (Blondeau et al., 2000; Petiot et al., 2000) is rescued by ablation of PI3KC3 (Velichkova et al., 2010), MTM1 was believed to control autophagosome formation. Indeed, MTM1 deficiency leads to autophagosome accumulation (Blondeau et al., 2000; G. S. Taylor et al., 2000). On the other hand, muscle-specific ablation of PI3KC2B but not PI3KC3 prevents or reverses the MTM1-KO phenotype (Reifler et al., 2014; Sabha et al., 2016). This suggests that muscle cells have two distinct pools of PI3P generated by PI3KC3 and PI3KC2B, respectively. Interestingly, PI3P is localized in both perinuclear regions, presumably lysosomes (Cabukusta & Neefjes, 2018; Korolchuk & Rubinsztein, 2011) and endosomes (Posor et al., 2022; Sabha et al., 2016)) in skeletal muscle; muscle-specific deletion of PI3KC3 reduces PI3P in endosomes, whereas muscle-specific deletion of PI3KC2B reduces perinuclear PI3P but not endosomal PI3P (Sabha et al., 2016). These data suggest that PI3KC3 may be responsible for producing PI3P in the early endosomes/recycling endosomes for autophagosome formation while PI3KC2B regulates PI3P production in the perinuclear region (Aung et al., 2019; Sabha et al., 2016) where PI3KC2B and MTM1 may coordinate PI3,5P2 homeostasis (Velichkova et al., 2010) to regulate autophagosome-lysosome fusion (Cabukusta & Neefjes, 2018; Korolchuk & Rubinsztein, 2011; Posor et al., 2022). In supporting this, both heterologous and endogenous perinuclear PI3KC2B

are highly co-localized with autophagosome marker LC3-II and less frequently with early endosome marker (Aung et al., 2019). This is also supported by previous studies showing a genetical interaction between MTM1 and PI3KC2B (Sabha et al., 2016; Velichkova et al., 2010) endolysosomal localization of MTM1 (Beggs et al., 2010; C. Cao et al., 2007; Tsujita et al., 2004a; Velichkova et al., 2010) and MTM1 binding to PI3,5P2 and reducing PI3,5P2 (Berger et al., 2002; Tronchère et al., 2004b; Tsujita et al., 2004a; Walker et al., 2001) Therefore, in XLMTM the loss of MTM1 may cause PI3,5P2 increase in the perinuclear region, leading to autophagosome-lysosome fusion defects (Hasegawa et al., 2016; Palamiuc et al., 2020).

The role of MTM1 in autophagy is not only related to phosphoinositide metabolism but is also suggested to be associated with mTORC1 modulation (Fetalvaro et al., 2013). Loss of MTM1 upregulates mTORC1 and inhibits autophagy in XLMTM (Fetalvero et al., 2013; Al-Qusairi et al., 2013). mTORC1 upregulation could be associated with PI3P and PI3,5P2 dysregulation in the perinuclear region or another pathway that is yet to be discovered.

1.6 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disorder that is characterized by the deterioration of motor function due to the rapid loss of motor neurons in the brain and spinal cord (Al-Chalabi & Hardiman, 2013). The progressive degeneration of these motor neurons leads to denervation at the neuromuscular junction, and muscular atrophy, paralysis, and death are most commonly caused by respiratory failure. The global incidences of ALS are estimated to be 1-3/100,000 within the ages of 50-75 years old (Chia et al., 2018). This fatal disease progresses rapidly and has a 2-4 years average survival time post-diagnosis (Zarei et al., 2015). ALS appears sporadically in the majority of cases, with suggested causatives to be infectious disease, neuroinflammation, injury, or exposure to toxins. However, approximately 10% of ALS cases have a familial history (Evans and Holzbaur, 2018). Various genetic loci have been discovered to increase understanding of ALS's pathophysiology in recent years. A number of mutated genes encode RNA-binding proteins such as transactivation response DNA-binding protein 43 kDA (TDP43) and fused in sarcoma (FUS) (Suk & Rousseaux, 2020), mitochondrial associated protein, SOD1 (Turner & Talbot, 2008) and genes related to autophagy including ubiquitin protein such as TANK-binding kinase 1 (TBK1), P62, optineurin (OPTN), as well as functional autophagic genes such as Ubiquilin-2 (UBQLN2), Valosin Containing Protein (VCP),

and C9orf72 (Al-Chalabi & Hardiman, 2013; Nassif et al., 2017). The genetic causatives of ALS suggest an alteration in mitochondrial function, intracellular transport, and proteolysis. The disease commences with the degeneration of motor neurons that innervate muscle fibers and extends to pathological changes in other neurons and glial cells (Kanning et al., 2010), likely due to protein misfolding, fragmented mitochondria, impaired autophagy, and damaged cytoskeleton (J. P. Taylor et al., 2016).

Autophagic defect is a common feature of ALS pathogenesis as the accumulation of P62 positive degradative material, termed as inclusion bodies, has been reported in several cases such as SOD1, TDP43, FUS, and C9orf72 (Neumann et al., 2006; Kwiatkowski et al., 2009; May et al., 2014), and accumulation of wild type TDP43 in the cytoplasm has also been implicated in sporadic ALS cases (Hergesheimer et al., 2019). Alongside mutant protein accumulation, there is apparent dysregulation of the protein quality control system, which could be due to the profuse number of ALS genes associated with autophagy, such as p62, VCP, OPTN, and TBK1 (Chia et al., 2018). The dysfunctional ubiquitination and accumulation of misfolded mutant proteins (Madinas et al., 2017) validate the significance of impaired autophagy in the progression of ALS.

1.6.1 SOD1 Mutation in ALS

Despite an abundant number of genetic models of ALS, SOD1 mutations have received the most attention due to the availability of rodent models recapitulating the pathological characteristics of human ALS (Turner & Talbot, 2008). The various SOD1 mutations account for 20% of familial ALS and 2-7% of sporadic ALS cases (Chiò et al., 2020). It was the first discovered familial ALS mutation genetic loci, which further aided in establishing the genetic link to generate transgenic mice lineage for *in vivo* studies (Turner & Talbot, 2008). At least 200 mutations in the SOD1 gene are associated with ALS; some have been used to generate transgenic mice models. $SOD1^{G93A}$ harbors a single amino acid mutation of glycine to alanine at residue 93 (Kreilaus et al., 2020). The notable characteristics of the $SOD1^{G93A}$ mice are rapid disease progression, significantly increased oxidative stress levels, and neuroinflammation by glial cells (Gurney et al., 1994).

SOD1 is a cytosolic redox protein that catalyzes ROS and prevents harmful oxidative stress to neurons. Expression of G93A mutant induces susceptibility to oxidative stress (Richardson et al., 2013; Rosen et al., 1993). Other characteristics of $SOD1^{G93A}$ include the accumulation of

insoluble protein due to autophagic inhibition, which furthers ALS pathogenesis. Several studies suggest that mSOD1 may contribute to the hyperactive induction of autophagy (Kitamura et al., 2014; Wei, 2014), but it could be associated with the type of SOD1 mutation. On the one hand, ULK1 phosphorylation was significantly reduced in motor neurons of $SOD1^{G85R}$ rodents (Bandyopadhyay et al., 2014), indicating uninhibited initiation of autophagy. On the other hand, increased LC3-II turnover was also observed *in vitro* cell cultures of $SOD1^{G93A}$ expressing motor neurons (Wei, 2014) and spinal cord and brain fractions of $SOD1^{G93A}$ mice (Li et al., 2008; An et al., 2014), suggesting increased autophagy induction but decreased autophagic flux. The detrimental effects of SOD1 are thought to involve autophagosome maturation and fusion with lysosome steps in autophagy as evidence suggests that mSOD1 acquires gain-of-interaction with dynein and impairs autophagosome transport process in neurons (Zhang et al., 2007). Similar results were observed in $SOD1^{G93A}$ mice where mutant proteins colocalize with dynein, a microtubule adaptor protein, in motor neurons, disrupting the transport of autophagosomes at the early symptomatic stage of ALS (Bilsland et al., 2010). This inhibition of autophagosome transport results in the accumulation of autophagosomes carrying damaged mitochondria and harmful material, which fail to reach the perinuclear of neurons and fuse with lysosomes (Lee et al., 2011). These findings support the key phenotypical characteristics of SOD1-ALS pathogenesis, as the accumulation of P62 and SOD1 positive inclusion bodies and increased LC3-II are observed in all SOD1-ALS models. This suggests that autophagy initiation and autophagosome formation are relatively not a problem here, and we might need to study the later stages, i.e., autophagosome-lysosome fusion and lysosomal degradation.

1.6.2 Autophagy in ALS

Neurons, due to their large and widely distributed cellular structure, require active autophagic machinery for the removal of accumulated substances in dendrites and axons. The unique cellular structure and lack of cell division make neurons vulnerable to substance accumulation. Therefore, failure in autophagic clearance is implicated in various neurodegenerative disorders. Reportedly, the deletion of autophagic genes *Atg5* and *Atg7* in mice generated spontaneous symptoms associated with several neurodegenerative diseases (Hara et al., 2006; Komatsu et al., 2006). Downregulation of VPS34 has been observed in Alzheimer's (Lipinski et al., 2010), as evidenced by accumulated autophagosomes being seen in the neocortical region of patients (Nixon et al., 2005). Increased mTORC1 activity has also been reported in aging

mouse brains and human fibroblasts (Ott et al., 2016). Similarly, in Parkinson's Disease, nuclear translocation of TFEB aids in removing α-synuclein inclusion bodies (Lewy bodies) from the dopaminergic neurons (Decressac et al., 2013). Furthermore, autophagy is not only associated with the ubiquitination of damaged proteins but is also involved in other neuronal activities, such as presynaptic structure and function in dopaminergic neurons (Hernandez et al., 2012) and synaptic pruning in cortical neurons (Tang et al., 2014). Therefore, autophagic defects result in damaged organelles accumulation and compromise the function of neurons.

Proteotoxicity due to gene mutation leads to misfolding and accumulation in neurons, a common characteristic of many forms of ALS. Proper autophagic flux has been observed to have a neuroprotective role in this disease as it increases the removal of mutant protein aggregates (Decressac et al., 2013).

1.6.3 Microglia in ALS

Earlier research had been predominantly focused on motor neurons due to phenotypical evidence of degeneration of these cells in the brain and spinal cord and loss of motor function. However, some recent approaches have directed attention toward activated microglia cells. Microglia are resident immune cells of the central nervous system, and they constitute around 0.5-16.6% of the total cell population of the human brain (Lawson et al., 1992). From the early developmental stage to adult CNS, microglia are involved in multiple functions, such as clearance of cell debris and synaptic pruning (Marin et al., 2004). The microglia cells acquire phenotypically distinct "active" states while performing neuroprotective or neurotoxic tasks (Clark et al., 2015). Due to both the neuroprotective, termed "M2" phenotype, and neurotoxic, "M1" phenotype, microglia recently has gained much attention in studying the pathology of various neurodegenerative disorders. For example, *in vitro,* studies have shown that activated microglia cause motor neuron injury by increasing the susceptibility of the AMPA receptor to glutamate toxicity (Zhao et al., 2004) and upregulation of inflammatory transcription factor nuclear-factor kappa B (NF-κB) in $SOD1^{G93A}$ mice microglia resulted in motor neuron degeneration (Frakes et al., 2014; Park et al., 2015).

Several pieces of evidence have provided novel insight into the non-cell-autonomous effect of glial cells on motor neurons and ALS disease progression. Microglia isolated from $SOD1^{G93A}$ mutant mice were shown to have a neurotoxic response *in vitro* co-culture with motor neurons

compared to the wild type (Xiao et al., 2007). Reportedly, motor neurons contribute to glial inflammation in a non-cell-autonomous manner, probably by the secretion of mSOD1 (Rudnick et al., 2017), as extracellular mSOD1 was enough to stimulate microglia in vitro. These findings could be further supported by the neurotoxic effect of microglia isolated from symptomatic SOD1 transgenic mice *in vitro* co-culture with motor neurons (Liao et al., 2012). Comparably, selective expression of $SOD1^{G93A}$ in microglial cells had little effect on disease onset (Boillee et al., 2006). In contrast, decreased $SOD1^{G93A}$ expression in later stages in microglial cells slowed disease progression (Beers et al., 2006). The study further revealed that selective expression of $SOD1^{G93A}$ in microglia increased neuron death and oxidative stress. These results suggest microglia may contribute to the propagation of disease rather than onset, both by motor neuron-mediated activation of the neurotoxic state of microglia and accumulation of mSOD1 in microglia. As mentioned earlier, the role of microglia in ALS has generated a new direction of ALS studies, foregrounding the microglia-mediated neurotoxicity, pathogenicity, detrimental effects at later stages of the disease, and disease progression.

Autophagy contributes to glial function, precisely the innate immune response of microglial cells, by mediating the release of soluble inflammatory effectors and phagocytosis (Sierra et al., 2013). Microglia autophagy not only contributes to the maintenance of the subcellular environment but also to the degradation of misfolded proteins and other harmful contents produced by neurons. Therefore, microglial autophagy modulates the intercellular homeostasis for neurons and processes inflammatory activation of microglial cells to M1 or M2 phenotype (M. Lin et al., 2022; Matarin et al., 2015). The M1 phenotype of microglia is predominant in promoting neurotoxicity in neurodegenerative disorders such as Alzheimer's disease, Huntington's disease, and ALS. Microglial neurotoxicity is detrimental to ALS pathogenesis, and there is growing evidence supporting the activation of microglia due to impaired autophagy (Ilieva et al., 2009; Valenzuela et al., 2018). Interestingly, both extrinsic and intrinsic $SOD1^{G93A}$ mediate microglial autophagy. $SOD1^{G93A}$ mutation increased ROS stress in microglia, which activates the pro-inflammatory M1 phenotype by downregulating autophagy (Méndez-lópez et al., 2021; Richardson et al., 2013; Rudnick et al., 2017; Yuan et al., 2019). In agreement with this study, another group demonstrated that overexpression of mutant $SOD1^{G93A}$ and $SOD1^{A4V}$ results in autophagic-mediated microglial activation and neurotoxicity (Massenzio et al., 2018a). Together, intracellular expression of mSOD1 mediates cellular stress conditions by ROS, autophagy

inhibition, and toxic protein accumulation, which results in microglia-led neurodegeneration. Interestingly, extracellular SOD1^{G93A} secreted by motor neuron in the later disease stage also promote the autophagic-dependent conversion of microglia to inflammatory phenotype (Ma et al., 2020; K. Zhang et al., 2021; Zhao et al., 2010). Collectively, there is increasing evidence that autophagic defects in microglia play an important role in ALS disease progression, and manipulation of the microglial autophagic pathway could be a promising therapeutic target.

CHAPTER 2: TRPML1 REGULATES X-LINKED MYOTUBULARIN MYOPATHY BY CONTROLLING AUTOPHAGIC FLUX IN SKELETAL MUSCLE

2.1 Rationale and Hypothesis

Because (A) XLMTM is a severe form of myopathy that is caused by the lipid phosphatase, myotubularin (MTM1), and MTM1 is responsible for the dephosphorylation of PI3,5P2 (Y. Liu & Bankaitis, 2010; Tronchère et al., 2004b; Tsujita et al., 2004b), (B) PI3,5P2 is essential for autophagic flux, i.e., autophagosome-lysosome fusion, (C) Basal autophagy is especially important in postmitotic cells such as muscles cells for differentiation and cell maintenance (Masiero et al., 2009; Mizushima & Levine, 2010). Autophagy is required not only for muscle mass maintenance in adults but also for muscle development before adulthood and injury (Fortini et al., 2016b; McMillan & Quadrilatero, 2014; Raben et al., 2008), and (D) TRPML1 serves as an ion channel on the lysosomal membrane, which is endogenously activated by PI3,5P2 (Cheng et al., 2010; Dong et al., 2010). Indeed, both the activation of TRPML1 and an increase in PI3,5P2 has been show to result in autophagosome accumulation (Ferguson et al., 2009; Hasegawa et al., 2016; Qi et al., 2021; Scotto Rosato et al., 2019b; T. Sun et al., 2011; X. Zhang, Cheng, et al., 2016). We proposed that loss of MTM1 may increase TRPML1 activity due to the dysregulation of PI3,5P2 metabolism and play an important role in muscle cell (myoblast) differentiation and fusion by regulating autophagic flux.

In this study, we demonstrated that upregulating, but not suppressing, TRPML1 inhibited myogenesis by compromising the last phase of autophagy, i.e., autophagosome-lysosome fusion. In agreement with this, we found that inhibiting autophagosome-lysosome fusion by deleting Syntaxin-17 (STX17), the autophagosomal SNARE that facilitates the fusion between autophagosomes and lysosomes (Itakura et al., 2012b), compromised myogenesis. In addition, the impaired myogenesis induced by upregulating TRPML1 was rescued by overexpressing STX17. Significantly, deleting MTM1 activated TRPML1 and suppressed autophagosome-lysosome fusion and, subsequently, myogenesis. The defects in MTM1 deficient cells were corrected by inhibiting TRPML1 or overexpressing STX17. Mechanistically, TRPML1 upregulation or MTM1 deficiency activated TFEB to increase DNM2 expression, further compromising autophagy and

myogenesis; suppressing DNM2 rescued the defective myogenesis and autophagy in both cells with TRPML1 upregulation and MTM1 deletion. Consistently, DNM2 overexpression inhibited autophagosome-lysosome fusion and myogenesis. Our study suggests that TRPML1 activates the TFEB-DNM2 pathway to inhibit autophagosome-lysosome fusion in skeletal muscle. This further compromises myogenesis. In XLMTM, TRPML1 is overactivated due to an increase in PI3,5P2. This leads to aberrant activity of TFEB-DNM2, which further inhibits autophagosome-lysosome fusion and myogenesis. Therefore, inhibiting TRPML1 could be a therapeutic approach for XLMTM.

2.2 Methods

2.2.1 Cell Culture and Differentiation

C2C12 cells were cultured in the growth medium, i.e., high glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37ºC and 5% CO2. When C2C12 cells reached 80-90% confluence, they were switched to a differentiation medium, i.e., high glucose DMEM medium containing 2% horse serum, to induce myoblast differentiation and myotube formation. Cells were grown in differentiation medium for 3-4 days until myotubes can be seen. Cells on day 4 in the differentiation medium were used for myotube quantification, cells on day 2 in the differentiation medium were used for myogenin and DNM2 western blot, and cells on day 1 in the differentiation medium were used autophagosome quantification. HEK293 cells were grown in DMEM with 10% FBS.

2.2.2 Chemicals, Antibodies, and Plasmids

Chemicals used in this study include ML-SA1 (44820, Princeton BioMolecular Research Inc), ML-SI1 (BML-CR112-0010, Enzo Life Sciences Inc), To-Pro-3 iodide (T3605, Life Technologies), Propidium Iodide (P3566, Invitrogen), GPN (sc-252858, Santa Cruz Biotechnology), Ionomycin (11932-1, Cayman), Bafilomycin A1 (1334, Tocris Bioscience Inc, and EAA (11130051, Gibco).

Myogenin antibody (F5D), MHC antibody (MF 20), and Lamp1 antibody (1D4B) were obtained from developmental studies hybridoma bank (DSHB) at the University of Iowa. LC3 antibody (NB100-2220) was purchased from Novus Biochemicals Inc. Antibodies against GAPDH (sc166574) and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology Inc. STX17 (HPA001204) antibodies were purchased from Sigma Aldrich. Antibody against dynamin 2 was purchased from Abcam (ab3457).

Plasmids of TRPML1-GFP, TRPML1-mCherry, TRPML1-HA, and Lamp1-GFP [52] were obtained from Dr. Haoxing Xu at the University of Michigan. mRFP-EGFP-LC3 (or ptfLC3, Plasmid #21074), wild-type HA-DNM2 pcDNA3.1 (Plasmid #34684) and K44A HA-DNM2 pcDNA3.1 (Plasmid #34685), FLAG-STX17 (Plasmid #45911) were obtained from addgene. GECO-TRPML1, LC3-mCherry, and STX17-mCherry were home-made. Plasmids were introduced into C2C12 cells using a Neon electroporation system (Life Technologies) following the manufacturer's instructions. Transfection efficiency reaches 90%, as observed after 24 hours.

2.2.3 Gene deletion of TRPML1, MTM1, STX17, and TFEB

For the knockout of mouse *TRPML1*, *STX17*, and *MTM1*, the CRISPR/Cas9 system was used following the published protocol. Guide RNA sequence was designed by CRISPR design online software (http://crispr.mit.edu/). After being annealed with their reverse complementary sequences, the designed gDNA sequence was modified to link with Bbs I digested pX330-2A-GFP (modified from pX330-U6-pSpCas9, Addgene #42230) or pX459-2A-Puro (Addgene #62988). pX330-2A-GFP/*TRPML1* (and *STX17*) gDNA or pX459-2A-Puro/*MTM1* was electroporated into C2C12 cells using Neon Transfection System. The gDNA sequences used in this study are shown in Table 1.

Table 1: gDNA sequences for gene knockdown

Gene	gDNA sequence
TRPML1-KO1	ATCCTGGCACTGCTCGATGT
TRPML1-KO2	GACATAGGCATACCGGCCC
STX17-KO1	GCGCTCCAATATCCGAGAAA
STX17-KO2	GGTCCCATCGGCCTCCTCGC
MTM1-KO1	CAACGTTTAGATCCCGAAAT
MTM1-KO2	ATGGGAGGCGCGACAAGTAG

We constructed pLKO shRNA vectors (addgene #8453) encoding shRNAs that target mMTM1 for knockdown studies. Plasmids were linearized using AgeI and EcoR1 to facilitate directional cloning. The sequences for mouse *MTM1* were: KD1, 5'-CCTCTAGTCAATTCGCCTTAT-3'; KD2, 5'- CTTTGAGATATTGGTACAGAA-3'. Mouse TFEB shRNA was purchased from Sigma (clone: TRCN0000085548). Lentivirus was made using a using a 2nd generation lentiviral packaging system. Briefly, shRNAs in the pLKO vector were co-transfected into HEK293T cells along with psPAX2 and pMD2G. Lentivirus was harvested 48h after transfection, filtered through a 0.45 μm sterile filter, and stored at -80 °C. C2C12 were infected with harvested lentivirus along with 8 μg/ml polybrene for 24 hours, and positive clones containing shRNA were selected using 2μg/ml puromycin.

2.2.4 RNA isolation and RT-PCR analysis

Total RNA isolation was carried out with TRIzol Reagent from Life Technologies Inc. C2C12 cells cultured in a 6 cm dish at 90% confluence were collected in 1 ml TRIzol. Detailed

operation followed instructions of the manufacturer. cDNA was obtained from 2 μg total RNA through iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (1725037 Bio-Rad). Real-time PCR was performed with Go Taq® qPCR Master Mix (A6001, Promega) and CFX96 Real-Time PCR Detection System (BioRad). Relative quantification of gene expression is calculated via formula $2^{-\Delta Ct}$ normalized to GAPDH. Following primers were applied in RT-TRPML1F: AAGCCCTGCAAGCTGATGC, RT-TRPML1R: TGCTCCTGTGTGTAGGCTGC, RT-GAPDHF: TGTCCGTCGTGGATCTGAC, RT-GAPDHR: GGAGTTGCTGTTGAAGTCGC. MTM1-F1: GCCCAATCACCATTGGAGGA, MTM1-R1: GACTGCAGCGCATAATGACC, MTM1-F2: TCCTTGGCCATGCTGATGTT, MTM1-R2: TTCGACATCTGCCACACACA.

2.2.5 Immunofluorescence

For muscle differentiation and fusion experiments, C2C12 cells were grown on glass coverslips and washed with PBS twice before fixation with 4% paraformaldehyde at room temperature for 10 minutes. Fixed cells were permeabilized with 0.3% Triton X-100 (T8787, Sigma Aldrich) for 10 minutes and then blocked with 2.5% BSA in PBS buffer for 60 min at room temperature. After 3 times washing with PBS, cells were incubated with MHC antibody (1: 200) at 4 °C overnight. After three more washes with PBS, cells were incubated with Alexa Fluor 488 goat anti-mouse IgG (1: 1000) for 60 minutes at room temperature in the dark. Nuclear stain TO-PRO-3 iodide (1: 500, Life Technologies) was added and washed three times with PBST 15 minutes before imaging. Images were acquired using an upright confocal microscope (LSM710, Zeiss) with a 20 X plain objective lens (for C2C12 differentiation analysis) or 63X oil-immersion objectives (for other analysis) and captured using ZEN 2009 software (Zeiss). Cells were counted via nucleus, and MHC-positive cells are myotubes. The differentiation index was defined as the number of nuclei in myotubes expressing MHC divided by the total number of nuclei in a field. The fusion index was calculated as the percentage of nuclei in MHC-positive myotubes containing at least 2 nuclei. Experiments were repeated with triplicated samples for differentiation and fusion index analysis for all conditions. More than 10 fields and more than 20 cells were counted for each condition.

For autophagosome-lysosome fusion experiments, cells were co-transfected with mRFP-GFP-LC3 and TRPML1-HA/empty-HA or DNM2-K44A and DNM2-WT according to experimental requirements.

2.2.6 TFEB Translocation

To generate a TFEB stable cell line, lentivirus was made using a 2nd generation lentiviral packaging system. Briefly, lentiviral plasmids pLJM1-EGFP (Addgene #19319) or pLJM1-EGFP-N1-TFEB (EGFP cDNA was replaced by inserting the EGFP-N1-TFEB cDNA between NheI and EcoRI site of pLJM1-EGFP vector) were co-transfected into HEK293T cells along with psPAX2 and pMD2.G. Lentivirus was harvested 48h after transfection, filtered through 0.45-µm sterile filter and stored at -80 °C. C2C12 cells were infected with harvested lentivirus along with 8 µg/ml polybrene for 24 hours and then selected by 2 µg/ml puromycin until resistant colonies were identified. TFEB stable cell line was maintained in DMEM medium supplemented with 10% FBS (Life Technologies) and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO2 incubator. To examine TFEB nuclear translocation in MTM1-KD cells, a TFEB stable cell line was infected with lentivirus expressing pLKO shRNA vector (addgene, 8453) or pLKO-mMTM1 shRNA along with 8 µg/ml polybrene for 24 hours. Cells were then changed to a growth medium and cultured for 4 days. Cells were treated with DMSO or 10 µM SA1 in differentiation medium or 0.1 µM SI1 in DMEM supplemented with 0.1% EAA (Gibco) and 0.05% FBS for 2 hours before confocal images were collected to show nuclear localization of TFEB.

2.2.7 GECO-Based Calcium Imaging

GECO-TRPML1 was made as described before (Q. Cao et al., 2015). C2C12 cells transfected with the indicated constructs were trypsinized and plated onto glass coverslips for 4–6 hours prior to experiments. The fluorescence intensity at 470 nm (F470) was monitored using the EasyRatioPro system. Lysosomal Ca^{2+} release was measured under a 'low' external Ca^{2+} solution containing 145 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 10 mM glucose, 1 mM EGTA, 20 mM HEPES, pH 7.4). Ca^{2+} concentration in the nominally free Ca^{2+} solution is estimated to be 1-10 mM. With 1 mM EGTA, the free Ca^{2+} concentration is estimated to be <10 nM based on the Maxchelator software.

2.2.8 Western Blot

Cells were washed with cold phosphate saline buffer (PBS) and lysed with lysis buffer containing protease and phosphatase inhibitors. After high-speed centrifugation, the protein in the supernatant was subjected to protein quantification with the BCA method. For each sample, 10-20 µg of total protein was loaded into SDS-PAGE. Protein on the SDS-PAGE was transferred to the PVDF membrane after electrophoresis. Specific primary antibodies and coordinated second

antibodies (HRP conjugated) were applied for blotting. The membrane was carried on to autoradiography after incubation in ECL solution for 1 minute. The films were scanned, and protein bands were quantified by Image J.

2.2.9 Data Analysis

Data are presented as mean ± SEM from at least three independent experiments. Statistical comparisons were made using a two-way analysis of variance (ANOVA) or Student's t-test. P values of < 0.05 were considered statistically significant; *: $P < 0.05$; **: $P < 0.01$, ***: $P < 0.005$.

2.3 Results

2.3.1 Upregulating TRPML1 Inhibits Muscle Differentiation and Fusion

Myosin Heavy Chain (MHC) is a motor protein that constitutes the thick filaments of skeletal muscles (Schiaffino et al., 2015). This protein is used as a myomarker to visualize fused myoblasts *in vivo*. In a similar manner, myogenin is a muscle-specific transcription factor that promotes the transcription of muscle-specific target genes involved in the coordination of skeletal muscle development. Myogenin expression is elevated during muscle differentiation and fusion (Benavente-Diaz et al., 2021). In this study, we used these two myomarkers of differentiation, i.e., MHC-positive myotubes and myogenin levels, to determine myogenesis (Ganassi et al., 2018; Millay et al., 2013).

It was first essential to establish whether activating TRPML1 facilitates muscle differentiation; therefore, we treated C2C12 cells, myoblasts from mice that are commonly used to study muscle differentiation, with ML-SA1, a synthetic agonist of TRPML1. Myotubes were indicated by MHC staining, and cell number was monitored by labeling the nucleus with To-Pro-3 iodide. ML-SA1 significantly decreased muscle differentiation and fusion, as demonstrated by reduced differentiation index and fusion index (Fig. 2.1A-C).

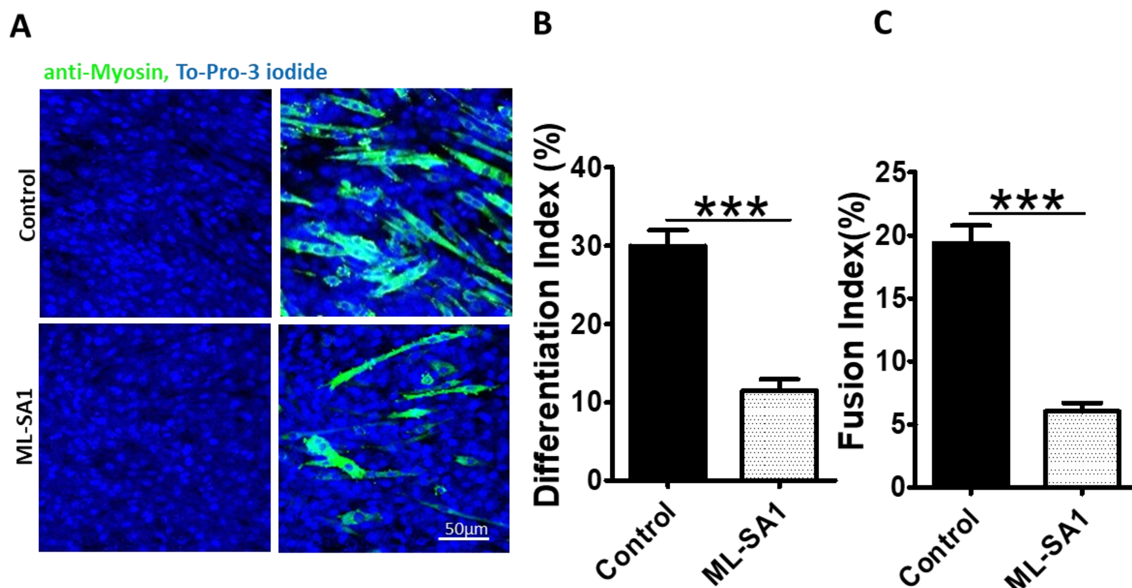

Figure 2.1: TRPML1 activation decreases differentiation of C2C12 cells. (A-C) ML-SA1 (10 µM) treatment reduced C2C12 cell differentiation. Myotubes were stained with myosin heavy chain antibody. Nucleus were labeled with To-Pro-3 iodide. Muscle cell differentiation was indicated by differentiation index and fusion index. For differentiation and fusion index analysis, experiments were repeated three times with triplicated samples each time for all conditions. Totally, more than 10 fields were counted. The data represent the mean ± SEM. NS: not significant; *: P < 0.05, **: P < 0.01; ***: P < 0.005.

ML-SA1 could act on targets other than TRPML1 to inhibit myogenesis. To exclude this possibility, we generated *Trpml1* knockout (TRPML1-KO) C2C12 cells using the CRISPR/Cas9 genome editing system (Cong et al., 2013). We found that the effect of ML-SA1 on muscle differentiation and fusion was prevented by TRPML1-KO (Fig. 2.2A-C), suggesting that ML-SA1 inhibits muscle differentiation by activating TRPML1. In agreement with MHC staining data, myogenin levels were markedly reduced by ML-SA1 during muscle differentiation, and this was corrected by TRPML1-KO (Fig. 2.2D). However, TRPML1-KO itself had little effect on muscle differentiation. This agrees with previous reports showing normal muscle development in TRPML1-KO mice (Cheng et al., 2014).

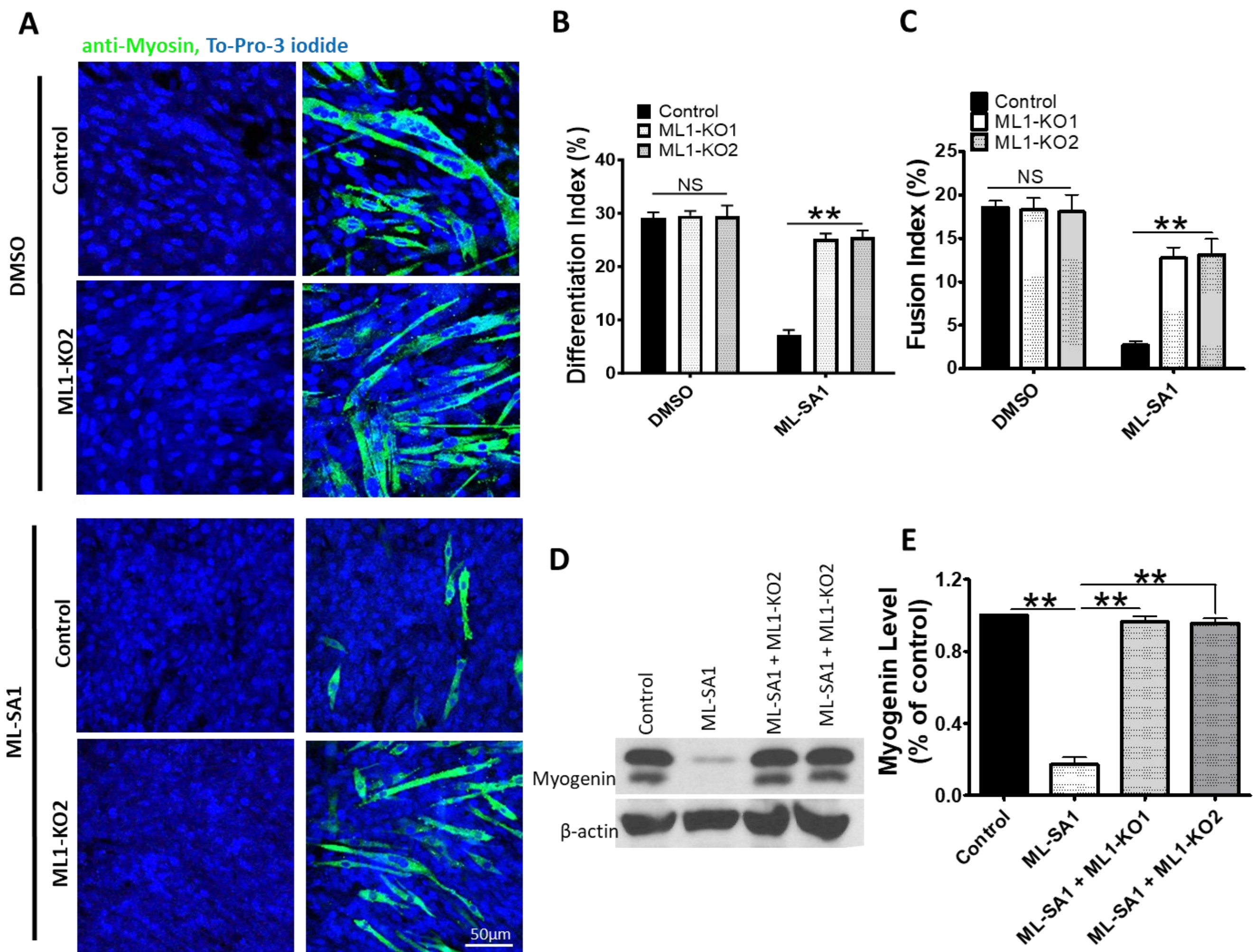

Figure 2.2: TRPML1 specific effect of ML-SA1 (A-C) The effect of ML-SA1 was inhibited by TRPML1-KO, suggesting that ML-SA1 regulates muscle differentiation is through activating TRPML1. Note that TRPML1-KO had no effect on muscle differentiation (D) ML-SA1 treatment reduced myogenin expression in C2C12 cells during cellular differentiation. ML-SA1's effect on myogenin expression was eliminated by TRPML1-KO. Myogenin western blotting experiments were repeated three times for all conditions. The data represent the mean ± SEM. NS: not significant; *: $P < 0.05$. **: $P < 0.01$.

Consistently, ectopic expression of TRPML1-mCherry (Q. Cao et al., 2015; Dong et al., 2008) also reduced muscle differentiation and fusion (Fig. 2.3A-C). TRPML1-GFP also reduced myogenin expression (Fig. 2.3D). Taken together, these data suggest that upregulating, but not downregulating, TRPML1 inhibits muscle differentiation and fusion.

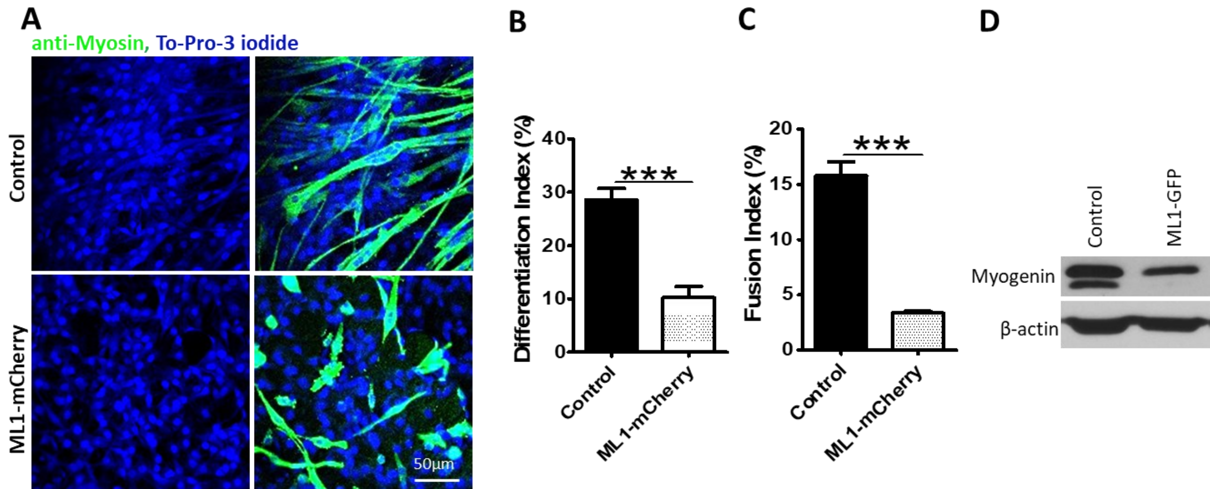

Figure 2.3: TRPML1 overexpression decreases C2C12 cell differentiation. (A-C) In accordance with ML-SA1 data, TRPML1-mCherry overexpression reduced differentiation and fusion index. Please note that images from red channel were not presented because mCherry signal was lost after 3-day in fusion medium. (D) TRPML1-GFP overexpression reduced myogenin expression levels. Myogenin western blotting experiments were repeated three times for all conditions. The data represent the mean ± SEM. NS: not significant; *: $P < 0.05$, **: $P < 0.01$; ***: $P < 0.005$.

2.3.2 TRPML1 Upregulation Inhibits Autophagosome-Lysosome Fusion

To test whether TRPML1 inhibiting muscle differentiation is attributed to defective autophagy (Fortini et al., 2016b; Masiero et al., 2009), we measured LC3-, which indicates autophagosome accumulation. We found that ML-SA1 treatment increased LC3-II in C2C12 cells (Fig. 2.4A). This is in agreement with the previous report that TRPML1 upregulation increases LC3-II levels (Cheng et al., 2014; Qi et al., 2021).

Efficient autophagy is represented by the autophagic flux, which is often defined as a measure of autophagic degradation activity but not autophagosome accumulation. A series of events, including autophagy initiation, autophagosome-lysosome fusion, and autolysosome degradation, regulate autophagic flux. LC3-II increase could be caused by an elevation in autophagosome formation and a reduction in autophagosome-lysosome fusion and/or autolysosome degradation (Klionsky et al., 2021) To differentiate these possibilities, Bafilomycin A1 (Baf-A1), an inhibitor of V-ATPase, was adopted to inhibit autolysosomal degradation. A

comparable LC3-II between the control and ML-SA1 group in the presence of Baf-A1 would suggest that TRPML1 upregulation does not affect autophagosome formation. As shown in Figure 2.4A, Baf-A1 treatment normalized LC3-II (lower band in the western blot image), suggesting that ML-SA1 does not affect autophagosome formation. Consistently, although TRPML1-GFP overexpression increased LC3-II levels, a comparable LC3-II level between the control and TRPML1-GFP group was detected in the presence of Baf-A1 (Fig. 2.4B). These data suggest that an increase in LC3-II (autophagosomes) by TRPML1 upregulation is likely due to a reduction in autophagosome-lysosome fusion and/or autolysosomal degradation but not autophagosome formation and autolysosomal degradation. Because TRPML1 upregulation does not affect lysosome function (Cheng et al., 2010; W. Wang et al., 2015a).

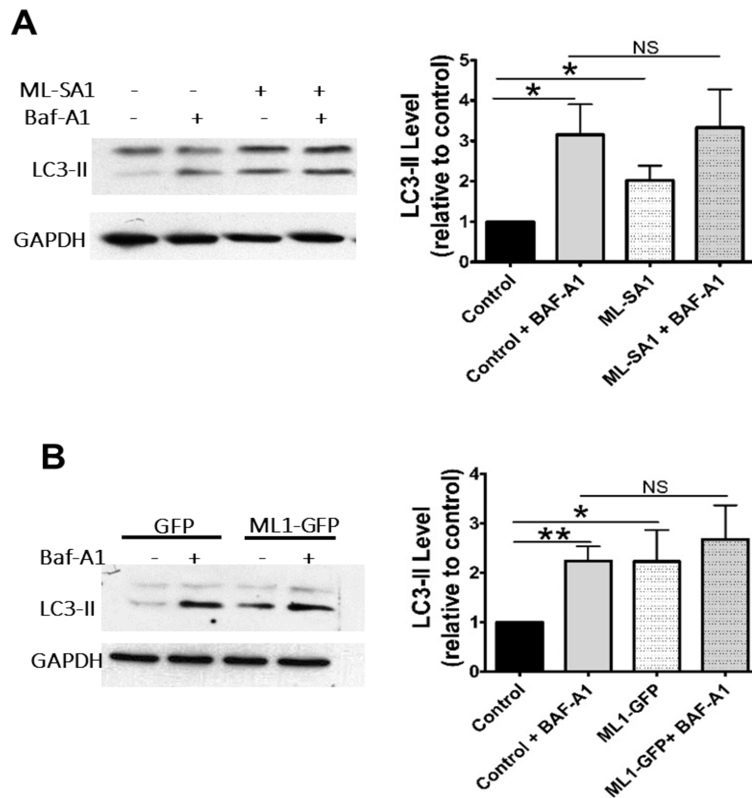

Figure 2.4: TRPML1 upregulation inhibits autophagy. (A) ML-SA1 (10 μM) treatment increased LC3-II expression in C2C12 cells compared with DMSO treated cells. Although ML-SA1 increased LC3-II expression, a comparable LC3-II expression between the control and ML-SA1 group in the presence of Baf-A1 (200 nM), suggesting that TRPML1 upregulation has no effect on autophagosome formation. LC3-II expression levels were normalized to GAPDH. (B) Although TRPML1-GFP overexpression increased LC3-II levels, comparable LC3-II levels between the control and TRPML1-GFP group were detected in the presence of Baf-A1. LC3-II expression levels were normalized to GAPDH. LC3-II western blotting experiments were repeated three times for all conditions. The data represent the mean ± SEM. NS: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$.

We suspected that TRPML1 activation might impair autophagosome-lysosome fusion and lead to autophagosome accumulation. To test this hypothesis, C2C12 cells expressing mRFP-GFP-LC3 (Kaizuka et al., 2016; Kimura et al., 2007) were used to quantitate autophagosome and autolysosome. Because the low pH of autolysosome quenches the fluorescent signal of GFP but not RFP, autophagosomes (high pH) and autolysosomes (low pH) will be labeled with yellow (i.e., RFP and GFP) and red (i.e., RFP only) signals, respectively. An increase in the yellow-to-red puncta ratio represents a blockade of autophagosomes and lysosome fusion. As shown in Figure 2.5A-B, ML-SA1 treatment significantly increased the ratio of autophagosomes to autolysosomes. Consistently, TRPML1 overexpression significantly increased the ratio of autophagosomes to autolysosomes (Fig. 2.5C-D). Altogether, these data suggest that TRPML1 upregulation results in a compromised autophagosome-lysosome fusion but not autophagosome formation.

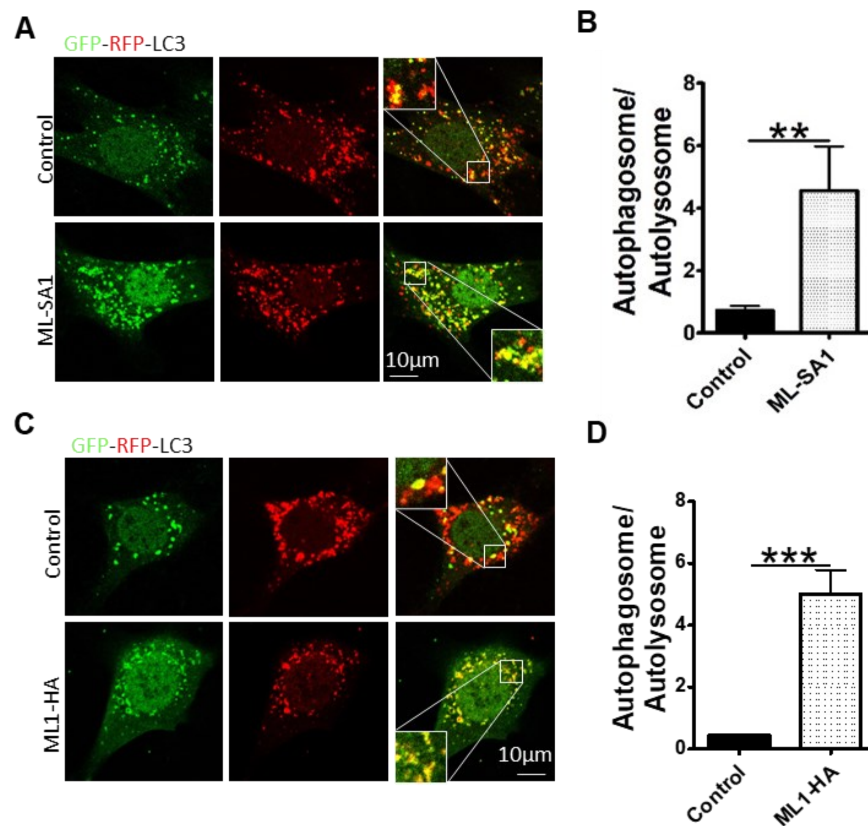

Figure 2.5: TRPML1 upregulation inhibits autophagosome-lysosome fusion. (A-B) C2C12 cells expressing mRFP-GFP-LC3 were used to quantitate autophagosome and lysosome fusion. ML-SA1 treatment increased the ratio of autophagosomes (yellow) vs autolysosomes (red), suggesting a blockade of autophagosomes and lysosomes fusion by ML-SA1. (C-D) TRPML1-HA overexpression increased the ratio of autophagosomes (yellow) vs autolysosomes (red). More than 20 cells were counted for each condition. The data represent the mean ± SEM. NS: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$.

2.3.3 Impaired Autophagosome-Lysosome Fusion Leads to Decreased Cell Differentiation

To test whether the compromised muscle differentiation induced by upregulating TRPML1 is associated with the later stages of autophagy, we inhibited autophagosome-lysosome by deleting *Stx17* (Itakura et al., 2012b). As shown in Fig. 2.6A-D, *Stx17* knockout (STX17-KO) inhibited muscle cell differentiation and fusion and caused a decrease in myogenin expression. These phenocopied C2C12 cells with TRPML1 upregulation (Fig. 2.1). Importantly, STX17 overexpression was able to rescue the defective muscle differentiation induced by both ML-SA1

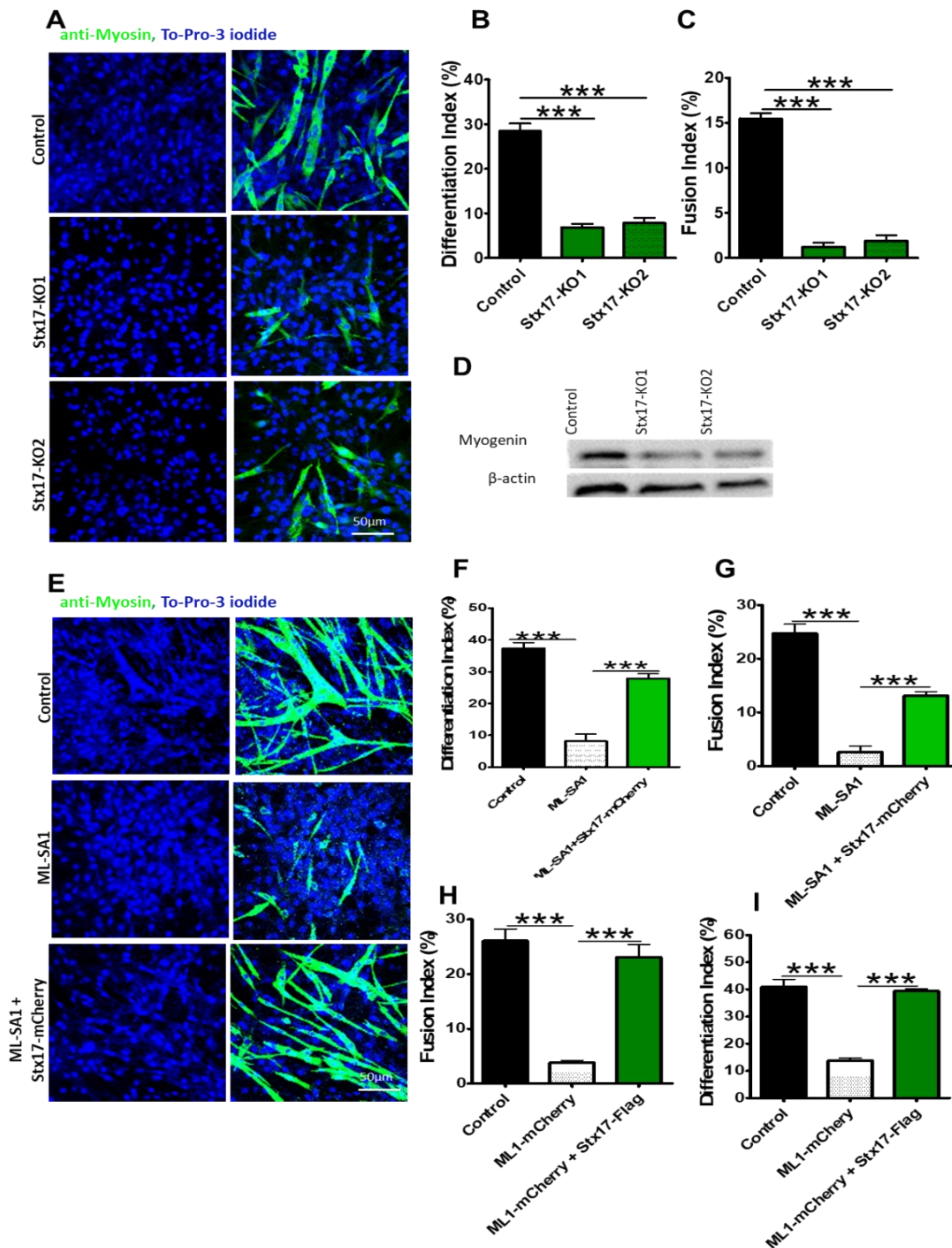

Figure 2.6: Impaired autophagic flux suppresses C2C12 cell differentiation. (A-C) Loss of STX17 decreased differentiation index and fusion index in C2C12 cells. (D) Suppression of autophagosome-lysosome fusion by STX17-KD reduced myogenin expression. Myogenin expression was measured after 2 days in differentiation medium. (E-G) Reduced differentiation index and fusion index induced by ML-SA1 were rescued by STX17-mCherry overexpression. (H-I) Reduced differentiation index and fusion index induced by TRPML1-mCherry overexpression were rescued by STX17-Flag overexpression. For differentiation and fusion index analysis, experiments were repeated three times with triplicated samples each time for all conditions. Totally, more than 10 fields and more than 20 cells were counted for each condition. The data represent the mean ± SEM. NS: not significant; *: $P < 0.05$; ***: $P < 0.005$.

treatment (Fig 2.6E-G) and TRPML1 overexpression (Fig. 2.6H-I). Thus, we demonstrate that proper autophagic flux is essential for muscle differentiation and provide genetic support that TRPML1 upregulation inhibits muscle differentiation and fusion by suppressing autophagosome-lysosome fusion.

2.3.4 MTM1 Deficiency Activates TRPML1 and Impairs Muscle Cell Differentiation

Because MTM1 deficiency may lead to elevated PI3,5P2 (Dong et al., 2010; Tronchère et al., 2004b; Tsujita et al., 2004b), the endogenous agonist of TRPML1 (Dong et al., 2010) and because TRPML1 activation inhibits muscle differentiation (Fig. 2.1) through suppressing autophagic flux (Fig. 2.2) that is fundamental for muscle development (Dowling et al., 2010; Fetalvero et al., 2013a; Masiero et al., 2009) (Fig. 2.3), we reasoned that PI3,5P2 accumulation due to MTM1 deficiency in XLMTM may activate TRPML1 to cause myopathy by inhibiting autophagic pathway. As the first step toward understanding the role of TRPML1 in XLMTM, we monitored PI3,5P2 in MTM1 knockdown (MTM1-KD) cells using the genetically encoded fluorescent probe GFP-ML1N*2 (X. Li et al., 2013). MTM1-KD significantly increased GFP-ML1N*2 signals in lysosomes, suggesting an increase of lysosomal PI3,5P2 (Fig. 2.7A-B).

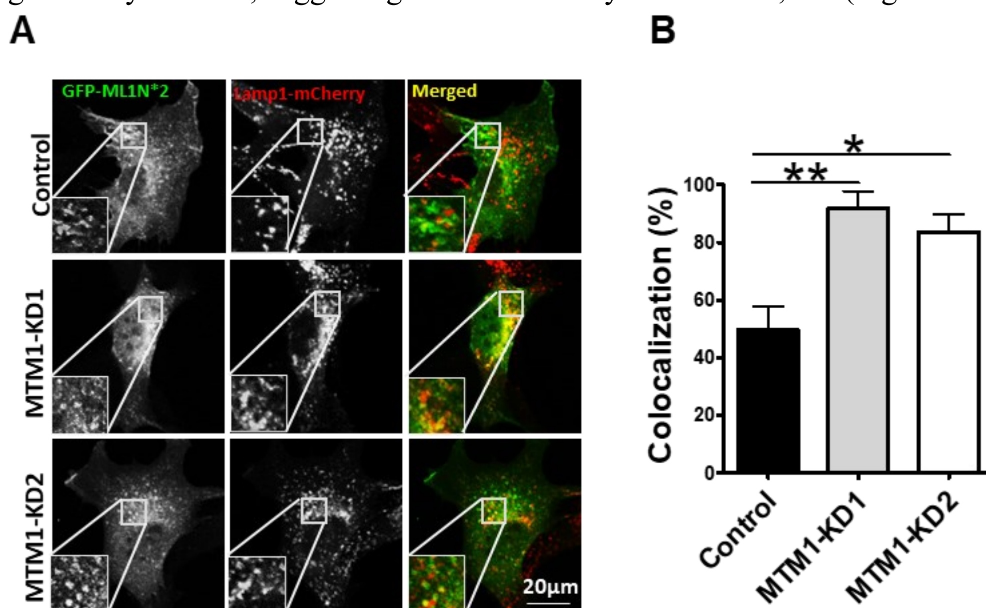

Figure 2.7: MTM1 deficiency elevates PI3,5P2 in lysosomes. MTM1-KD cells were co-transfected with Lamp1-mCherry and GFP-ML1N*2, a PI3,5P2 probe, and maintained in growth media. Confocal images were taken 24 hours post transfection. The data represent the mean ± SEM (n = 3). *: P < 0.05. **: P < 0.01.

Next, we generated *Mtm1* knockout (MTM1-KO) C2C12 cells using CRISPR/Cas9 method. As expected, MTM1 deficiency in C2C12 cells markedly reduced muscle cell differentiation and fusion index (Fig. 2.8A-C) and myogenin expression (Fig. 2.8D). Consistently, MTM1-KD cells also showed defects in muscle differentiation and fusion and reduced myogenin expression (Fig. 2.8E-H).

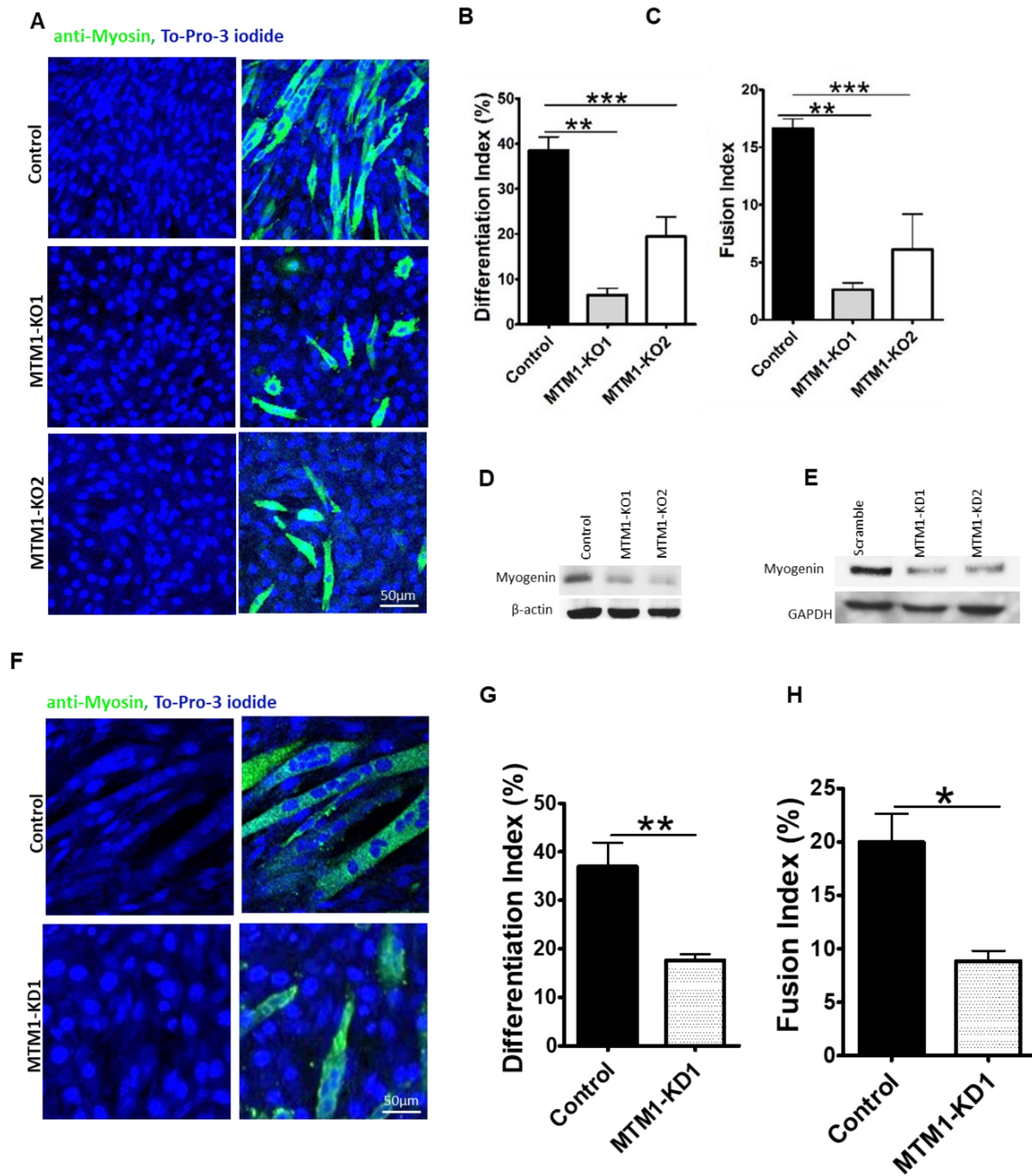

Figure 2.8: Loss of MTM1 suppresses muscle differentiation. (A-C) MTM1 deletion inhibited muscle differentiation as indicated by reduced differentiation index and fusion index. (D) MTM1-KO reduced myogenin expression in C2C12 cells. Myogenin expression was measured after 2 days in differentiation medium.(D-G) MTM1 deficiency inhibited C2C12 cell differentiation and fusion. (H) MTM1 deficiency decreased myogenin expression in C2C12 cells. The data represent the mean ± SEM (n = 3). *: $P < 0.05$; **: $P < 0.01$.

Since TRPML1 is a lysosomal Ca^{2+} permeable channel, to understand the mechanism underlying the compromised muscle differentiation and fusion induced by MTM1 deficiency, we measured TRPML1 activity using the lysosome-targeted genetically encoded Ca^{2+} indicator, i.e., GECO-TRPML1, that we generated before (Q. Cao et al., 2015). We found that MTM1 deficiency resulted in increased TRPML1 activity (Fig. 2.9A-B) without altering lysosomal Ca^{2+} content (Fig. 2.9C). Meanwhile, comparable GECO-TRPML1 signals were detected between the control and MTM1-KO cells, suggesting that the increased GECO-TRPML1 signals in MTM1-KO cells were not caused by elevated GECO-TRPML1 expression. Altogether, we suggest that increased PI3,5P2 levels due to MTM1 deficiency activate TRPML1, subsequently leading to compromised muscle differentiation and fusion.

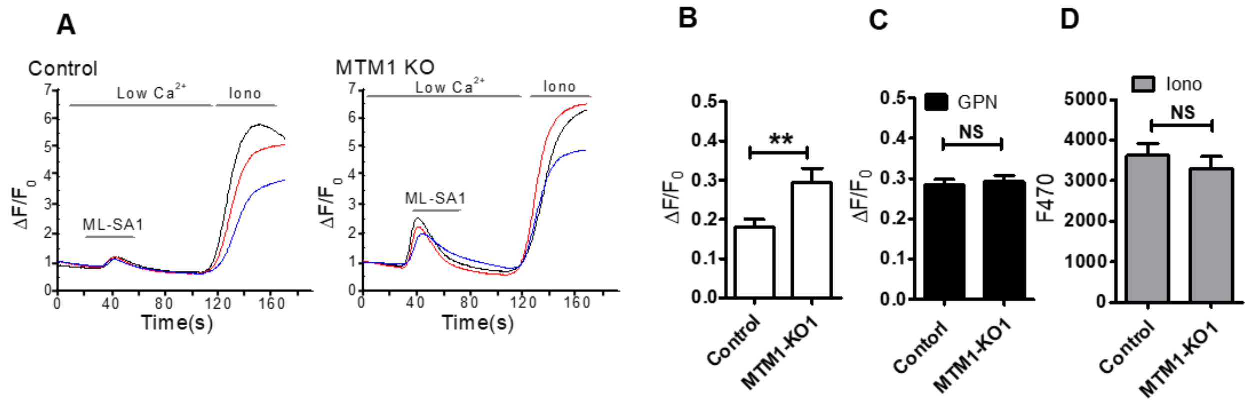

Figure 2.9: MTM1 deficiency activates TRPML1. (A-B) Larger TRPML1 activity in MTM1-KO C2C12 cells. TRPML1 activity was indicated by ML-SA1-induced GECO-TRPML1 signal. (C) Comparable GECO signals induced by GPN between the control and MTM1-KO cells, suggesting MTM1-KO has no effect on lysosomal Ca^{2+} content and the elevated TRPML1 activity in MTM1-KO cells is not due to an increased lysosomal Ca^{2+} content. (D) Comparable GECO signals induced by ionomycin between the control and MTM1-KO cells, suggesting elevated GECO signals in MTM1-KO cells is not due to an increased GECO-TRPML1 expression in MTM1-KO cells. Experiments were repeated three times and more than 100 cells were counted for each condition. The data represent the mean ± SEM. NS: not significant; **: $P < 0.01$; ***: $P < 0.005$.

2.3.5 Downregulation of TRPML1 Corrects the Impaired Muscle Differentiation and Autophagy Induced by MTM1 Deficiency

If compromised muscle differentiation and fusion induced by MTM1 deficiency is attributed to elevated TRPML1 activity, suppressing TRPML1 is expected to correct the phenotypes in MTM1 deficient cells. Indeed, TRPML1-KO corrected the defects in muscle differentiation and fusion (Fig. 2.10A-C) and the reduction in myogenin expression (Fig. 2.10D) induced by MTM1 deficiency. To leverage the mechanistic understanding gained from our experiments into drug development, we treated MTM1-KO C2C12 cells with TRPML1 inhibitor ML-SI1. As expected, ML-SI1 rescued the impaired muscle differentiation and fusion in MTM1-KO cells (Fig. 2.10E-G). Consistently, ML-SI1 also rescued the reduced myogenin expression (Fig. 2.10H) in MTM1-KO cells.

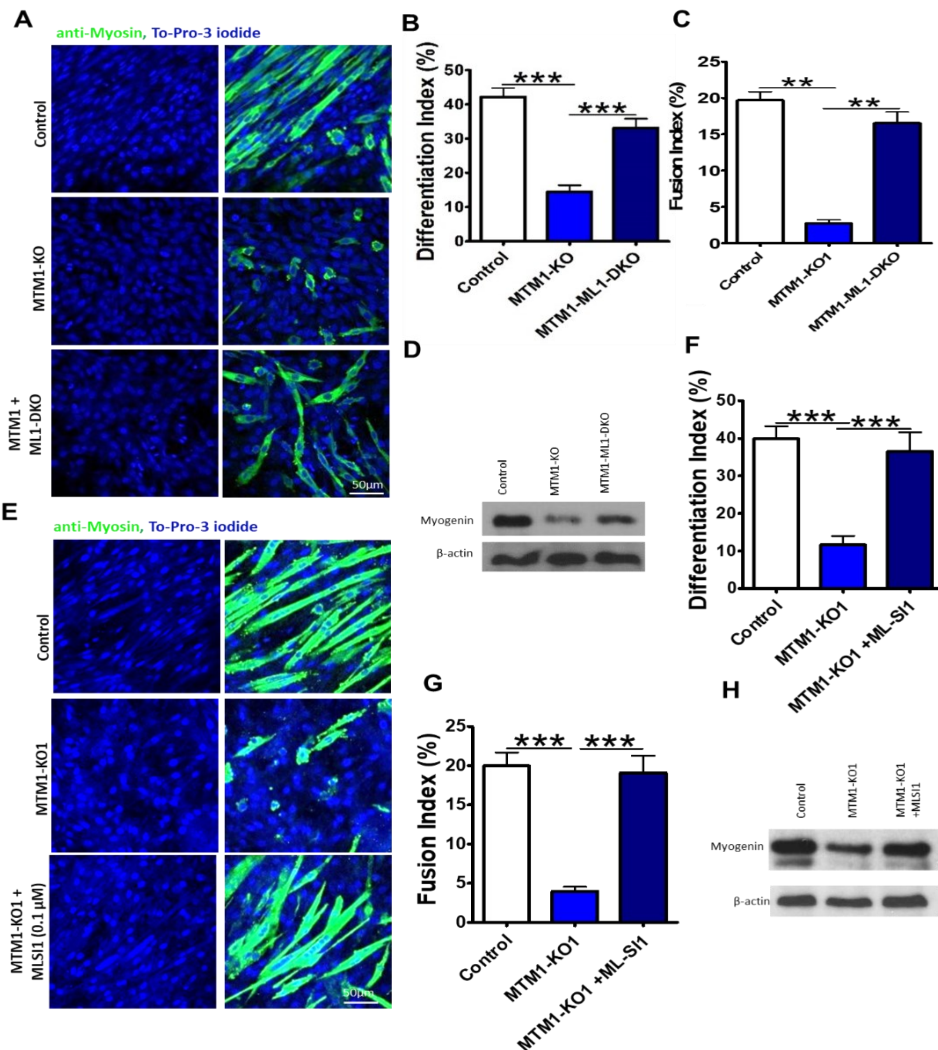

Figure 2.10: Compromised muscle differentiation by MTM1 deficiency is rescued by TRPML1 inhibition. (A-C) TRPML1-KO rescued the compromised muscle differentiation induced by MTM1-KO. DKO: double knockout. (D) Myogenin expression was reduced by MTM1-KO, and this was rescued by TRPML1-KO. MTM1-KO1 and TRPML1-KO2 were used to generate MTM1-TRPML1-DKO. (E-G) The impaired muscle differentiation induced by MTM1 deficiency was rescued by inhibiting TRPML1 using ML-SI1 (0.1 μM). (H) Reduced myogenin expression was rescued by ML-SI1 (0.1 μM). Totally, more than 10 fields were counted for each condition. The data represent the mean ± SEM. NS: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$.

As we noticed elevated TRPMl1 activity reduced autophagy, we measured autophagic flux in MTM1-KO cells. MTM1 deficiency increased LC3-II levels, which was normalized by Baf-A1 (Fig. 2.11A-B). Notably, ML-SI1 also corrected the increased LC3-II in MTM1-KO cells, which was normalized by Baf-A1 (Fig. 2.11A-B). In addition, autophagosome-lysosome fusion was monitored using mRFP-GFP-LC3 in MTM1-KO cells. We found that MTM1 deficiency inhibited autophagosome-lysosome fusion, which was rescued by ML-SI1 (Fig. 2.11C-D).

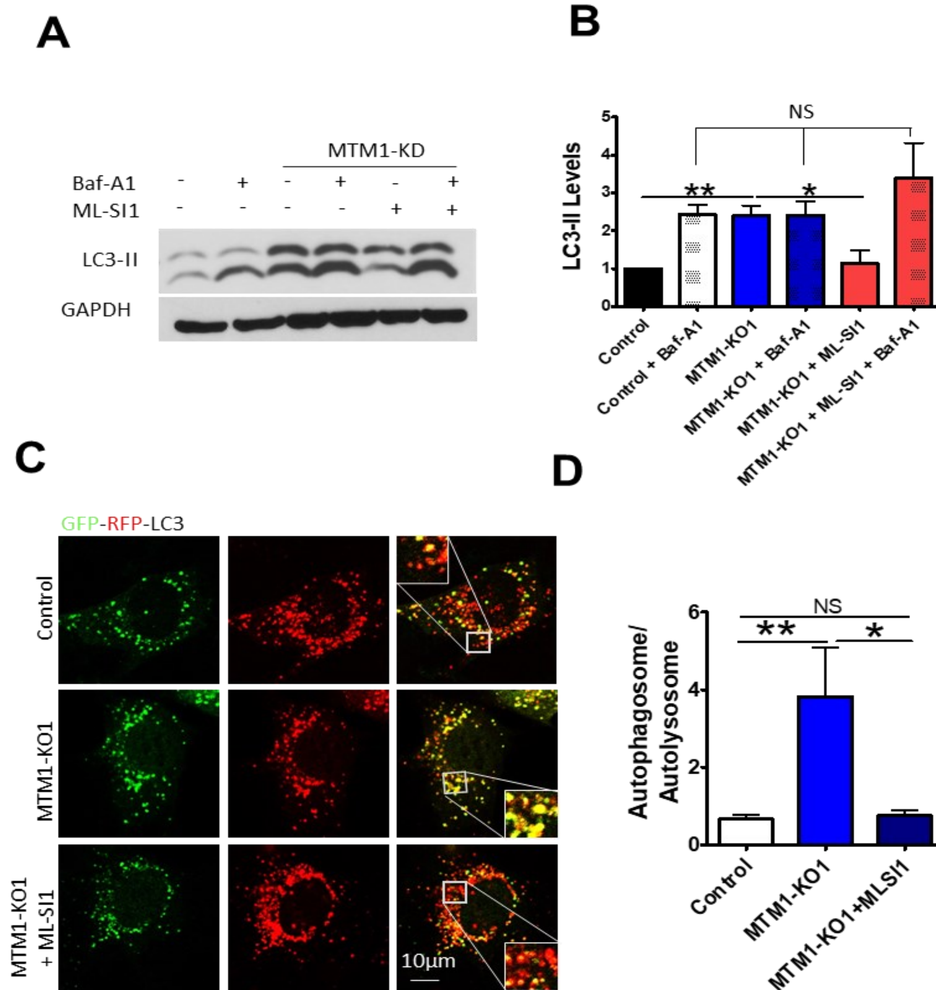

Figure 2.11: Compromised autophagy in MTM1-KO cells was rescued by TRPML1 inhibition. (A-B) MTM1 deficiency increased LC3-II levels, and this was normalized by Baf-A1; ML-SI1 (1 μM) corrected the increased LC3-II levels induced by MTM1 deficiency. (C-D) MTM1 deficiency increased the ratio of autophagosome vs. lysosome, and this was rescued by ML-SI1 (1 μM). These data suggest that loss of MTM1 inhibits autophagosome-lysosome fusion by activating TRPML1. LC3-II western blotting experiments were repeated three times. For the mRFP-GFP-LC3 imaging, more than 20 cells were counted for each condition. The data represent the mean ± SEM. NS: not significant; *: P < 0.05; **: P < 0.01; ***: P < 0.005.

2.3.5 STX17 Overexpression Corrects the Impaired Muscle Differentiation and Autophagy Induced by MTM1 Deficiency

If XLMTM is due to impaired autophagosome-lysosome fusion induced by MTM1 deficiency, promoting autophagosome-lysosome fusion is expected to rescue the phenotypes in MTM1 deficient cells. Indeed, STX17-mCherry overexpression corrected the compromised muscle differentiation and fusion (Fig. 2.12A-C) and the reduced expression of myogenin (Fig. 2.12D) induced by MTM1-KO. STX17 overexpression also corrected the defective autophagosome-lysosome fusion (Fig. 2.12E-F) in MTM1 deficient cells. Therefore, MTM1 deficiency activates TRPML1, subsequently inhibiting autophagosome-lysosome fusion, resulting in compromised muscle differentiation.

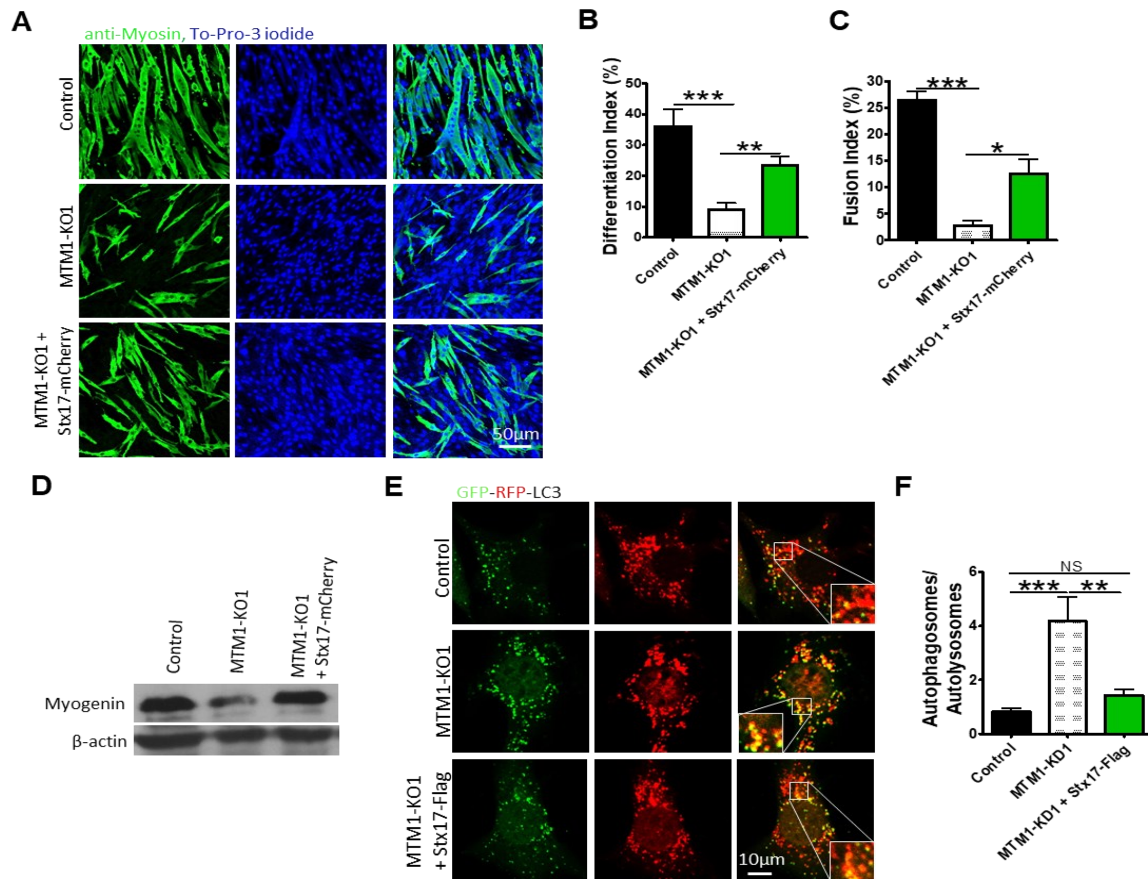

Figure 2.12: Compromised muscle differentiation by MTM1 deficiency is rescued by promoting autophagosome-lysosome fusion. (A-C) Impaired muscle differentiation in MTM1-KO cells were rescued by STX17 that promotes autophagosome-lysosome fusion. (D) STX17 overexpression rescued the reduced myogenin induced by MTM1 deficiency. (E, F) Impaired autophagosome-lysosome fusion in MTM1-KD cells was rescued by STX17 overexpression. For differentiation and fusion index analysis, experiments were repeated three times with triplicated samples each time for all conditions. Totally, more than 10 fields were counted for each condition. For the mRFP-GFP-LC3 imaging, more than 20 cells were counted for each condition. The data represent the mean ± SEM. NS: not significant; *: P < 0.05; **: P < 0.01; ***: P < 0.005.

2.3.6 MTM1 Deficiency Activates TRPML1-TFEB-DNM2 Pathway

Because 1) TRPML1 can stimulate autolysosomal protein expression by activating TFEB (Medina et al., 2015a), 2) DNM2 is an autolysosomal protein (Koutsopoulos et al., 2011; Schulze et al., 2013) that is upregulated by starvation (Durieux et al., 2012), and 3) DNM2 expression is elevated in XLMTM patients and a mouse model of XLMTM (Cowling et al., 2014; Tasfaout et al., 2018) and a reduction of DNM2 prevents and reverts myotubular myopathy in XLMTM mice (Tasfaout et al., 2017, 2018); we predicted that in XLMTM hyperactive TRPML1 may increase DNM2 by activating TFEB and eventually inducing impaired autophagosome-lysosome fusion and defective muscle differentiation. If this is the case, DNM2 is likely an autolysosomal protein that is regulated by TFEB upon starvation. To address the question, we performed immunoblot and immunostaining of endogenous DNM2 in C2C12 cells. Our western blot data showed that starvation significantly increased DNM2 protein levels, and this was reversed by deleting either TRPML1 or TFEB (Fig. 2.13A-B).

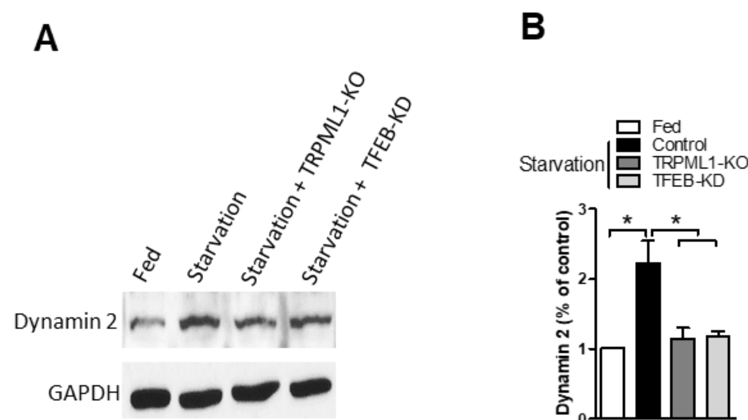

Figure 2.13: Starvation increases DNM2 expression, and this is rescued by TRPML1-KD and TFEB-KD. Cells were starved in DMEM supplemented with 10% EAA (Gibco) and 1% FBS for 24h before western blotting. Experiments were repeated three times. The data represent the mean ± SEM. *: P < 0.05.

In parallel, MTM1-KO activated TFEB (Fig. 14A), which was indicated by TFEB nuclear translocation, and this was inhibited by TRPML1 inhibitor ML-SI1 (Fig. 2.14 A-B). MTM1-KO also elevated DNM2 expression, and this was corrected by ML-SI1 (Fig. 2.14C-D), TRPML1-KO (Fig. 2.14E-F), and TFEB-KD (Fig. 2.14G-H).

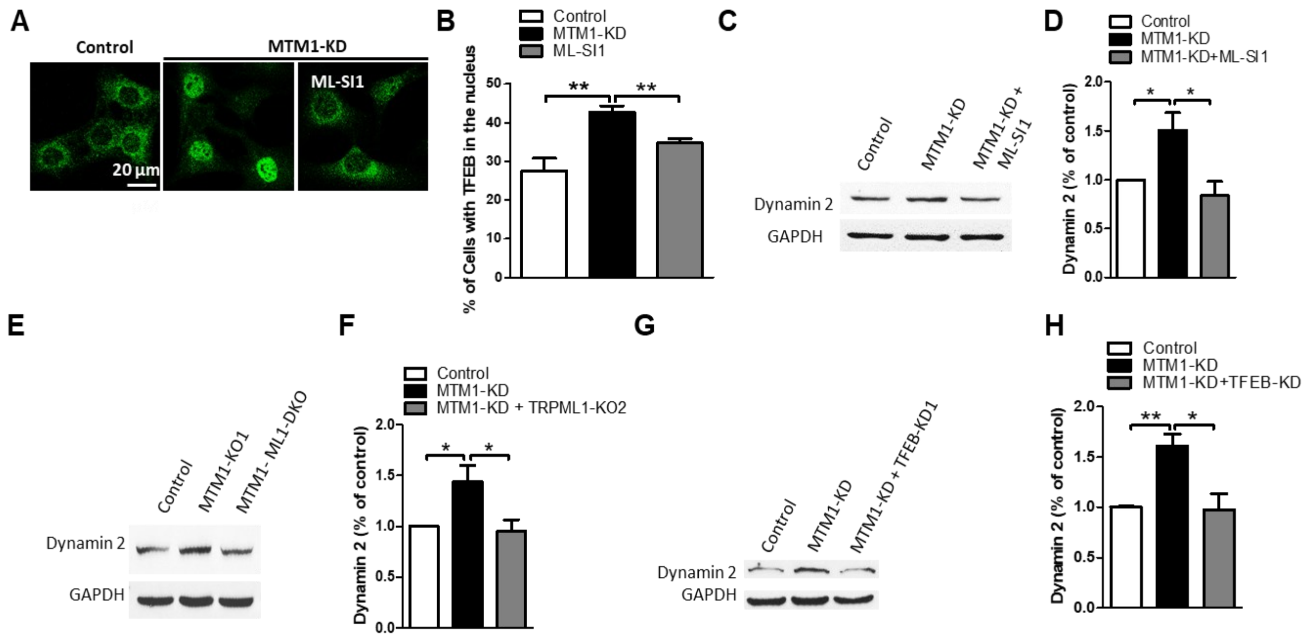

Figure 2.14: MTM1 deletion increases DNM2 in a TRPML1- and TFEB-dependent manner. (A, B) MTM1 deletion caused TFEB nuclear translocation, and this was corrected by ML-SI1 (0.1 µM), suggesting that ML-SI1 inhibits the loss of MTM1 activated TFEB. Cells were treated with 0.1 µM SI1 in DMEM supplemented with 0.1% EAA (Gibco) and 0.05% FBS for 2 hours before confocal images were collected to show nuclear localization of TFEB. (C, D) DNM2 protein expression was increased in MTM1-KD C2C12 cells, and this was corrected by ML-SI1. MTM1-KD cells were treated with ML-SI1 in DMEM supplemented with 10% EAA (Gibco) and 1% FBS for 24h before western blotting. (E, F) Increased DNM2 level in MTM1-KO C2C12 cells was corrected by TRPML1-KO. MTM1-KO1 and TRPML1-KO2 were used to generate MTM1-TRPML1-DKO. Cells were then cultured in DMEM supplemented with 10% EAA (Gibco) and 1% FBS for 24h before western blotting. (G, H) Increased DNM2 level in MTM1-KD C2C12 cells was corrected by TFEB-KD. The data represent the mean ± SEM. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$.

Furthermore, upregulating TRPML1 also activated TFEB (Fig. 2.15A-B) and increased DNM2 levels (Fig. 2.15C-D) in C2C12 cells. The upregulated DNM2 by TRPML1 was inhibited by TFEB-KD (Fig. 2.15C-D). Altogether, these data suggest that in MTM1-KO cells, TRPML1 activation stimulates TFEB to increase DNM2 expression.

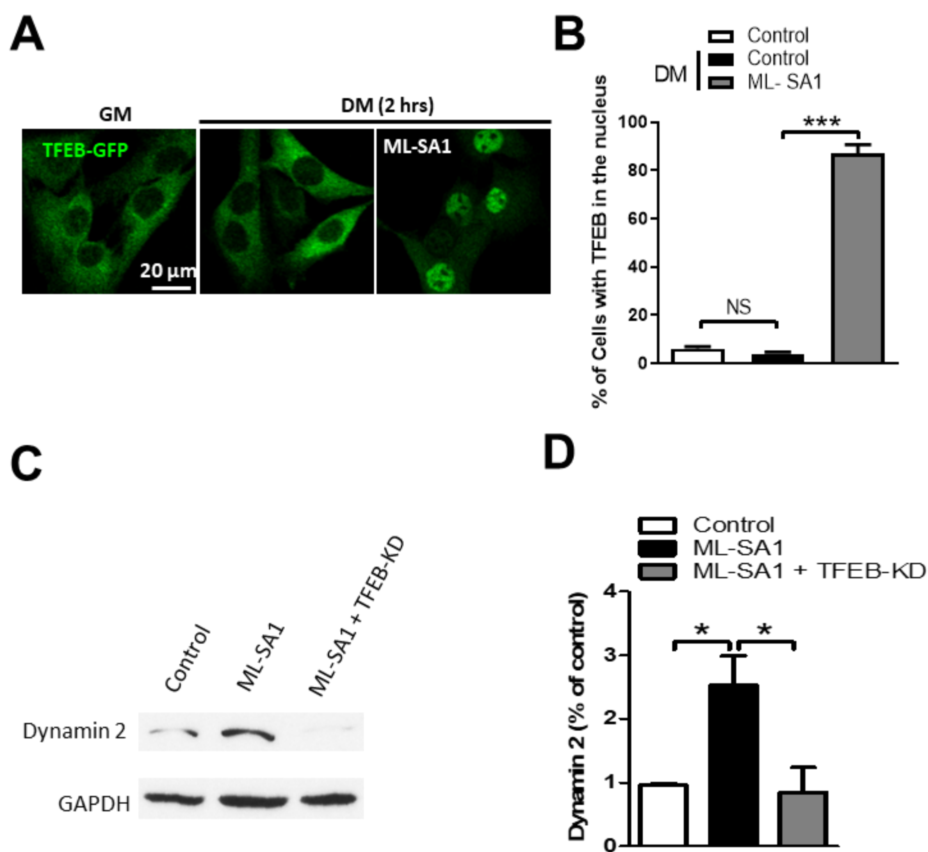

Figure 2.15: TRPML1 activation leads to TFEB nuclear translocation. Representative confocal images of GFP-TFEB localization in C2C12 cells treated with DMSO or ML-SA1 in growth medium or differentiation medium for 2 hours. (K, L) DNM2 expression was elevated by ML-SA1 in C2C12 cells, and this was rescued by TFEB-KD. Cells were then cultured in DMEM supplemented with 10% EAA (Gibco) and 1% FBS for 24h before western blotting) The data represent the mean ± SEM. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$.

2.3.7 Rescue of Defective Myogenesis in MTM1 Deficient Muscle by Inhibiting TRPML1-TFEB-Dynamin 2 Pathway

We first examined whether impaired muscle differentiation induced by TRPML1 overexpression was rescued by the dominant negative DNM2-K44A mutant (DNM2-DN). As shown in Figure 2.16 A-D, DNM2-DN explicitly corrected the reduced muscle differentiation and myogenin induced by TRPML1 overexpression. DNM2-DN rescued the impaired autophagosome-lysosome fusion in cells expressing TRPML1 (Fig. 2.16E-F). In line with these data, reduced myogenin induced by upregulating TRPML1 was also corrected by TFEB-KD (Fig. 2.16G)

To further support our hypothesis, we found that impaired muscle differentiation and fusion (Fig.2.16F-J) and autophagosome-lysosome fusion (Fig. 2.16K-L) in MTM1-KO cells were

rescued by DNM2-DN. Similarly, compromised myogenesis induced by MTM1-KD was corrected by TFEB-KD (Fig. 2.16M) and ML1-KO (Fig 2.10).

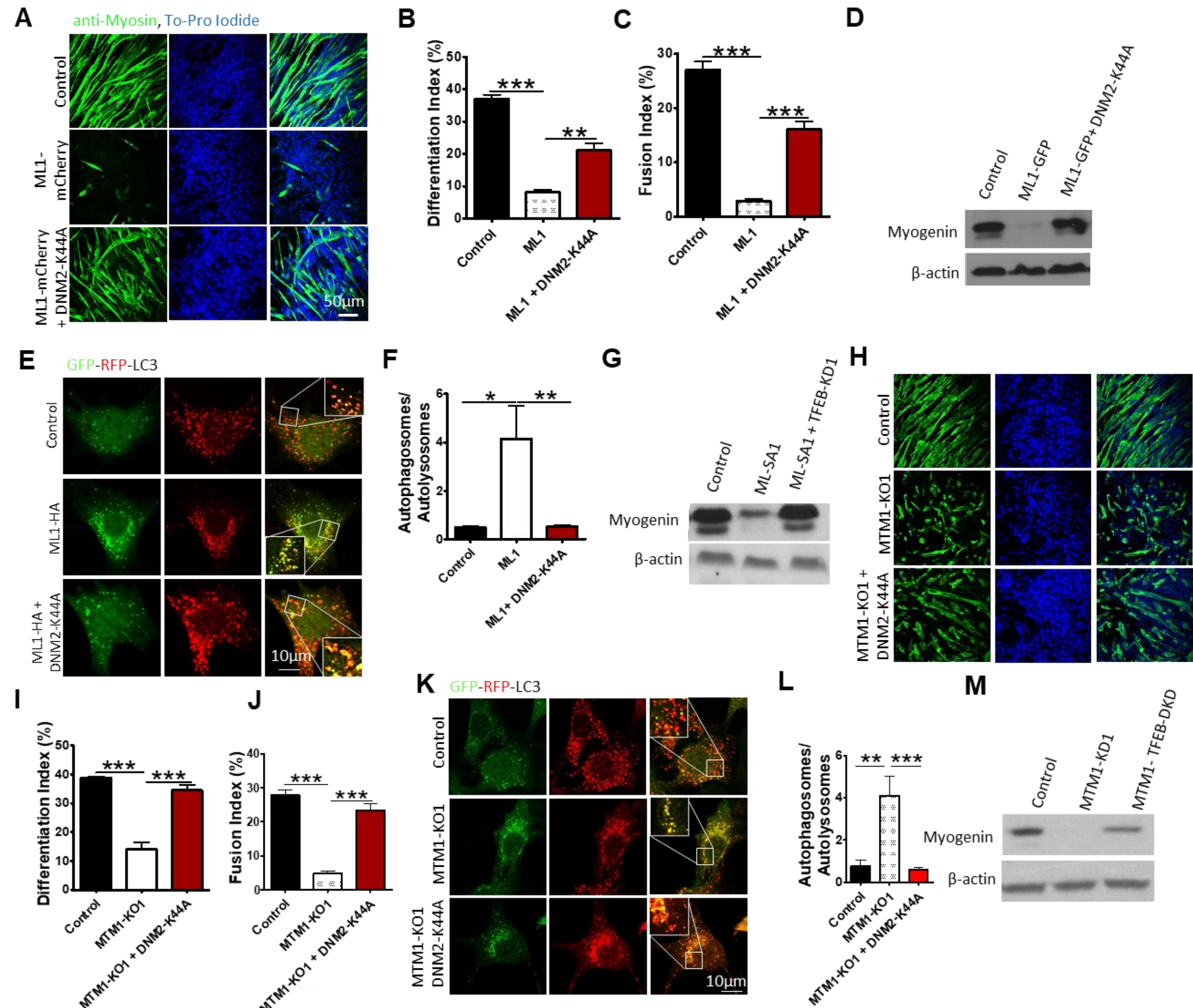

Figure 2.16: Suppressing DNM2 rescues the compromised muscle differentiation and autophagosome-lysosome fusion induced by TRPML1 upregulation and MTM1 deficiency. (A-C) Impaired muscle differentiation induced by TRPML1-mCherry overexpression was rescued by DNM2-DN. (D) DNM2-DN rescued the reduced myogenin induced by TRPML1 overexpression. (E, F) Impaired autophagosome-lysosome fusion induced by TRPML1-HA was corrected by DNM2-DN. (G) ML-SA1 reduced myogenin in C2C12 cells, and this was rescued by TFEB-KD. (H-J) Impaired muscle differentiation induced by MTM1 deficiency was rescued by DNM2-DN. (K, L) Impaired autophagosome-lysosome fusion induced by MTM1-KO was rescued by DNM2-DN. (M) Reduced myogenin expression in MTM1-KD cells were rescued by TFEB-KD. For differentiation and fusion index analysis, experiments were repeated three times with triplicated samples each time for all conditions. Totally, more than 10 fields were counted for each condition. For the mRFP-GFP-LC3 imaging, more than 20 cells were counted for each condition. The data represent the mean ± SEM. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$.

In addition, DNM2 overexpression inhibited autophagosome-lysosome fusion (Fig. 2.17A-B), muscle cell differentiation and fusion (Fig. 2.17C-E), and myogenin level (Fig. 2.17 F). Altogether, our data suggest that in XLMTM, MTM1 deficiency hyperactivates TRPML1 due to elevated PI3,5P2, further increasing DNM2 levels via TFEB. Eventually, increased DNM2 leads to impaired autophagosome-lysosome fusion and subsequent defects in muscle cell differentiation and fusion.

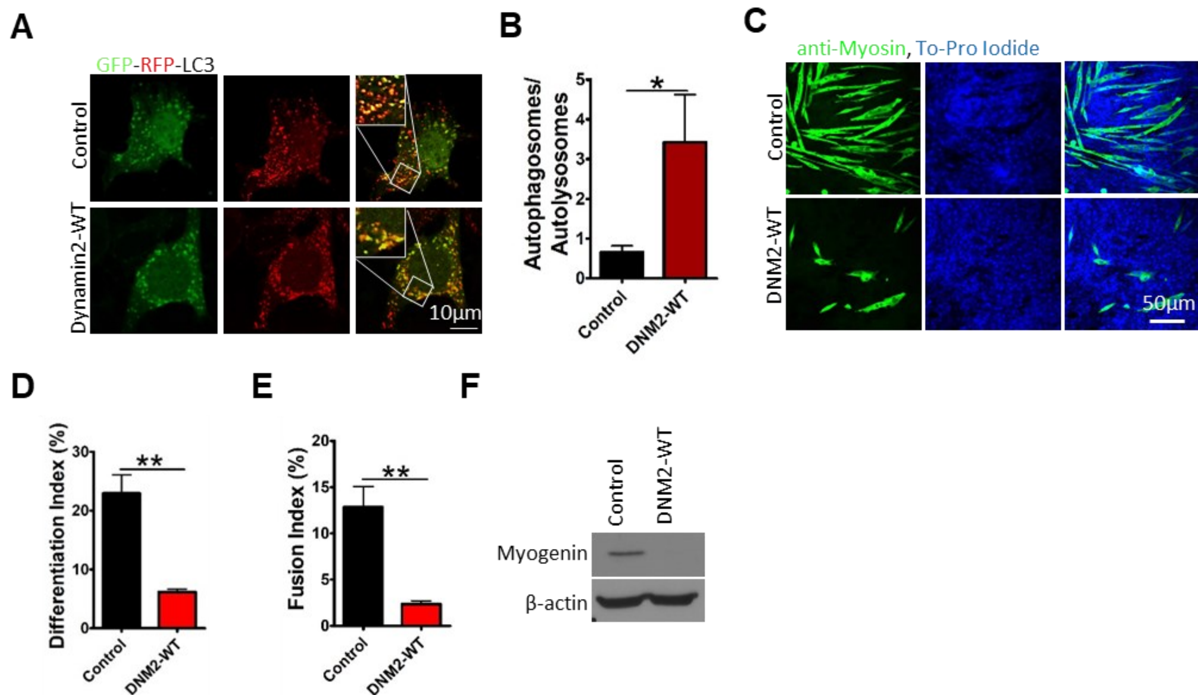

Figure 2.17: Dynamin 2 overexpression inhibits autophagosome-lysosome fusion and myogenesis. (A, B) Dynamin 2 overexpression inhibited autophagosome-lysosome fusion. The data represent the mean ± SEM (n = 3). (C-E) Dynamin 2 overexpression inhibited cell differentiation and fusion. The data represent the mean ± SEM (n = 3). (F) Dynamin 2 overexpression inhibited reduced myogenin level. *: P < 0.05; **: P < 0.01.

2.4 Discussion

The lysosome plays a vital role in autophagy that is involved in the development and differentiation of mammals (Mizushima & Levine, 2010). In this study, we found that upregulation of the lysosomal ion channel, TRPML1, compromised muscle differentiation by inhibiting the fusion between autophagosomes and lysosomes. This was rescued by promoting autophagosome-lysosome fusion with STX17 overexpression. Consistently inhibiting autophagosome and lysosome fusion by deleting STX17 (Itakura et al., 2012b) causes defective muscle differentiation. More importantly, deficiency in MTM1 led to elevated TRPML1 activity, impaired autophagosome-lysosome fusion, and defective muscle differentiation. The phenotypes in MTM1 deficient muscle cells were rescued by inhibiting TRPML1 or expressing STX17. Mechanistically, either TRPML1 upregulation or MTM1 deficiency caused elevated DNM2 by activating TFEB. Suppressing DNM2 rescued compromised autophagosome-lysosome fusion and impaired muscle differentiation induced by MTM1 deficiency and TRPML1 upregulation. These results suggest that hyperactivation of TRPML1 and subsequently elevated DNM2 levels due to the loss of MTM1 could contribute to muscle defect in XLMTM and that inhibiting TRPML1 and DNM2 may be a strategy to cure XLMTM.

Previous studies have generated controversial results regarding TRPML1's role in autophagy. Earlier studies suggest that TRPML1 deletion impaired autophagic flux with characteristics of accumulated autophagosomes (T. Sun et al., 2011; Venkatachalam et al., 2015). On the contrary, a later study suggests that TRPML1 upregulation causes autophagosome accumulation by increasing autophagosome formation (Scotto Rosato et al., 2019a). Interestingly, the present study and a recent report (Qi et al., 2021) demonstrate that TRPML1 upregulation also causes autophagosome accumulation by compromising the fusion between autophagosomes and lysosomes. The observation of autophagosome accumulation induced by both upregulation and downregulation of TRPML1 is in line with recent reports showing that both excess and inadequate PI3,5P2 levels on lysosomes hamper autophagy (Ferguson et al., 2009; Hasegawa et al., 2016; S. Martin et al., 2013). Therefore, we suspect that PI3,5P2/TRPML1 may regulate multiple steps of autophagy, and an optimal level of lysosomal PI3,5P2/TRPML1 is likely essential for the cell to maintain high autophagic flux and adapt to environment changes (P. Huang et al., 2020).

Earlier studies showed that autophagy is required for muscle maintenance (Masiero et al., 2009; Mizushima & Levine, 2010). New evidence suggested a role of the canonical autophagy

pathway in myogenesis (Fortini et al., 2016a; McMillan & Quadrilatero, 2014; Rashid et al., 2015; Sin et al., 2016). Consistently, we also show that inhibiting autophagy by ablating STX17 in C2C12 cells results in compromised muscle differentiation and fusion (Figure 2.4). Therefore, the canonical autophagy pathway (Codogno et al., 2011) is important not only for muscle maintenance (Masiero et al., 2009; Mizushima & Levine, 2010; Raben et al., 2008) but also for muscle development (Fortini et al., 2016a; McMillan & Quadrilatero, 2014; Rashid et al., 2015)

Previous studies also suggested that MTM1 deficiency leads to autophagosome accumulation (Al-Qusairi et al., 2009; Childers et al., 2014; Fetalvero et al., 2013a). In this study, we further suggested that the autophagosome accumulation-induced MTM1 deficiency is attributed to the inhibition of autophagosome-lysosome fusion, likely by activating the TRPML1-TFEB-DNM2 pathway. Currently, it is unclear how elevated DNM2 inhibits autophagosome-lysosome fusion. Given that DNM2 functions as an actin-remodeling protein (Gu et al., 2010; Mooren et al., 2009; Ochoa et al., 2000) and regulates actin-dependent intracellular vesicular trafficking actin/cortactin-associated vesicular trafficking (Gu et al., 2010; Hasegawa et al., 2016; J. Y. Lee et al., 2010; Mooren et al., 2009; Nicoziani et al., 2000), DNM2 may inhibit autophagosome-lysosome fusion by impairing actin-associated mechanism. DNM2 may also inhibit muscle autophagosome-lysosome fusion by regulating the SNARE system, as in yeast (Peters et al., 2004). These await further investigation.

The role of MTM1 in muscle development is also a long-standing debate. *In vivo* studies suggest that MTM1 is essential for skeletal muscle maintenance but not for muscle development in mice (Sarikaya et al., 2022). Recently, a comprehensive longitudinal study of MTM1-KO mice suggested an essential role of MTM1 in muscle development (Sarikaya et al., 2022). Myogenesis. In this study, we show that MTM1 deficiency results in impaired muscle differentiation (Dowling et al., 2010; Fetalvero et al., 2013a; Masiero et al., 2009; Vergne & Deretic, 2010) by elevating TRPML1 activity and subsequent DNM2 expression. Given that during myogenic differentiation, the expression of MTM1 (Buj-Bello et al., 2002; Gupta et al., 2013; Hnia et al., 2011) and MTM1-related proteins (Buj-Bello et al., 2002; Gupta et al., 2013) is elevated, and some myotubularin-related 1 proteins have been implicated in myogenesis, we reason that the lack of myogenesis phenotypes in MTM1 mutant mice could be attributed to the compensation of other MTM1 related phosphatases (Hnia et al., 2011; S. A. Kim et al., 2002; Sarikaya et al., 2022).

Adult skeletal muscle has a remarkable regenerative capacity attributable to satellite cells (SCs) (Jan et al., 2016; Le Grand & Rudnicki, 2007). In response to muscle injury or certain diseases, quiescent SCs become activated and then proliferate and differentiate into the mature skeletal muscle to recover muscle function(Bryson-Richardson & Currie, 2008; Le Grand & Rudnicki, 2007). Because during regeneration, many of the same transcription factors that control embryonic myogenesis are redeployed to regulate SC differentiation and muscle regeneration (Bryson-Richardson & Currie, 2008; van Wijngaarden et al., 1969; Wagers & Conboy, 2005), our study suggests that the MTM1-TRPML1-TFEB-DNM2 pathway may also play an important role in muscle regeneration in XLMTM (Buj-Bello et al., 2002; Childers et al., 2014; Hasegawa et al., 2016; Spiro et al., 1966; van Wijngaarden et al., 1969). Altogether, we suspect that MTM1-TRPML1-TFEB-DNM2 is essential not only for skeletal muscle maintenance (Buj-Bello et al., 2002) but also for myogenesis. MTM1 activation leads to a reduction in TRPML1 activity in myoblasts due to a decrease in PI3,5P2 level, promoting muscle biogenesis and regeneration. In XLMTM, PI3,5P2 accumulation due to MTM1 deficiency may hyperactivate TRPML1, inhibiting autophagy, myogenesis, and regeneration.

CHAPTER 3: TRPML1 REGULATES AMYOTROPHIC LATERAL SCLEROSIS (ALS) PROGRESSION BY MODULATING AUTOPHAGIC FLUX IN MICROGLIA

3.1 Rationale and Hypothesis

Amyotrophic lateral sclerosis is a late-onset fatal neurodegenerative disorder characterized by the selective loss of both upper and lower motoneurons in the CNS. Currently, the precise molecular mechanisms leading to motoneuron degeneration in ALS are unclear, and no cure has yet been developed for ALS. However, it is generally accepted that abnormal aggregation and toxic gain of function of the mutant SOD1 proteins are important contributors to the neuronal death process (Chiò et al., 2020; Teng et al., 2012). While it is believed that the disease onset takes place inside motoneurons, different cell types in the CNS may control the progression during distinct disease phases (Boillée, Vande Velde et al., 2006; Clement et al., 2003; J. Lee et al., 2016). Microglia are activated by infection, neuronal injury, and inflammation. Because neuroinflammation is one of the most striking hallmarks of ALS, much effort has been devoted to understanding the role of microglia in ALS progression. Although it is widely accepted that microglia contributed to ALS pathogenesis, the molecular mechanisms underlying microglial-neuronal interactions that lead to motoneuron degeneration remain elusive. Cumulative evidence suggests that mSOD1 accelerates ALS pathogenesis by inducing microglial Interleukin 1 β (IL-1β) (Y. Hu et al., 2017; Meissner et al., 2010) and tumor necrosis factor α (TNF-α) secretion (Frakes et al., 2014; Hu et al., 2017; Y. Liu et al., 2009), which further causes neurotoxicity (Takeuchi et al., 2006).

Mounting evidence has suggested that autophagy regulates ALS progression by affecting both motoneurons and microglia. In motoneurons, autophagy is important for the secretion and clearance of abnormal aggregation of mutant SOD1 protein (Al-Chalabi & Hardiman, 2013; Redmann et al., 2017; Tedeschi et al., 2019). In microglia, $SOD1^{G93A}$ results in the inhibition of autophagic flux (Massenzio et al., 2018a), potentially by producing excessive ROS, thereby promoting TNF-α and (Beers et al., 2006; Henkel et al., 2006; Q. Li et al., 2011; D. C. Wu et al., 2006; Xiao et al., 2007) IL-1β secretion and subsequent motoneuron damage (Joshi et al., 2019; X. Peng et al., 2019a; Saitoh et al., 2008a, 2008b). A direct link between autophagy genes and ALS

has also been suggested. For example, mutations in p62 account for approximately 1% of ALS cases (Le Ber et al., 2013; Rea et al., 2014). Similarly, autophagic core proteins OPTN and TBK1 (Toth & Atkin, 2018; Wong & Holzbaur, 2014) and C9orf72 (Balendra & Isaacs, 2018; Sellier et al., 2016; Sivadasan et al., 2016; Sullivan et al., 2016) and UBQLN2 (Deng et al., 2011) have also been associated with ALS. Therefore, autophagy has become an attractive therapeutic target for preventing and treating ALS-associated neurodegeneration. Because 1) microglia $SOD1^{G93A}$ results in the inhibition of autophagic flux (Fabbrizio et al., 2017a; Massenzio et al., 2018a; Rudnick et al., 2017; X. Zhang et al., 2011), potentially by producing excessive ROS (Joshi et al., 2019; Obrador et al., 2020), 2) TRPML1 is activated by elevated ROS (X. Zhang, Cheng, et al., 2016), and 3) TRPML1 activation inhibits autophagic flux (Qi et al., 2021; Xing et al., 2022). Therefore, we hypothesized that in $SOD1^{G93A}$ microglia, elevated ROS activates TRPML1 to promote IL-1β and TNF-α release by disrupting autophagic flux, thereby promoting ALS progression.

In this study, we reported that $SOD1^{G93A}$ progressively activates TRPML1 in microglia. $SOD1^{G93A}$-mediated TRPML1 activation is eliminated by the antioxidant N-acetylcysteine (NAC). $SOD1^{G93A}$ microglia also display higher mTORC1 activities and impaired autophagic flux, which are inhibited by downregulating TRPML1 and NAC. Because ROS activates TRPML1 and TRPML1 activation increases mTORC1, we suggest that $SOD1^{G93A}$ activates TRPML1 by increasing ROS, subsequently increasing mTORC1 and inhibiting autophagic flux. We also showed that increased cytokine release from $SOD1^{G93A}$ microglia was corrected by inhibiting TRPML1. Altogether, our data suggest that TRPML1 plays an essential role in microglial activation in $SOD1^{G93A}$ microglia and that the ROS-TRPML1-mTORC1-autophagy-cytokine release pathway may be an important factor regulating ALS progression.

3.2 Methods

3.2.1 Mice

The Protocol was approved by the Dalhousie University Committee on Laboratory Animals (UCLA). All experiments were carried out according to the Canadian Council on Animal Care (CCAC) principles and the National Institute of Health Guide to the Care and Use of Experimental Animals. Mice were obtained from Jackson Lab (catalog # 004435), C57Bl/6 transgenic hemizygous mice which express human $SOD1^{G93A}$ (B6.Cg-Tg(SOD1-G93A)1Gur/J). Mice were housed in the LSRI animal facility in an Allentown IVC rack with irradiated corncob bedding, autoclaved RMH 3500 feed, and municipal (chlorinated tap) water with 3% Colyte. In accordance with the literature, mice were monitored for disease symptoms. Typically, SOD1-G93A mice develop symptoms after 3 months (91 days). For experiments, we used adult mice distributed in two categories, i.e., asymptomatic (30 days-60 days) and symptomatic (90 days and onwards). Mice were euthanized when they reached a score of 3.

Evaluation of Disease Progression in the $SOD1^{G93A}$:

Disease scoring was established to monitor mice for symptoms and humane endpoint.

0: No apparent signs

1: Weakness or trembling of hindlimbs during tail suspension or weakness in one hind limb with a normal gait.

2: Swaying/weakness of back end when walking and/or dragging any part of foot/feet along cage bottom or table.

3: Moderate to severe weakness of both hind limbs or paralysis of one hind limb or inability to perform rearing motion

4: Mouse cannot right itself within 15 seconds after being placed on either side or functional paralysis of both hind limbs.

3.2.2 Chemicals

Chemicals used in this study include ML-SA1 (44820, Princeton BioMolecular Research Inc), ML-SI1 (BML-CR112-0010, Enzo Life Sciences Inc), Ionomycin (11932-1, Cayman), Bafilomycin A1 (1334, Tocris Bioscience Inc and N-Acetyl-L-cysteine (A7250-5G).

ML1-SA1, ML2-SA2, and ML3-SA1 were kind gifts of Dr. Christian Grimm, Walther Straub Institute of Pharmacology and Toxicology, Faculty of Medicine, Ludwig-Maximilians-University, Munich, Germany (Kilpatrick, Yates, et al., 2016; Plesch et al., 2018; Spix et al., 2022).

3.2.3 Primary Microglia Culture

As reported, the spinal cord dissection protocol was followed (Yip et al., 2009). Briefly, 1- to 4-month-old mice were anesthetized with Pentobarbital (100mg/kg) and transcardially perfused with ice-cold 0.9% Saline. The whole spinal cord was dissected and placed in chilled Hibernate A Media supplemented with 1 xB27 and 2 mM Glutamine. The spinal cord was cut into small pieces before resuspending into a dissociation solution composed of Papain and Hibernate A Media (25U/ml protein) and incubated at 30°c for 30 minutes on a shaking platform. After dissociation, media was removed, and DMEM supplemented with 2 mM GlutaMAX, 15% FBS, and 1% P/S (culture media, named DFP) was added. Tissue was triturated until a turbid single-cell suspension was visible before centrifuging at 400xg for 5 minutes. Media was changed after 24 hours of culture to allow attachment of mixed glial cells.

A confluent cell layer can be observed after 10 days, while floating microglia are visible after 2-3 weeks of culture. Media was changed 24 hours prior to trypsin separation. The mixed glial culture was washed with PBS before the addition of Trypsin-DMEM (0.25% Trypsin diluted in serum-free DMEM, 1:3 ratio) and placed in an incubator at 37°C and 5% CO_2. After 30 minutes, the upper mixed glial layer, composed mainly of astrocytes and fibroblasts, separating from the lower pure microglia cell layer, can be observed under the microscope (Fig 1A). The detachment process takes approximately 30-45 minutes; the detached layer was discarded, and 0.25% Trypsin was added to obtain pure microglia cells.

3.2.4 Cell Culture and Lentiviral Transduction

HEK293T and BV-2 cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% Penicillin/Streptomycin. Cells were maintained at 37°c in a 5% CO_2 incubator. For certain imaging experiments, cells were seeded on Poly-L-Lysine coated slides 24 hours before the experiment.

To prepare the BV-2 cell line expressing $SOD1^{WT}$ and $SOD1^{G93A}$, we used plv-AcGFP-SOD1-WT (add gene #27138) and plv-AcGFP-SOD1-G93A (addgene #27142), a kind gift from Dr. Elizabeth Fisher. Lentivirus was made using a three-plasmid packaging system. Briefly, vectors

were co-transfected into HEK293T cells along with psPAX2, and pMD2.G. Lentivirus was harvested 48h after transfection and filtered. BV-2 cells were transduced with collected lentivirus media for 24 hours, and the efficiency was determined by GFP-positive cells.

For knockdown studies, we constructed pLL3.7 shRNA vectors (addgene #11795) encoding shRNAs that target *mTRPML1*. The sequences for mouse *TRPML1* were: KD1, 5'-GCCTCAAGTACTTCTTTATGA-3'; KD2, 5'- GGTCACTGTGCAGCTCATTCT-3'. Lentivirus was made using a 2nd generation lentiviral packaging system as mentioned above.

3.2.5 Calcium Imaging

Cells were loaded with 1mM Fura-2 AM and 20% Pluronic F-127 (1:1 ratio). Calcium imaging was accomplished using a UV source connected to a monochromator system. Fluorescence was recorded at two wavelengths (340 nm to 380 nm) using the EasyRatioPro system (PTI) software. The ratio of F340 and F380 was used to determine intracellular calcium release from lysosomes. TRPML1 channel-mediated calcium released was measured in low calcium buffer composed of 145 mM NaCl, 5 mM KCl, 3 mM MgCl2, 10 mM glucose, 1 mM EGTA, and 20 mM HEPES (7.4 pH) ML-SA1 (50 µM). Ionomycin (1 µM) was added at the conclusion of all experiments to induce a maximal response for comparison. Cells were plated 24-72 hours before imaging.

3.2.6 Western Blot

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors. Total protein in cell lysate was measured using BCA Protein Assay (or Smith Assay) to normalize different samples. Cell lysates (1-4 mg/ml) were reconstituted in 4x Laemmli Sample Buffer and boiled to linearize the proteins. Samples were resolved by SDS-PAGE and subjected to immunoblotting. Standard Western analysis methods analyzed proteins.

3.2.7 ELISA

Pro-inflammatory cytokines, TNF-α, and IL-1β, were detected from cell culture media. Cells were cultured at equal density in 24-well plates, and media was changed 24 hours and 3 days before collection. Total protein was also calculated using BCA at the end of the experiment to normalize ELISA results.

TNF-α and IL-1β quantities were measured using Mouse TNF-alpha Quantikine ELISA Kit (MTA00B) and Mouse IL-1 beta/IL-1F2 Quantikine ELISA Kit (MLB006) by R&D systems, following the manufacturer's protocol.

3.2.8 ROS Detection

Reactive oxygen species in microglia cells were detected using the Cellular ROS Assay Kit (Deep Red) (ab186029), following the manufacturer's protocol. Briefly, cells cultured on glass cover slips were treated with NAC (5mM) 30 minutes prior to staining with deep red dye. Images were acquired using an upright confocal microscope (LSM710, Zeiss) with 40X oil-immersion objective and captured using ZEN2009 software (Zeiss).

3.2.9 Data Analysis

Data are presented as mean ± SEM from at least three independent experiments. Statistical comparisons were made using a two-way analysis of variance (ANOVA) or Student's t-test. P values of < 0.05 were considered statistically significant; *: $P < 0.05$; **: $P < 0.01$, ***: $P < 0.005$.

3.3 Results

3.3.1 Isolation of Functional Primary Microglia from Adult Mice Spinal Cord

Primary microglia were isolated from mice's spinal cord at different ages and disease stages. The main concern is purifying microglial cells from a mixed glial culture containing astrocytes, oligodendrocytes, and fibroblasts. Figure 3.1A demonstrates the sequence of microglia isolation by mild trypsinization from mixed confluent glial culture after separation of the upper layer containing astrocytes and isolated microglial cells after trypsinization (40 minutes). The purity of microglia was also confirmed using Iba-1, microglial marker, GFAP, astrocytes marker, and staining (Fig 3.1B). After isolation, detecting whether cells were functionally viable was important, so we adopted LPS-mediated microglial activation. Treatment with LPS (100ng/ml) induced amoeboid morphology of cells, as reported before (Y. He et al., 2021; Saura et al., 2003). This suggested that microglia isolated with our protocol can be used for further experiments.

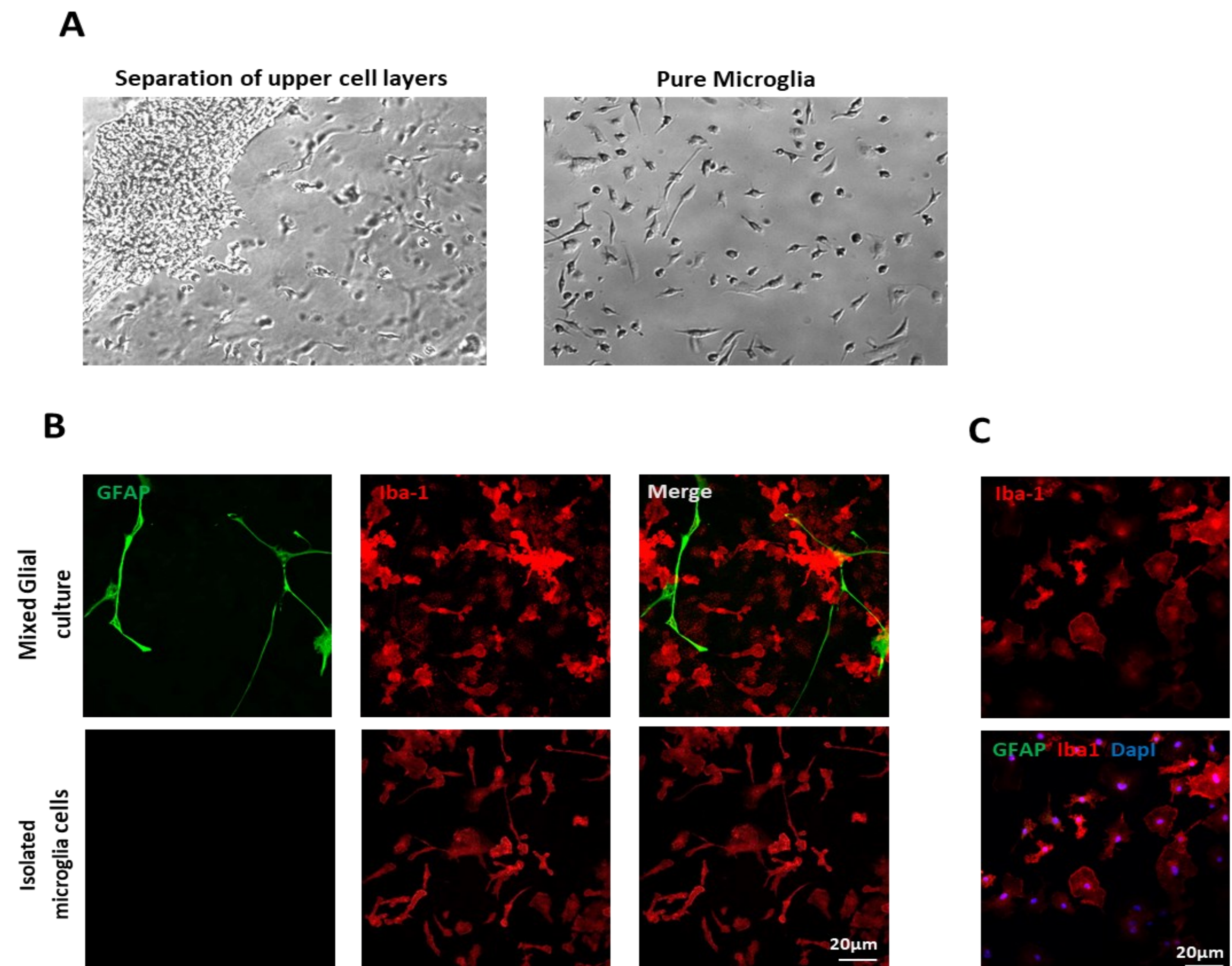

Figure 3.1: Purified Primary Microglia cells from adult mice. (A) Microglia separated from mixed glial culture. 90% of the purified cells were microglial cells. (B) Majority of the isolated cells were microglia as shown by the Iba-1, microglia marker, and GFAP, astrocyte marker, staining. (C) Primary microglia responded to LPS stimulation.

3.3.2 Mutant SOD1 Activates TRPML1 in Primary Microglia as the Disease Progresses

To test whether TRPML in microglia is involved in ALS progression, we measured TRPML1 activity in primary microglial cells from $SOD1^{G93A}$ mice (Fig. 3.2). TRPML1 activity was examined using Fura-2-based Ca^{2+} imaging because TRPML1 activation induces lysosomal Ca^{2+} efflux. ML-SA1, a synthetic TRPML agonist, activates TRPML1 (Dong et al., 2010; W. Wang et al., 2015b). As in Figure 3.2 E-H, in the absence of LPS, TRPML1 activity was remarkedly upregulated in $SOD1^{G93A}$ microglia at both late asymptomatic and symptomatic stages but not early asymptomatic stages P30 (Fig 3.2A-C). When microglia were activated using LPS, a bacterial endotoxin, TRPML1 activity was increased in $SOD1^{G93A}$ at all three stages (Fig. 3.2B, E, and H). Additionally, LPS was also able to increase ML-SA1-induced response in WT, indicating that TRPML1 could be involved in the activated state of microglia. Increased TRPML activity could be attributed to elevated endolysosomal Ca^{2+} content or cell status changes. To exclude this possibility, Fura-2 signals induced by Ionomycin were measured. We found comparable Ionomycin-induced Fura-2 signals between WT and $SOD1^{G93A}$ microglial cells (Fig. 3.2C, F, and I), suggesting that the more considerable TRPML activity in $SOD1^{G93A}$ microglia was not attributed to a change in cell status induced by $SOD1^{G93A}$. Altogether, these data indicate that $SOD1^{G93A}$ generates a toxic factor that activates TRPML in microglia as the disease progresses. LPS can also induce this harmful factor at an early stage of the disease.

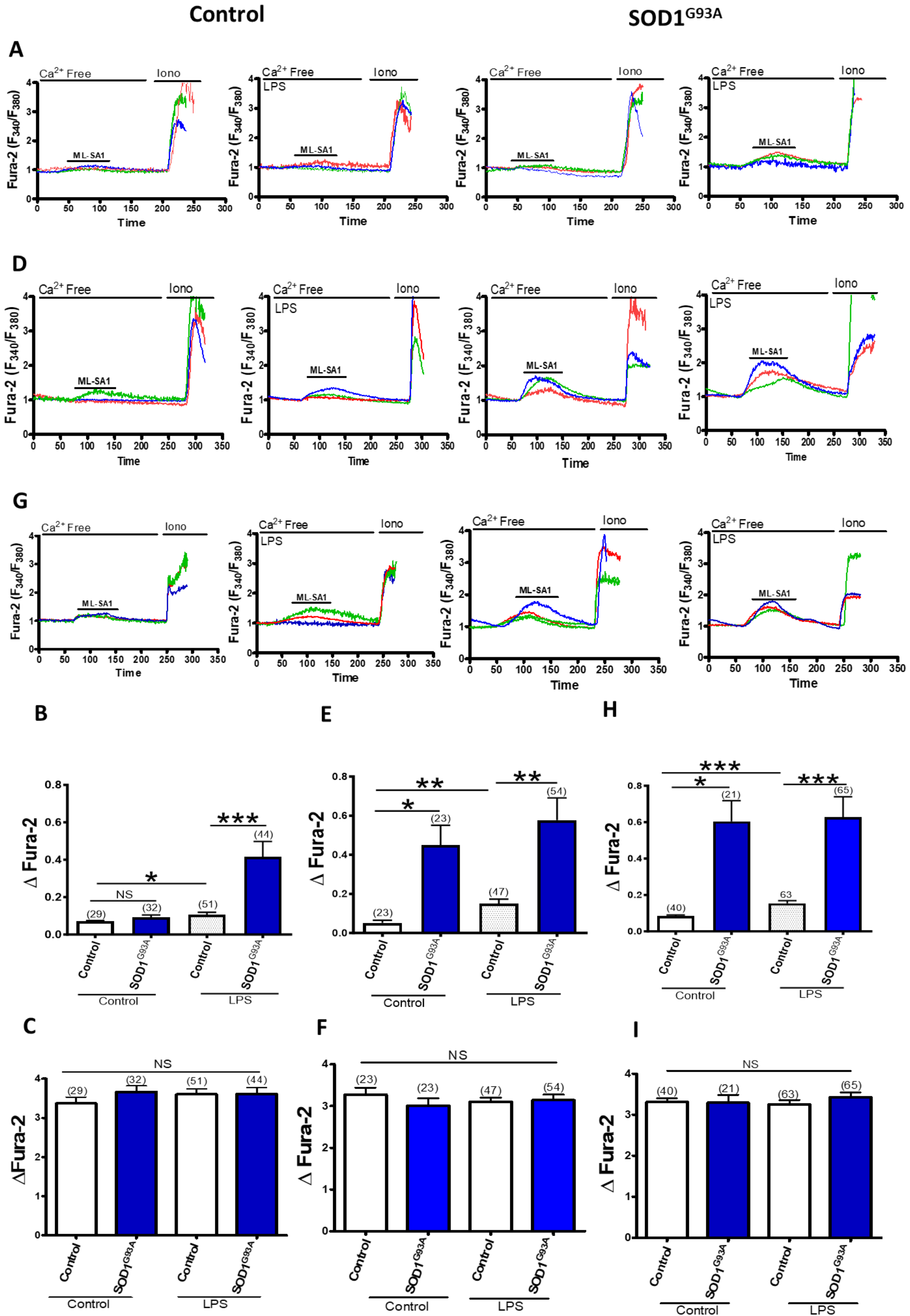
Control
SOD1G93A
A
D
G
B
E
H
C
F
I
Ca2+ Free
Iono
LPS
ML-SA1
Fura-2 (F340/F380)
Time
Δ Fura-2
NS
Control
SOD1G93A
LPS

Figure 3.2: TRPML1 activity is increased in SOD1^{G93A} microglia. (A-C) A synthetic TRPML agonist ML-SA1 induced comparable Ca^{2+} signals between the control and SOD1^{G93A} primary microglia at the early asymptomatic stage (P30). However, when microglia were stimulated with Lipopolysaccharide (LPS) (100 ug/ml, 30 min) SOD1^{G93A} significantly increased TRPML activity. (D-F) Late asymptomatic (P60) showed increased Ca^{2+} response. (G-I) symptomatic (P100) stages TRPML activity was dramatically increased in SOD1^{G93A} microglia in both the absence and presence of LPS. Experiments were repeated three times for each condition. The data represent the mean ± SEM. NS: not significant; *: $P < 0.05$, **: $P < 0.01$; ***: $P < 0.005$.

To differentiate which TRPML is regulated by SOD1^{G93A} in microglia, we adopted three specific TRPML agonists targeting TRPML1-3, respectively. We found that in symptomatic microglia, TRPML2-specific agonist ML2-SA1 (Plesch et al., 2018) induced little Ca^{2+} response in both wild-type (WT) and SOD1^{G93A} microglia (Fig. 3.2A-B). TRPML3-specific agonist ML3-SA1 (Spix et al., 2022) generated Fura-2 signals. However, a comparable Ca^{2+} response was detected between WT and SOD1^{G93A} microglia. Notably, TRPML1-specific agonist ML1-SA1 (Spix et al., 2022) induced significantly larger Ca^{2+} responses in SOD1^{G93A} microglial cells compared with WT microglial cells. In addition, ionomycin-induced Fura-2 signals were comparable between WT and SOD1^{G93A} microglial cells (Fig. 3.2C). Altogether, we suggest that SOD1^{G93A} activates TRPML1 but not TRPML2 and TRPML3 in microglia.

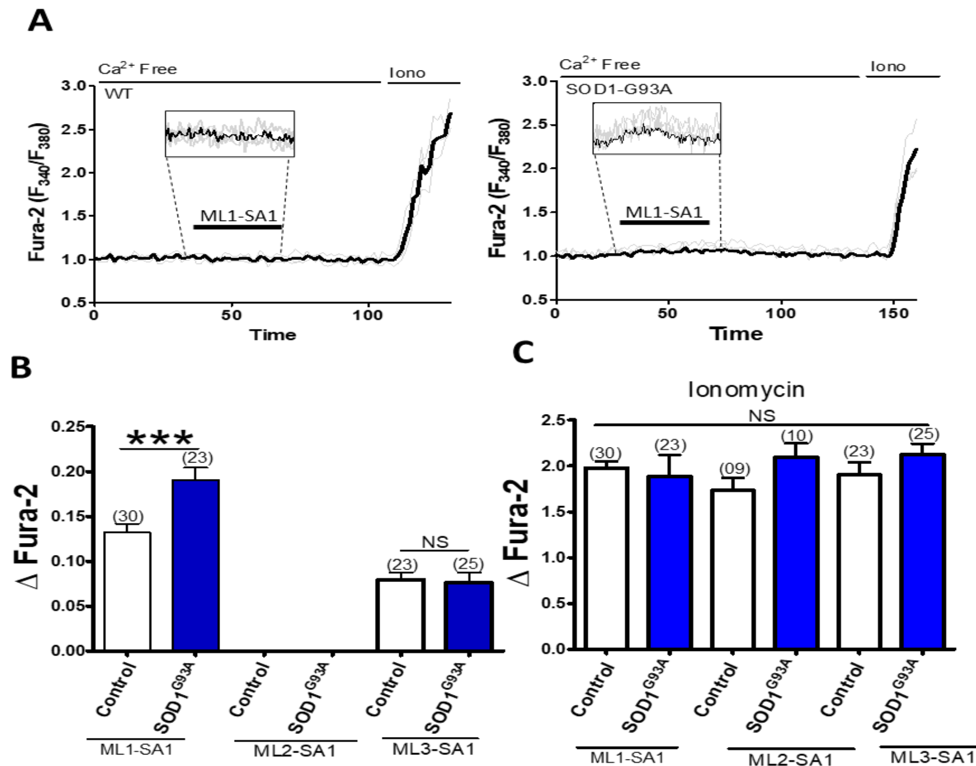

Figure 3.3: TRPML1 is responsible for increased Ca^{2+} response in SOD1^{G93A} microglia. (A) Fura 2-AM showed increased endogenous TRPML1 response in SOD1^{G93A} primary cells. (B) TRPML1 agonist ML1-SA1 induced increased response in mutant SOD1^{G93A}, TRPML2 agonist ML2-SA1 induced no response whereas TRPML3 agonist had no difference between SOD1^{G93A} and WT. (C) Ionomycin-induced Fura-2 signals were comparable between WT and SOD1^{G93A} microglial cells. Experiments were repeated three times for each condition. The data represent the mean ± SEM. NS: not significant; *: $P < 0.05$, **: $P < 0.01$; ***: $P < 0.005$.

Furthermore, a commonly used microglial cell line BV-2 cell was adopted to consolidate our conclusions. Consistent with those in primary microglial cells, ML-SA1 induced larger Fura-2 responses in SOD1^{G93A}-expressing cells than cells expressing SOD1^{WT} without altering endolysosomal Ca^{2+} content and cell status (Fig 3.4A-B). To confirm ML-SA1 induced response was through TRPML1, we knockdown TRPML1 in SOD1^{G93A} expressing BV-2 cells and observed that KD abolished ML-SA1 induced response in SOD1^{G93A}. Collectively, these data suggest that TRPML1 is upregulated in SOD1^{G93A} microglia. We also suggest that intrinsic but not extrinsic SOD1^{G93A} activates TRPML1 in microglia.

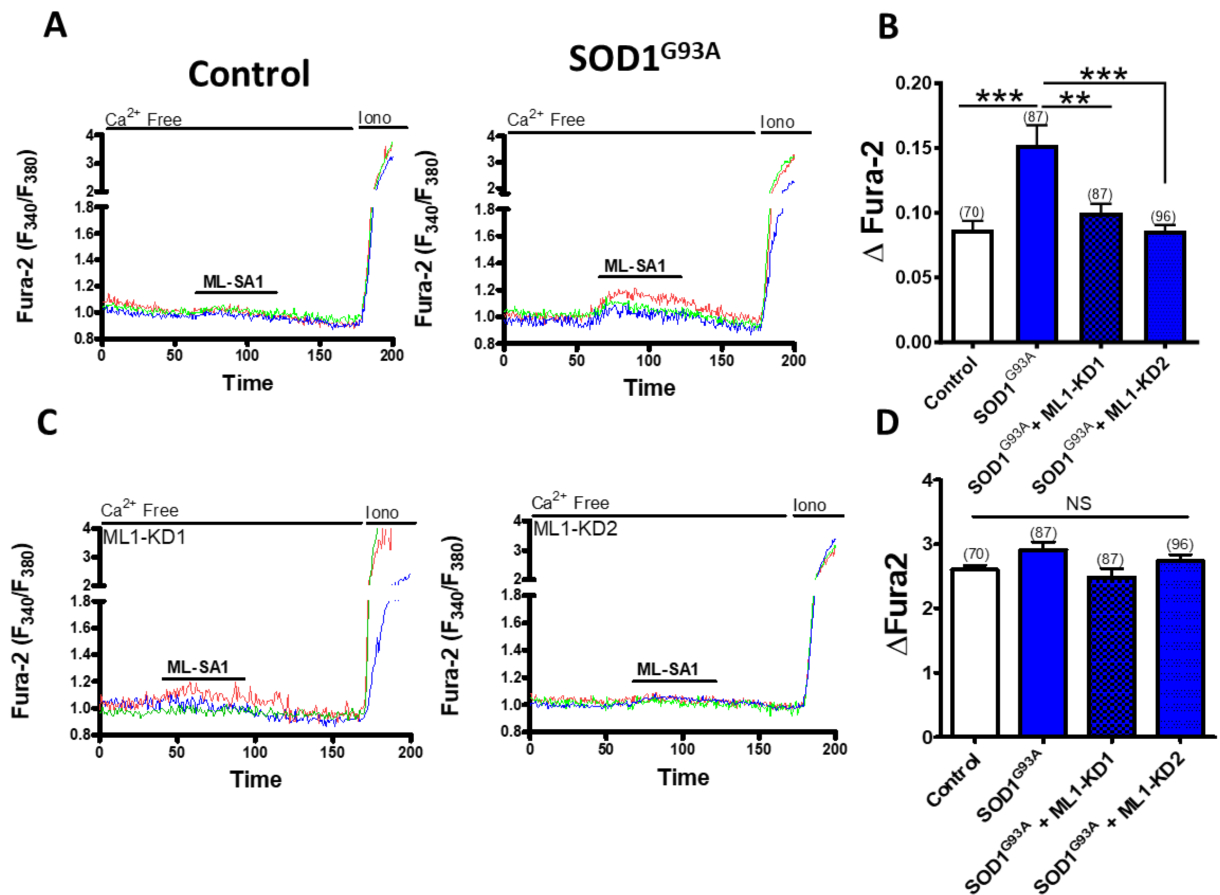

Figure 3.4: TRPML1 knockdown abolishes increased TRPML1 response in SOD1^{G93A} microglia. (A) SOD1^{G93A} expression induced larger Fura-2 responses to ML-SA1 compared with SOD1WT. (B-C) Increased Ca^{2+} signals induced by SOD1^{G93A} were eliminated by TRPML1-KD. (D) Comparable Ca^{2+} signal was induced by ionomycin between control and SOD1$^{G93A.}$ The experiment was repeated three times. The data represent the mean ± SEM. NS: not significant; *: $P < 0.05$, **: $P < 0.01$; ***: $P < 0.005$.

3.3.3 SOD1^{G93A} Activates TRPML1 by Increasing ROS

The data above suggest that SOD1^{G93A} in microglia induces a toxic factor to activate TRPML1. Recently, PI3,5P2 (Dong et al., 2010) and ROS (M. Xu et al., 2019; X. Zhang, Cheng, et al., 2016) have been identified as two endogenous agonists of TRPML1. Because 1) ROS actively affect microglia-associated neurodegenerative diseases through their role as pro-inflammatory molecules and modulators of pro-inflammatory processes (Bordt & Polster, 2014; Rojo et al., 2014; Shi et al., 2015a); 2) ROS is involved in microglia activation (Bordt & Polster, 2014; Joshi et al., 2019; Simpson & Oliver, 2020) 3) ROS is progressively elevated in SOD1^{G93A} microglia, and microglia of SOD1^{G93A} mice secrete more ROS and NO than wild-type microglia and kill co-cultured motoneurons (Beers et al., 2006; D. C. Wu et al., 2006), 4) ROS is also induced by LPS (De Bont et al., 2006; Y. He et al., 2021), 5) ROS activates only TRPML1 but not TRPML2 and TRPML3 (X. Zhang, Cheng, et al., 2016)whereas all three TRPMLs are activated by PI3,5P2 (Dong et al., 2010), we suspected that ROS is the toxic factor that activates TRPML1 in SOD1^{G93A} microglia. In support of our hypothesis, we compared ROS levels between the control and SOD1^{G93A} microglia. As shown in Figure 5, SOD1^{G93A} induced significantly higher ROS signals than the control in both primary microglia (Fig 3.5A) and BV-2 cells (Fig 3.5B).

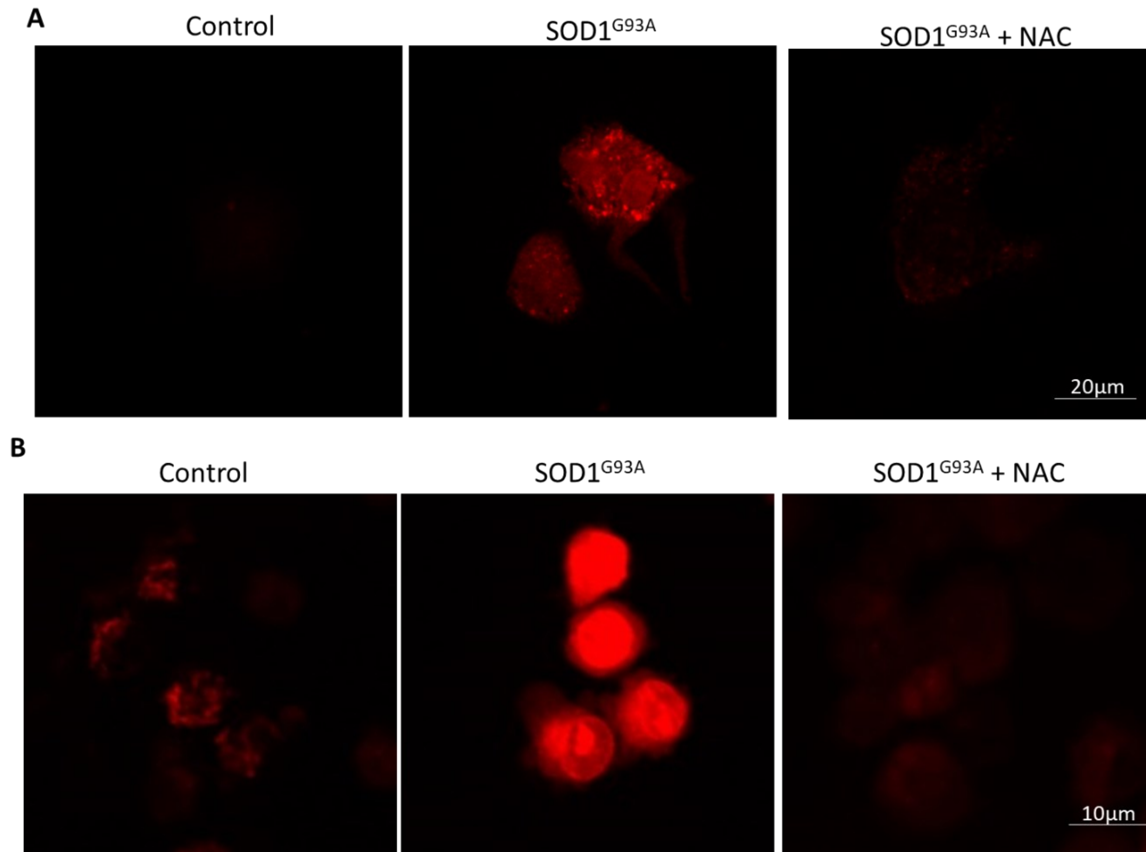

Figure 3.5: SOD1^{G93A} increases ROS in microglia. (A) Primary cells isolated from symptomatic SOD1^{G93A} mice showed increased fluorescence intensity of ROS detection dye, which was suppressed by 30 minutes treatement of NAC (5mM), an antioxidant, treatment. (B) BV-2 cells expressing SOD1^{G93A} had notably increased fluorescence of ROS detection dye than SOD1^{WT}. The data is representative of three independent experiments.

Because we had suggested that ROS activates TRPML1 in SOD1^{G93A}, we conducted Fura-2 imaging after treating SOD1^{G93A} cells with NAC. We found NAC was able to reduce TRPML1 response in both BV-2 cells significantly (Fig 3.6A-C) and primary microglia (Fig 3.6-D-F) expressing SOD1^{G93A}. Altogether, we suggest that SOD1^{G93A} accumulation in microglia increases ROS (Joshi et al., 2019), subsequently activating TRPML1.

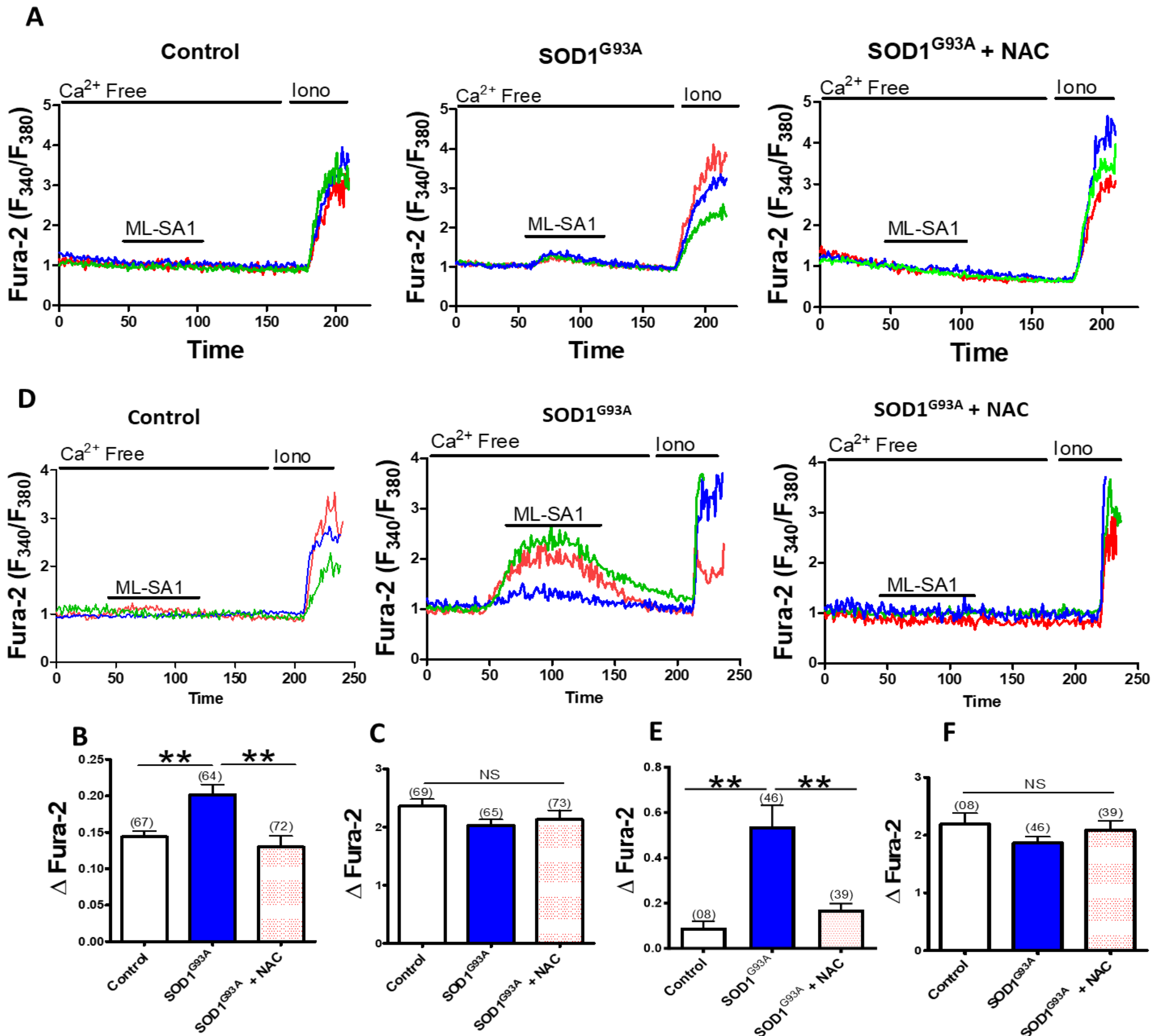

Figure 3.6: NAC reduces the ROS induces TRPML1 activity in SOD1^{G93A} microglia. (A-C) BV-2 cells expressing SOD1^{G93A} were pretreated with NAC before Fura-2 imaging, the cells showed decreased TRPML1 activity (D-F) Pretreatment of primary microglia cells with NAC reduced TRPML1 activity in SOD1^{G93A} cells. The experiment was repeated three times. The data represent the mean ± SEM. NS: not significant; *: $P < 0.05$, **: $P < 0.01$; ***: $P < 0.005$.

3.3.4 SOD1^{G93A} Activates TRPML1 to Increase MTORC1 Activity

TRPML1 has been shown to upregulate mTORC1 activity in stress conditions (X. Sun et al., 2018; M. Xu et al., 2019). Similarly, mTORC1 has been shown to be upregulated in SOD1^{G93A} astrocytes (Granatiero et al., 2021), and its dysregulation has been reported in neurodegenerative disorders (Querfurth & Lee, 2021). To test whether mTORC1 is dysregulated in microglia, we used the phosphorylation state of P70S6-kinase, a downstream target of mTORC1. Increased phosphorylated P70S6K was observed in SOD1^{G93A} expressing BV-2 cells (Fig. 3.7A) and primary

microglia (Fig. 3.7C). This was abolished by TRPML inhibitor ML-SI1 (Fig 3.7A-F) and TRPML1-KD (Fig 3.7B and 3.7E). NAC rescued hyperactive mTORC1 induced by SOD1G93A (Fig. 3.7C and 3.7F). These results suggest that mTORC1 is upregulated in SOD1^{G93A} mutant microglia due to hyperactive TRPML1.

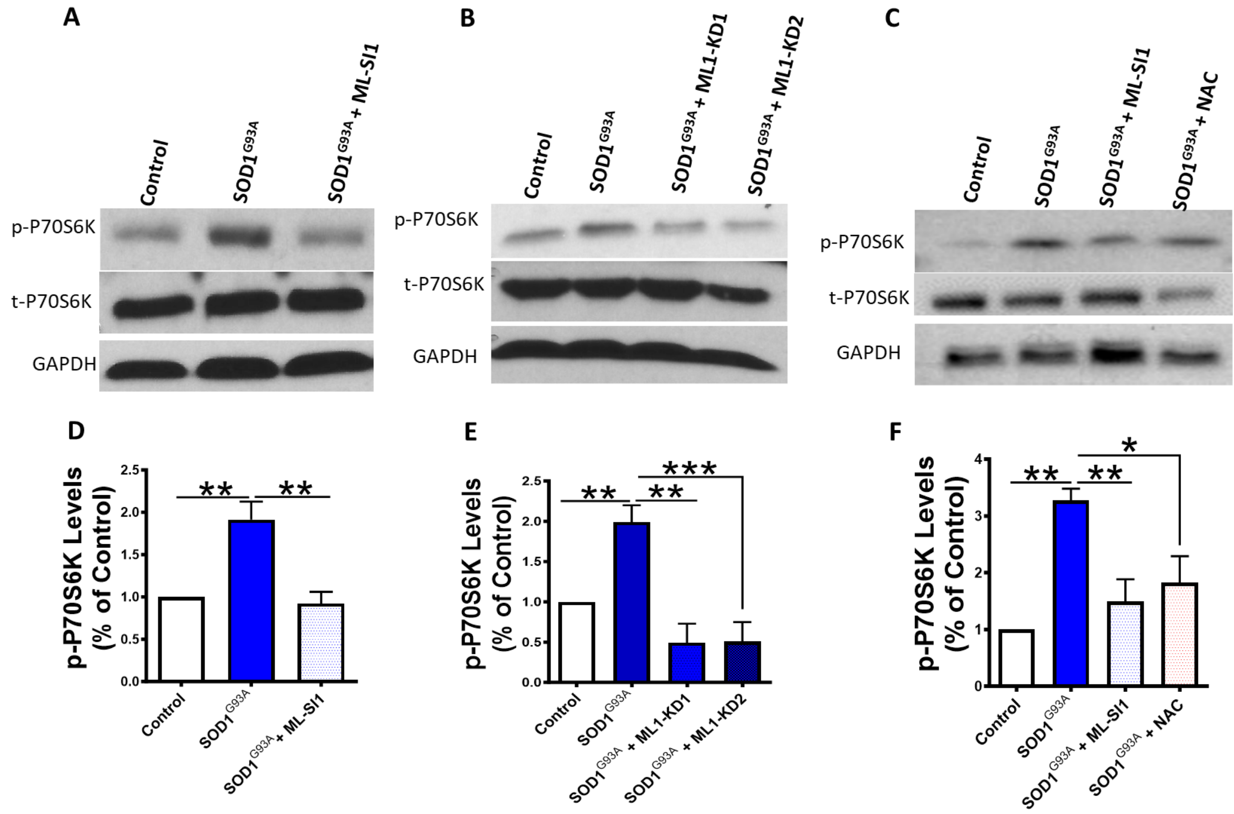

Figure 3.7: TRPML1 inhibition reduces increased mTORC1 in SOD1^{G93A} microglia. (A-B) TRPML1 inhibition through ML-SI1 (10µM) and TRPML1-knocdown reduced hyperactive mTORC1 in SOD1^{G93A} expressing BV-2. (C) Both ML-SI1 (10µM) and NAC (5mM) reduced mTORC1 in primary microglia isolated from symptomatic SOD1^{G93A} mice. (D) Statistical representation of figure A. (E) Statistical representation of figure B. (F) Statistical representation of figure C. Cells were treated with DMEM (no amino acid), 10% FBS for 30 minutes prior to protein collection. The data represent the mean ± SEM. NS: not significant; *: $P < 0.05$, **: $P < 0.01$; ***: $P < 0.005$.

3.3.5 Inhibition of TRPML1 Rescues Autophagic Defects in SOD1^{G93A}

LC3 lipidation to LC3-II enables it to recruit ubiquitin p62 to degradative cargo. LC3-II is present on the autophagosomal membrane; once autophagosomes fuse with lysosomes to form autolysosomes, LC3-II is degraded by the lysosomal hydrolyses (Nath et al., 2014; Tanida et al., 2008). Therefore, increased LC3-II is indicative of defective autophagy and accumulation of LC3-II positive vesicles in cells. In addition, mTOR suppresses autophagy initiation. The increased mTORC1 in SOD1^{G93A} microglia suggests reduced autophagy. Surprisingly, LC3-II levels were

increased by SOD1^{G93A}, and this was eliminated by both ML-SI1 and TRPML1-KD (Fig 3.8A-B), suggesting that SOD1^{G93A} may inhibit autophagosome maturation and degradation but not autophagy induction in a TRPML1-dependent manner. Additionally, P62 accumulation is associated with autophagy inhibition (Bjørkøy et al., 2009; Kumar et al., 2022) and ALS-linked pathology (Trist et al., 2022). We monitored P62 levels in the SOD1^{G93A} microglia cells and found an accumulation of P62. This phenotype was rescued by inhibition of TRPML1 through knockdown and ML-SI1 (Fig 8C-D).

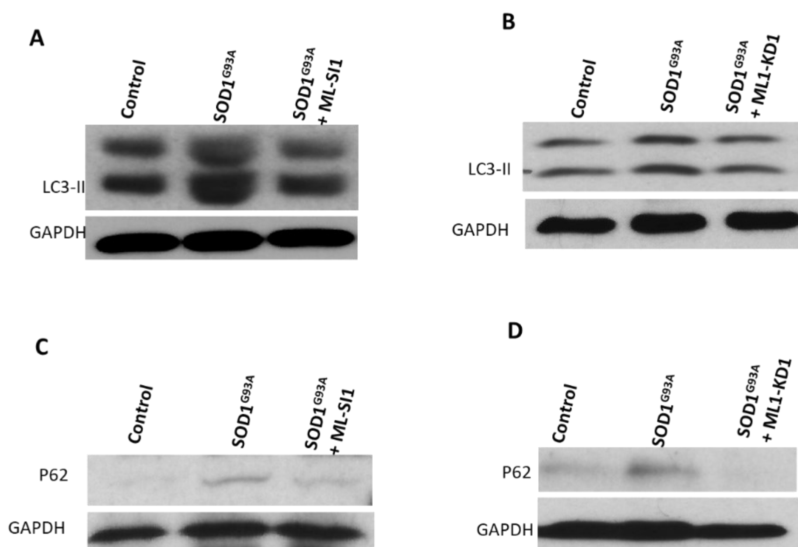

Figure 3.8: Defective autophagy in SOD1^{G93A} is corrected by TRPML1 inhibition (A-B) Increased LC3-II levels in SOD1^{G93A} were corrected by ML-SI1 (10µM) and TRPML1-KD1 (C-D) P62 accumulation in SOD1^{G93A} was rescued by ML-SI1 (10µM) treatment and TRPML1-KD1. Cells were treated with DMEM (no AA), 10% FBS for 30 minutes prior to protein collection.

To further test whether elevated TRPML1 in ALS inhibited autophagy, we adopted an autophagic flux assay by measuring LC3-II levels in the presence of bafilomycin A1, which inhibits autolysosomal degradation as well as the fusion of lysosomes with autophagosomes (S. W. Kim et al., 2022; Mauvezin & Neufeld, 2015). We found that SOD1^{G93A} impaired autophagic flux BV-2 cells (Fig. 3.9A-B), and both ML-SI1 and NAC corrected this.

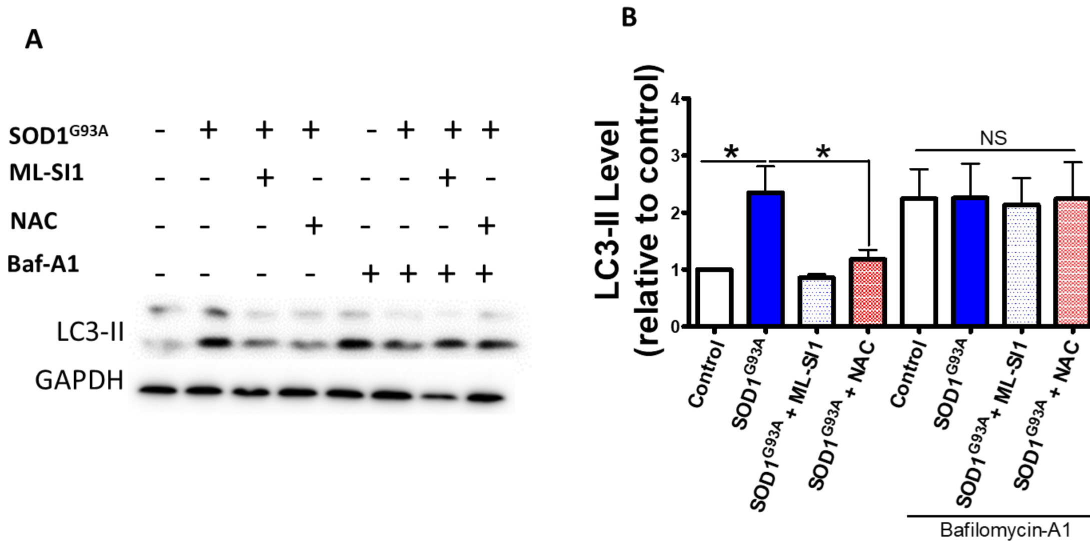

Figure 3.9: Inhibition of TRPML1 and ROS corrects autophagic defects in SOD1^{G93A} microglia. (A) BV-2 cells stably expressing SOD1^{G93A} were treated with ML-SI1 (10µM) and NAC (5mM), both reduced increased LC3-II levels which were comparable to Baf-A1 (200nM) treated SOD1^{WT} and SOD1^{G93A}. (B) Statistical representation of A. Cells were treated with DMEM (no AA), 10% FBS for 30 minutes prior to protein collection. The data represent the mean ± SEM. NS: not significant; *: $P < 0.05$, **: $P < 0.01$.

3.3.6 TRPML1 Regulates Pro-Inflammatory Cytokine Release from SOD1^{G93A} Microglia

Microglia secret neuroprotective or neurotoxic cytokine in response to specific stimuli (Orihuela et al., 2016). The phenotypical polarization is critical for neurons, and the classic "M1" phenotype is associated with pro-inflammatory mediators, while "M2" polarization promotes neural repair and anti-inflammation (Guo et al., 2022; Orihuela et al., 2016). It has been noted that autophagy is a crucial regulator of the immune system and microglial polarization (Redmann et al., 2017). Decreased autophagy has emerged as a significant factor in brain diseases (Vivekanantham et al., 2015; Wyss-Coray & Rogers, 2012). In ALS, microglial activation has been shown to modulate disease progression. Neurotoxic microglia in later disease stages secrete pro-inflammatory effectors (Frakes et al., 2014), leading to the activation of other cells, including astrocytes, and rapid motor neuron death (Banerjee et al., n.d.; Joshi et al., 2019; J. Lee et al., 2016). Because in microglia, TRPML1 is progressively activated by ROS as the disease develops, and because TRPML1 activation inhibits autophagy, we suspected that TRPML1 might be involved in microglial activation and cytokine secretion in SOD1^{G93A} microglia. We monitored TNF-α and IL-1β levels in the culture medium to test our hypothesis. Symptomatic SOD1^{G93A} microglia released more cytokines than the control, which was eliminated by ML-SI1 (Fig. 3.10A-B). Similarly, in BV-2 cells, SOD1^{G93A} significantly increased cytokine release, which was

corrected by ML-SI1 (Fig 3.10C-D) and TRPML1-KD (Fig 3.10 E). Taken together, these data suggest that SOD1^{G93A} promotes cytokine release by activating TRPML1.

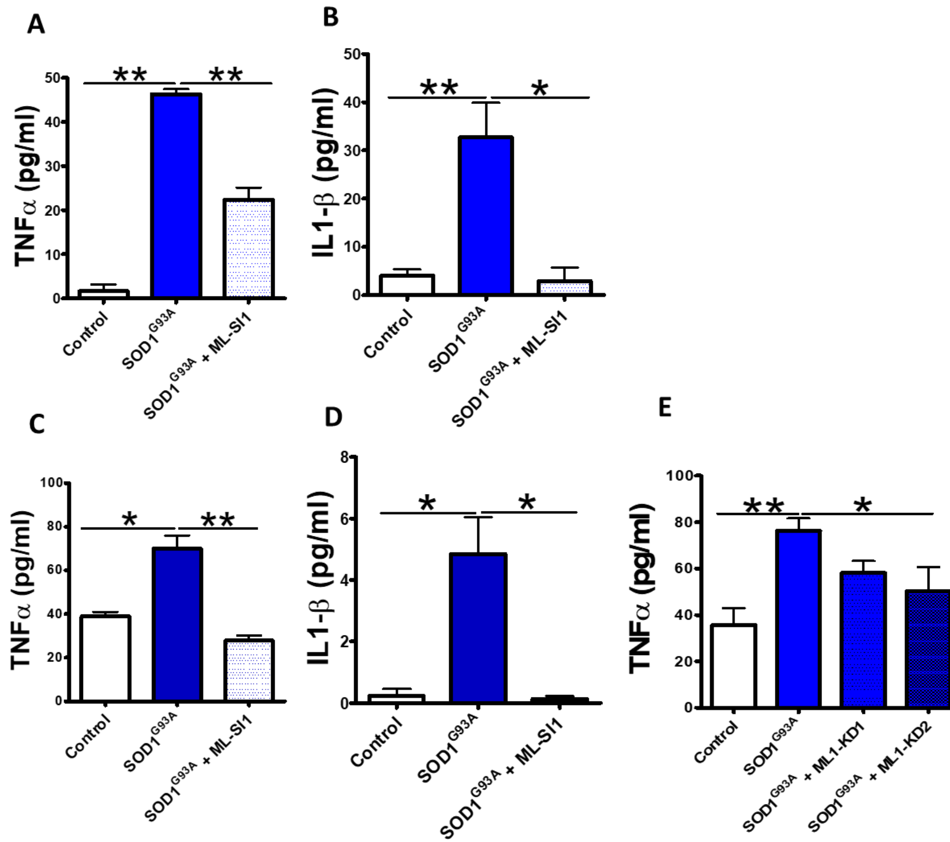

Figure 3.10: Elevated pro-inflammatory cytokines in SOD1^{G93A} are reduced by TRPML1 inhibition. (A-B) Both pro-inflammatory cytokine TNFα and IL-1β were increased in primary microglia from symptomatic SOD1^{G93A} which were rescued by ML-SI1 (10µM). The cytokines were measured from cell culture media collected after 3 days. (C-D) SOD1^{G93A} increased TNFα and IL-1β in BV-2 cells, which was corrected by ML-SI1 and (D) TNFα was also decreased by TRPML1-KD. The cytokines were measured from cell culture media collected after 24 hours. The data represent the mean ± SEM. NS: not significant; *: $P < 0.05$, **: $P < 0.01$; ***: $P < 0.005$.

3.4 Discussion

In the nervous system, microglial cells outnumber neurons by a 10:1 ratio (Herculano-Houzel, 2014). They provide the necessary support to neural function under normal conditions. However, microglia could be neurotoxic depending on cell conditions and stimuli (Rasband, 2016). Earlier studies have provided evidence of microglial toxicity in the symptomatic stage of ALS, i.e., activated microglia increase the release of pro-inflammatory cytokines, accelerating disease progression (Alexianu et al., 2001; Andrus et al., 1998; Frakes et al., 2014; Henkel et al., 2006; Meissner et al., 2010). By using an ALS mouse model and a cell model, in this study, we found that SOD1^{G93A} progressively activates TRPML1 in microglia due to an increase in ROS. TRPML1 activation increases mTORC1 activity, thereby suppressing autophagic flux. This further facilitates cytokine release. Inhibition of TRPML1 rescued the increased cytokine release in SOD1^{G93A} microglia by correcting the impaired autophagy.

The neurotoxic potential of mSOD1 microglia in ALS has been well documented. Early studies suggest that mSOD1 released from motoneuron leads to microglial mSOD1 accumulation, and this further promotes cytokine release from microglia to facilitate ALS progression (Frakes et al., 2014; J. Lee et al., 2016; Meissner et al., 2010). Later studies highlighted that microglia are activated in later disease stages rather than early, as the conditional expression of mSOD1 in microglia did not cause disease onset (Boillée, Yamanaka, et al., 2006) or *in vitro* co-culture of neonatal SOD1^{G93A} microglia with motor neurons did not cause neurotoxicity (Frakes et al., 2014). In agreement with these findings, we observed that, in the absence of motorneurons, microglial SOD1^{G93A} is sufficient to activate microglia and causes cytokine release in a TRPML1-dependent manner. One of the important features of ALS is a progressive disease (Boillée, Vande Velde, et al., 2006; Huber & Yeo, 2022). Using the SOD1^{G93A} mouse model, we observed that TRPML1 was activated at late asymptomatic and symptomatic disease stages but not early asymptomatic (Fig 3.2A-I). These data suggest that TRPML1 may be involved in the progressive development of ALS, i.e., progressive activation of TRPML1 by SOD1^{G93A} in microglia increases cytokine release to execute their neurotoxic effect.

Mechanistically, we suggest that SOD1^{G93A} activates TRPML1 by increasing ROS. Interestingly, TRPML1 is also activated by LPS (30 min) to a lesser extent than SOD1^{G93A}. Because LPS induces ROS within several minutes (Hsu & Wen, 2002a; Qin et al., 2005) and LPS-

activated WT microglia are cytotoxic to motoneurons owing to the production of ROS (Zhao et al., 2004), we believe that LPS (30 min) may activate TRPML1 by producing ROS (Hsu & Wen, 2002b; L. Li et al., 2010; Park et al., 2015). Considering that TRPML1 activation induces the M1 phenotype of macrophages (D. Chen et al., 2018), TRPML1 could be a player in microglial M1 polarization that is commonly induced by LPS (M. M. Jin et al., 2018; Ribeiro et al., 2018; Ye et al., 2020a). In alignment with this, LPS phenocopies TRPML1 (W. Wang et al., 2015a; Xing et al., 2022) (Fig. 3.7A-F and 3.9A-B) to increase mTORC1 activity and inhibit autophagy (M. M. Jin et al., 2018; Park et al., 2015; Ribeiro et al., 2018; Ye et al., 2020b).

Although our data suggest an intrinsic $SOD1^{G93A}$ activates TRPML1 in microglia, the contribution of extrinsic $SOD1^{G93A}$ may also make a significant contribution to the activation of TRPML1 in microglia *in vivo* because microglia reportedly internalize $SOD1^{G93A}$ that was released from motoneurons and other cell types (Sargsyan et al., 2009; Zhao et al., 2010). Treating WT microglia with purified $SOD1^{G93A}$ proteins may help address this question. Similarly, although we suggest that microglial TRPML1 is activated by ROS generated in microglia, under an *in vivo* setting, ROS generated by surrounding cells may also contribute to microglial TRPML1 activation and ALS progression. On the other hand, in addition to cytokines, free radicals generated by activated microglia may spread and initiate motoneuron injury by activating the signaling pathway in motoneurons (Xiao et al., 2007b; Zhao et al., 2004)

Although ALS-linked $SOD1^{G93A}$ typically increases the production and release of cytokines such as IL-1β (Joshi et al., 2019; Meissner et al., 2010) and TNF-α to damage motoneurons, it remains unclear how TNF-α and IL-1β are released to extracellular space. Emerging evidence suggests that autophagy/lysosome-based unconventional secretory pathway for extracellular delivery of IL-1β and TNF-α is attracting more attention. On the one hand, autophagy activity facilitates autolysosomal degradation of cytokines (Andrei et al., 1999a; Harris et al., 2011; Lopez-Castejon & Brough, 2011a), thus impaired autophagic flux increases cytokine release from microglia (Andrei et al., 1999a; Harris et al., 2011; Netea-Maier et al., 2016). In this scenario, autophagy-mediated exosome formation and release have taken a large part in controlling IL-1β and TNF-α secretion from microglia (X. Peng et al., 2019b; Pu et al., 2017; Shi et al., 2015b; Zheng et al., 2017). In agreement with this, a growing body of evidence has suggested increased exosomes in ALS tissues and microglia (McCluskey et al., 2022; Pinto et al., 2017; Silverman et al., 2019; Vaz et al., 2019). Therefore, $SOD1^{G93A}$ may activate TRPML1 to secrete IL-1β (Joshi et al., 2019;

Meissner et al., 2010) and TNF-α (Frakes et al., 2014; Joshi et al., 2019; Tortelli et al., 2020). On the other hand, cytokines can be translocated into lysosomes through the autophagy pathway (Andrei et al., 1999b; Lopez-Castejon & Brough, 2011b), where lysosomal enzymes process them. Mature TNF-α (Han et al., 2013; Jiang et al., 2016) and IL-1β (Andrei et al., 1999b; Lopez-Castejon & Brough, 2011c) are then released into the extracellular space via lysosomal exocytosis(Andrei et al., 2004; Monif et al., 2016; Rubartelli et al., 1990; Takenouchi et al., 2011). Interestingly, new evidence suggests that both exosome release (M. S. Kim et al., 2019; G. Li et al., 2019) and lysosomal exocytosis (Samie & Xu, 2014) are regulated by TRPML1. Therefore, more work must be done to determine which release pathway is regulated by TRPML1 in $SOD1^{G93A}$ microglia.

Increased release of cytokines from mSOD1 microglia could also be due to increased cytokine biogenesis induced by mSOD1. Nuclear factor-κB (NF-κB), a master regulator of inflammation, is a ubiquitously expressed transcription factor regulating the expression of many inflammatory genes upon various stimuli such as nutrient starvation, bacterial and viral infection, neuronal injury, inflammation, and oxidative stress. Previous studies suggest that the expression of IL-1β and TNF-α (Carta et al., 2013; M. M. Hu et al., 2014; Monif et al., 2016; Renard et al., 1997; Semino et al., 2018) is modulated by transcription factor NF-κB (Hayden & Ghosh, 2014; T. Liu et al., 2017), and NF-κB is upregulated in spinal cords of ALS patients and $SOD1^{G93A}$ mice (Frakes et al., 2014). By using primary microglia isolated from ALS mice, it is further suggested that NF-κB in adult microglia but not in neonatal microglia or astrocytes promotes motoneuron death in AL S (Crosio et al., 2011; Frakes et al., 2014; Xiao et al., 2007b), and selective NF-κB inhibition in ALS microglia but not in ALS astrocytes is sufficient to rescue motoneuron death (Frakes et al., 2014). Interestingly, impairing autophagy by deleting *ATG* genes results in enhanced NF-κB activation and proinflammatory cytokines production (X. Peng et al., 2019a; Saitoh et al., 2008b; Shi et al., 2015b). Because TRPML1 has been shown to direct the M1 phenotype of macrophages by modulating NF-κB nuclear translocation (D. Chen et al., 2018), and because TRPML1 inhibits autophagic flux, TRPML1 may also regulate cytokine biogenesis via activating NF-κB in microglia. In addition, microglial TRPML1 may also activate TFEB pathway (Crosio et al., 2011; Iyer et al., 2022; Medina et al., 2015b) to increase cytokine expression because TFEB is required for the expression and secretion of several pro-inflammatory cytokines in response to lipopolysaccharide (LPS) (Pastore et al., 2016) and the function of microglia (Iyer et al., 2022).

Overall, our data suggest that TRPML1 plays an important role in microglial activation. In ALS microglia, elevated ROS stimulates TRPML1, which activates microglia to release cytokines by disrupting autophagy; activated microglia with impaired autophagy, in turn, produce more ROS (Wolf et al., 2017), forming a positive feedback loop to promote cytokine release. Excess cytokines eventually lead to motoneuron degeneration in ALS. Therefore, inhibiting microglial TRPML1 could break the feedback loop, thereby alleviating ALS progression. Considering TRPML1 is ubiquitously expressed in all cell types, TRPML1 in motoneurons and other cell types, such as astrocytes, could also contribute to ALS progression.

CHAPTER 4: DISCUSSION

Earlier studies have suggested that TRPML1 positively regulates autophagy (Tedeschi, Petrozziello, Sisalli, et al., 2019a) by modulating the Ca^{2+} efflux from the lysosomal lumen. It is reported that Ca^{2+} release through TRPML1 binds to calmodulin to activate mTORC1 and to calcineurin to activate TFEB, respectively (R.-J. Li et al., 2016; Medina et al., 2015b; Scotto Rosato et al., 2019a). TRPML1 also regulates VPS34 activation by activating Ca^{2+}/calmodulin-dependent kinase (Scotto Rosato et al., 2019a). By regulating these Ca^{2+}-dependent downstream factors, TRPML1 controls autophagosome formation (Medina, 2023; Medina et al., 2015b), autophagosome-lysosome fusion (Scotto Rosato et al., 2019a), lysosomal degradation (Kendall & Holian, 2021), and autophagic lysosome reformation (L. Yu et al., 2010a). Moreover, we and others (J. H. Lee et al., 2015; Qi et al., 2021; Xing et al., 2022) suggest that TRPML1 negatively regulates autophagy when hyperactivated in disease conditions.

4.1 TRPML1 in Myopathies

Myogenesis is a fine-tuned process that governs skeletal muscle development and maintenance. Quiescent myoblasts respond to intrinsic or extrinsic stimuli and undergo a metabolic switch to activate proliferation, followed by differentiation (Bentzinger et al., 2012; D. Yu et al., 2021). Herein, autophagy fulfills the energy requirements of cells for differentiation and fusion (Le Grand & Rudnicki, 2007; Masiero et al., 2009; Raben et al., 2008; Xia et al., 2021). As we have shown, TRPML1 seems to be dispensable for muscle differentiation and fusion in normal conditions (Fig. 2.2). However, nutrient stress and disease conditions such as XLMTM (Fig 2.5 and Fig 2.11) lead to TRPML1 activation to inhibit autophagic flux, causing impaired muscle differentiation and fusion (X. Sun et al., 2018). This is in agreement with our previous studies showing that TRPML1 is activated explicitly in stress conditions but not in normal conditions.

Mechanistically, TRPML1 activation results in TFEB nuclear translocation and activation, which further induces the overexpression of DNM2. By an unknown mechanism, elevated DNM2 leads to defects in autophagosome-lysosome fusion and myoblast fusion (Fig. 2.16 and 2.17). This study not only shows how TRPML1 activation negatively regulates autophagy and myogenesis but also suggests a pathological mechanism of XLMTM, potentially offering a new therapeutic target for the disease, i.e., inhibiting TRPML1.

In addition to XLMTM, our studies also suggest that TRPML1 inhibition is a potential approach to mitigate centronuclear myopathies (CNM) and muscle defects that are associated with DNM2 overexpression or gain-of-function mutations (Gómez-Oca et al., 2022). Similarly, decreased autophagosome-lysosome fusion has been reported in Nonaka myopathy, characterized by increased ROS (Cho et al., 2017; T. Zhang et al., 2022, 2023). We predict that TRPML1 could be involved in the disease.

At present, no treatment for XLMTM is available, and preclinical trials are being conducted based on *MTM1* gene therapy (Childers et al., 2014), PI3KC2B inhibition (Sabha et al., 2016), and dynamin 2 downregulation (Cowling et al., 2014; Tasfaout et al., 2017, 2018). Interestingly, recent studies have suggested that two clinical drugs, estradiol and tamoxifen, could be beneficial to XLMTM (Gayi et al., 2018; Maani et al., 2018; McMillin et al., 2022), and both estradiol (Rühl et al., 2021) and tamoxifen (our unpublished data) inhibit TRPML1, suggesting a role of TRPML1 in estradiol- and tamoxifen-based XLMTM treatment. Moreover, because of the importance of TRPML1 in myopathies, some industries are interested in developing drugs targeting TRPML1. For example, Casma Therapeutics announced in 2022 that they are using a TRPML1-TFEB model in muscle therapies.

4.2 TRPML1 in Neurodegenerative Disorders

Lysosomal Ca^{2+} storage regulates the cellular process by timed and tuned Ca^{2+} efflux to the cytosol, and dysregulation of lysosomal Ca^{2+} has been associated with neurotoxicity. Abnormal lysosomal Ca^{2+} homeostasis causes autophagic defects, which are predominant pathological hallmarks of proteinopathic neurodegenerative disorders (Koh et al., 2019; Santoni et al., 2020; Udayar et al., 2022) such as Alzheimer's disease, Parkinson's disease, and ALS which are characterized by toxic protein accumulation. In Alzheimer's disease, hyperactivation of endogenous TRPML1 has been suggested to cause autophagic impairment in the PS1 deficit model due to abnormal Ca^{2+} efflux from lysosomes (J. H. Lee et al., 2015). Similarly, two models of Parkinson's disease (i.e., mutations in glucocerebrosidase (GBA-1), a lysosomal enzyme, and leucine-rich repeat kinase 2 (LRRK2), an endolysosomal protein) have shown depleted lysosomal Ca^{2+} levels and eventual defective toxic protein clearance (Hockey et al., 2015; Kilpatrick, Magalhaes, et al., 2016). These suggest that TRPML1 is probably hyperactive in both GBA-1 and

LRRK2 deficient cells to deplete lysosomal Ca^{2+}, similar to what has been reported in the PS1-KO model of AD.

Lysosome and mitochondria modulate cellular homeostasis and constitute a functional relationship; hence, mitochondrial defects impact lysosomal function (Burbulla et al., 2017; Obrador et al., 2020; W. Peng et al., 2020a, 2020b; Plotegher & Duchen, 2017; Siow et al., 2022). For example, damaged mitochondrial results in elevated ROS (Joshi et al., 2019; Siow et al., 2022) and disturbed Ca^{2+} signaling in lysosomal storage disorders (Burbulla et al., 2017; Plotegher & Duchen, 2017). Because ROS is an endogenous agonist of TRPML1 (X. Zhang, Cheng, et al., 2016), TRPML1 hyperactivation and subsequent autophagic defect are potentially involved in the neurodegenerative disorders exhibiting mitochondrial dysfunction. Therefore, it would be interesting to study if TRPML1 is hyperactivated in dopaminergic neurons derived from PD patients that exhibit mitochondria-induced oxidative stress and autophagic defects (Burbulla et al., 2017). Because motor neurons (Chamberlain et al., 2021; Duan et al., 2010; Obrador et al., 2020; Pollari et al., 2014; Xie et al., 2015) and astrocytes (Birger et al., 2019; Harlan et al., 2016; Hoang et al., 2019) in ALS also exhibit oxidative stress and decreased autophagy (Bordt & Polster, 2014; Fabbrizio et al., 2017; Granatiero et al., 2021; Simpson & Oliver, 2020; D. C. Wu et al., 2006; Ye et al., 2020b), investigation of TRPML1 in both motoneurons and astrocytes would provide a whole picture of TRPML1 in ALS.

Various studies have suggested autophagy being a prospective target in ALS treatment; however, the reproducibility of these approaches and potential clinical application cannot be stipulated because both beneficial and detrimental effects of autophagy inducers have been implicated in ALS progression. On the one hand, inducing autophagy using rapamycin has been shown to exacerbate disease progression in the $SOD1^{G93A}$ mice model (X. Zhang et al., 2011). On the other hand, another autophagy inducer, Trehalose, delays the disease pathogenesis and reduces motor neuron degeneration in $SOD1^{G93A}$ (Castillo et al., 2013). These differences in therapy targeting autophagy may be attributed to the stage of the disease and neuroinflammation. However, because rapamycin directly activates TRPML1 (X. Zhang et al., 2019), the unfavorable effect of rapamycin could be due to hyperactivation of TRPML1 and subsequent impairment of autophagosome-lysosome fusion and neurinflammation. Another mTOR-dependent drug, tamoxifen, has been tested in the TDP43 model of ALS, where it showed promising results in

rodents (I. F. Wang et al., 2012). Our unpublished data revealed that tamoxifen acts as an antagonist of TRPML1, highlighting that tamoxifen may target TRPML1 to mitigate ALS. Due to the important role of TRPML1 in autophagy and neurodegeneration, some pharmaceuticals have announced preclinical trials for TRPML1 in neurodegenerative disorders. One example is Caraway Therapeutics' TRPML1 program, although conclusive results have not been reported. We believe that TRPML1 antagonists could be an option to cure some diseases.

Future Direction

Overall, we have suggested that TRPML1 is hyperactivated to impair autophagic flux in both XLMTM and ALS, subsequently contributing to the disease progression. Inhibiting TRPML1 could be an approach to mitigate the conditions. Despite all the results, certain limitations and future directions are worth mentioning.

For the XLMTM project, most of our experiments were done in C2C12, a mouse myoblast cell line. Data from primary satellite cells and *in vivo* models to reconfirm our findings will be essential. We also need to focus on blocking upstream of PI3,5P2, such as PIKfyve and mTORC1, to evaluate the contribution of PI3,5P2 accumulation and mTORC1 activation in this disease. It is also essential to test whether lysosomal pH or other ion channels, such as TRPML2 and TRPML3, are compromised in XLMTM.

For the ALS project, it is important to exclude the contribution of PI3,5P2 and test our hypothesis using a microglia-motoneuron coculture system and an *in vivo* animal model. Additionally, since LPS activates TRPML1, more work is needed to establish its role in microglial M1 polarization and cytokine transcription and secretion regulation.

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