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## Current concepts in the neuropathogenesis of mucopolidosis type IV

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### Abstract

Mucopolidosis type IV (MLIV) is an autosomal recessive, lysosomal storage disorder causing progressively severe intellectual disability, motor and speech deficits, retinal degeneration often culminating in blindness, and systemic disease causing a shortened lifespan. MLIV results from mutations in the gene *MCOLN1* encoding the transient receptor potential channel mucolipin-1. It is an ultra-rare disease and is currently known to affect just over 100 diagnosed individuals. The last decade has provided a wealth of research focused on understanding the role of the enigmatic mucolipin-1 protein in cell and brain function and how its absence causes disease. This review explores our current understanding of the mucolipin-1 protein in relation to neuropathogenesis in MLIV and describes recent findings implicating mucolipin-1's important role in mTOR (mechanistic target of rapamycin) and TFEB (transcription factor EB) signaling feedback loops as well as in the function of the greater endosomal/lysosomal system. In addition to addressing the vital role of mucolipin-1 in the brain, we also report new data on the question of whether haploinsufficiency as would be anticipated in *MCOLN1* heterozygotes is associated with any evidence of neuron dysfunction or disease. Greater insights into the role of mucolipin-1 in the nervous system can be expected to shed light not only on MLIV disease but also on numerous processes governing normal brain function.

### Graphical abstract

Mucopolidosis type IV (MLIV) is a rare, autosomal recessive, lysosomal storage disorder caused by mutations in the endosomal/lysosomal protein, mucolipin-1. This review explores the roles of

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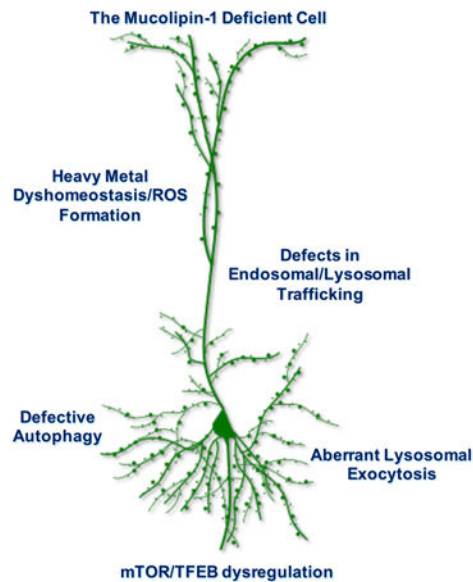
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aberrations to endosomal/lysosomal trafficking, autophagy, lysosomal exocytosis, mTOR (mammalian target of rapamycin) and TFEB (transcription factor EB) signaling, and heavy metal regulation in MLIV neuropathogenesis. We also share pilot data on the question of whether *Mcoln1* murine heterozygotes are associated with any evidence of neuron dysfunction or disease. The goal of this review is to provide greater insights into mucolipin-1's role in the nervous system and the multifaceted pathways contributing to MLIV disease pathogenesis.



## Keywords

TFEB; mTOR; heterozygote; lysosome; lysosomal disease; autophagy

## Introduction

### Lysosomal disorders and the mucopolidoses

While individually rare, when grouped together lysosomal disorders have a collective frequency of about 1 in every 7700 live births, presenting a significant health burden (Hoffmann and Mayatepek 2005). Lysosomal disorders were first described in the 1820s, yet it was not until the 20th century that the disorders were clinically differentiated and their underlying causes characterized. The discovery of lysosomes by Christian de Duve in 1955 was followed by the articulation of the lysosomal storage disease concept by H.G. Hers in 1965 (de Duve *et al.* 1955; Hers 1965). As a result, lysosomal disorders emerged as a class unifying a spectrum of over 50 individual diseases (Futerman and van Meer, 2004; Hers and Hoof, 1973; Platt and Walkley, 2004). Broadly described, most of these conditions result in intralysosomal accumulation of macromolecules arising from an inherited metabolic defect, hence the often used term “storage” disorders (Hers and Hoof 1973). Though tissue and organ penetrance varies across this class of diseases, the resulting lysosomal system dysfunction is often accompanied by neurological involvement (Platt and Walkley 2004).

One such lysosomal disease exhibiting severe neurological manifestations is the autosomal recessive disorder, mucopolipidosis type IV (Slaugenhaupt *et al.* 1999).

In 1974, a young male presenting with corneal clouding was first described as having a new variant of mucopolipidosis, termed MLIV (Berman *et al.* 1974). Until this point, the class of mucopolipidoses (MLs) included MLI (originally described as a sialidosis, caused by defects in neuraminidase), MLII (I-cell disease, caused by mutations in N-acetylglucosamine-1-phosphotransferase (GlcNAc phosphotransferase) leading to alterations in lysosomal enzyme targeting), and MLIII (pseudo-Hurler polydystrophy, also caused by defects in GlcNAc phosphotransferase) (Pshezhetsky *et al.* 1997; Bonten *et al.* 1996; Raas-Rothschild *et al.* 2000; Spranger and Wiedemann 1970; Kudo *et al.* 2006). The MLs are an interesting example of a class of lysosomal disorders grouped together due to similar clinical manifestations and storage pattern, yet arising from mutations in distinct genes (Spranger and Wiedemann 1970; Slaugenhaupt 2002). The 1974 case was described as a new variant of mucopolipidosis given the unique combination of neurologic deficits, neonatal corneal clouding, and ultrastructural evidence of electron dense inclusion bodies in the bone marrow, fibroblasts, and liver - in the absence of skeletal abnormalities and apparent lack of changes in enzymatic activity (Berman *et al.* 1974). Within the year of describing this new variant, additional patients presenting with similar features were described by Isabelle Rapin and colleagues (Tellez-Nagel *et al.*, 1976), and the name mucopolipidosis type IV for this new clinical variant became established in the literature (Tellez-Nagel *et al.* 1976; Berman *et al.* 1974).

### Natural history of MLIV and identification of MCOLN1

Much of what is known of MLIV's clinical progression comes from initial studies of the first MLIV patients and comprehensive analyses of clinical chart data and family interviews originating from the 1970s and 1980s (Amir *et al.* 1987; Berman *et al.* 1974; Tellez-Nagel *et al.* 1976; Chitayat *et al.* 1991). This seminal study provided the first and only brain biopsy tissues from an MLIV patient examined to date (Tellez-Nagel *et al.* 1976). MLIV disease results in intellectual disability, systemic pathologies such as kidney involvement and achlorhydria, motor and speech deficits, retinal degeneration, and a shortened lifespan (Amir *et al.* 2013; Schiffmann *et al.* 1998; Schiffmann *et al.* 2014). During the first year of life, children present with psychomotor delays and visual impairment, and storage accumulation can already be detected upon tissue biopsy (Amir *et al.* 1987; Tellez-Nagel *et al.* 1976). One of the most unique characteristics of MLIV is achlorhydria with increased plasma gastrin secretion, which has been utilized as a diagnostic factor even in infant patients (Goldin *et al.* 2008; Chandra *et al.* 2011; Schiffmann *et al.* 1998). Dysmyelination and reduced size of the corpus callosum have also been reported in very young MLIV patients indicating that there is likely a developmental component to the disease in addition to later neurodegeneration (Schiffmann *et al.* 2014; Frei *et al.* 1998). Affected individuals go on to exhibit developmental and motor milestone delays, and impaired fine motor movement (Amir *et al.* 1987). However, while some patients experience deteriorating motor function over time, others are able to develop motor skills such as walking, sitting upright, or even standing with support (Tellez-Nagel *et al.* 1976; Amir *et al.* 1987). There is currently no cure or corrective therapy for MLIV disease. Small molecule therapy using miglustat has recently been shown

to delay cerebellar disease in the MLIV mouse model, but has not yet been tested in human patients (Boudewyn *et al.* 2017). Bone marrow transplant (BMT) has also been explored in the mouse model, which was reported to amelio transmembrane domains 5 and rate motor phenotypes (Walker and Montell 2016). However, it remains unlikely that BMT would rescue clinical phenotypes overall in humans given that mucolipin-1 is a transmembrane protein (as described below) and thus would not be amenable to secretion for cross correction of diseased neurons.

Importantly, there is a need in the MLIV community for a comprehensive natural history study to chronicle MLIV disease symptoms and identify measurable clinical biomarkers over the course of life. It has been noted for example that many patients in the second to third decade of life suffer kidney disease and kidney failure, which as a major cause of morbidity in MLIV represents one of the significant knowledge gaps in MLIV research (personal communication, Dr. Rebecca Oberman, Director, ML4 Foundation). Such a study would provide information needed to establish measurable readouts for future clinical trials of emerging therapies, and to better understand pressing questions such as kidney disease etiology.

The majority of MLIV patients who have been described to date are of Ashkenazi Jewish descent (Slaugenhaupt *et al.* 1999; Raas-Rothschild *et al.* 1999; Gideon *et al.* 2005). In 1999, Slaugenhaupt and colleagues utilized genome scans from 13 Ashkenazi Jewish families to map the location of the MLIV gene (Slaugenhaupt *et al.* 1999). Using linkage analysis, the gene was mapped to chromosome 19p13.2-13.3, and two founder haplotypes were identified in this population (Slaugenhaupt *et al.* 1999; Acierno *et al.* 2001). Approximately 95% of all MLIV patients possess one of these two founder haplotypes, referred to as the major and minor mutations, localized to the *MCOLN1* gene (Bargal *et al.* 2000). *MCOLN1* contains 14 exons and encodes the 580-amino acid, 65 kDa mucolipin-1 protein. The most common mutation in MLIV accounts for 72% of MLIV cases and results in a splice mutation at IVS3-2A→G (Bargal *et al.* 2000; Edelmann *et al.* 2002). The second most common haplotype accounting for 23% of MLIV cases is caused by a 6434 base pair (bp) deletion including the first 6 exons and the first 12 bps of exon 7. The remaining cases of MLIV (~5%) can be traced to over 20 independent mutations (Slaugenhaupt 2002; Altarescu *et al.* 2002; Mirabelli-Badenier *et al.* 2014; Saijo *et al.* 2016; Bargal *et al.* 2000; Shiihara *et al.* 2018). The extent of protein dysfunction or absence has been reported to correlate with the severity of disease (Altarescu *et al.* 2002).

### **The TRP proteins and mucolipin-1 in the endosomal/lysosomal system**

The mucolipin-1 protein, or TRPML1, is a member of the transient receptor potential family of proteins (Montell 2005). The Transient Receptor Potential (TRP) channel family is comprised of seven subfamilies made up of over 20 different channel proteins, including the TRPC, TRPV, TRPM, TRPN, TRPA, TRPML, and TRPP (Clapham *et al.* 2001; Minke 2010). TRP proteins can be found widely distributed in mammalian tissues with a range of subcellular locations and functions, but are united by a similar structure built on 6 transmembrane domains (Clapham *et al.* 2001). The mammalian mucolipin TRP (TRPML) family consists of mucolipin-1, 2, and 3. Mucolipin-1 is predominantly localized to the

surface of late endosomes and lysosomes, and can be found in every tissue with the highest expression in brain, kidney, liver, spleen, and heart (Cheng *et al.* 2010). Like other TRP channels, mucolipin-1 contains 6 transmembrane domains, and harbors a serine lipase domain, a nuclear localization signal, and lysosomal targeting motifs comprised of di-leucine C-terminal motifs and two N-terminal proline rich sequences between the first and second transmembrane domains (Fig. 1) (Bach 2001; Kiselyov *et al.* 2005; Vergarajauregui and Puertollano 2006). The N- and C- terminal ends of mucolipin-1 extend into the cytosol (Fig. 1) (Sun *et al.* 2000). The N-terminal di-leucine motif is also purported to interact with clathrin adaptors AP1 and AP3, while the C-terminal di-leucine motif is predicted to interact with AP2, thus promoting plasma membrane internalization (Vergarajauregui and Puertollano 2006). The C-terminal end also possesses palmitoylation sites at cysteine residues, promoting membrane localization (Pryor *et al.* 2006; Vergarajauregui and Puertollano 2006; Miedel *et al.* 2006; Kiselyov *et al.* 2005; Venkatachalam *et al.* 2006). A loop is present between transmembrane domains 5 and 6 which serves as the channel's pore (Fig. 1) (Kiselyov *et al.* 2005; Zhang *et al.* 2017b; Chen *et al.* 2017). The intraluminal loop between transmembrane domains 1 and 2 also contains a serine-lipase region, a proline rich domain, and a proteolytic cleavage site at Arg<sup>200</sup> (Fig. 1) (Kiselyov *et al.* 2005). This site undergoes proteolytic cleavage in a post Golgi compartment (Kiselyov *et al.* 2005). It is thought that following cleavage, mucolipin-1 may form multimers and even heteromultimers (Zeevi *et al.* 2010; Cytia *et al.* 2009).

Currently, the general consensus in the field suggests mucolipin-1 is a nonselective, inwardly rectifying (i.e., lysosome to cytosol) cation channel, with permeability to calcium, iron, sodium, potassium, manganese, magnesium, zinc, and hydrogen ions (Fig. 1) (Cheng *et al.* 2010; Dong *et al.* 2009; Soyombo *et al.* 2006). Though mucolipin-1 is permeable to a number of cations, the channel property that appears to be most integrally related to our current understanding of the protein's cellular function is its permeability to calcium. Mucolipin-1's role as a lysosomal calcium channel potentially places this TRP channel at the crux of autophagic regulation, trafficking regulation, and even mTOR (mammalian [or mechanistic] target of rapamycin) and TFEB (transcription factor EB) signaling - yet as described below, these roles and mucolipin-1's permeability to other cations implicate multiple pathogenic pathways in the context of MLIV disease.

### Understanding neuronal dysfunction and degeneration in MLIV

While studies in recent years have provided new insights into the many roles played by mucolipin-1 in cells, as well as a better characterization of the underlying neuropathology of MLIV disease, the distinct connections between the loss of function of this protein and the neuronal phenotypes that result remain poorly understood. Here we provide a focus on five potential neuropathogenic pathways and how they may be related to the observed abnormalities in MLIV disease – specifically, aberrations in endosomal/autophagosomal/lysosomal trafficking, alterations to autophagy, dysregulation of lysosomal exocytosis, changes in the mTORC1/TFEB signaling axis, and altered regulation of heavy metal homeostasis.

## Endosomal Lysosomal Trafficking

Mucolipin-1 has long been implicated as having a role in the first pathogenic pathway: trafficking and fusion of late endosomes and lysosomes in neurons and other cell types. Early studies of MLIV model systems demonstrated the presence of enlarged late endosomes/lysosomes and accumulation of autophagosomes consistent with such trafficking defects in the absence of mucolipin-1 (Chandra *et al.* 2011; Venkatachalam *et al.* 2008; Venugopal *et al.* 2009). Studies more specifically dissecting the endosomal pathway indicate the absence of mucolipin-1 leads to malfunctions in retrograde lipid transport within the late endocytic pathway (Shen *et al.* 2012; Pryor *et al.* 2006; Ye *et al.* 2004; Miedel *et al.* 2008). The loss of this TRP channel's local, lysosomal calcium source has also been reported to lead to improper fission events needed to re-form lysosomes from autolysosomes and to recycle transport vesicles from the endosomal network (Miller *et al.* 2015; Dong *et al.* 2010). Defects in endosomal/lysosomal trafficking emerge as a logical candidate underlying the heterogenous lysosomal storage phenotype present in MLIV disease, particularly in regards to complex gangliosides (Micsenyi *et al.* 2009; Venugopal *et al.* 2007). Gangliosides are synthesized within the Golgi network, and rely on proper vesicular transport to be delivered to their destinations in the plasmalemma. Following residence in the plasmalemma, they are endocytosed and recycled via the lysosomal system, with precursors for subsequent ganglioside synthesis being made available by this process (Riboni and Tettamanti 1991; Ghidoni *et al.* 1989; Möbius *et al.* 1999). The described fusion and endocytic trafficking errors with the loss of mucolipin-1 may lead to a failure to efficiently catabolize gangliosides such as GM1 and GM2, with these becoming trapped within organelles as a result. Indeed, observed storage patterns in *Mcoln1*<sup>-/-</sup> brain tissues indicate that individual types of gangliosides (GM1, GM2 and GM3) are not always localized to the same storage compartment, a finding similar to other lysosomal diseases, and can even be found within vesicles separate from LAMP-positive late endosomes/lysosomes (Micsenyi *et al.* 2009). Neurons as post-mitotic cells may be uniquely sensitive to such perturbations to proper endosomal/lysosomal cycling, but whether such failure is uniquely contributing to storage in MLIV-affected neurons remains uncertain.

Interestingly, mucolipin-1's role in lipid trafficking may even be important in the pathogenic cascade of other disorders. In cultured Niemann-Pick type C (NPC) knockout CHO cell lines, mucolipin-1-mediated lysosomal calcium release is reduced and is inhibited by sphingomyelin (Shen *et al.* 2012; Wang *et al.* 2015). Further, in cultured human retinal epithelial cells (ARPE-19) and in the ABCA4 mouse model of Stargardt's retinal dystrophy, induced lysosomal accumulation via the sphingomyelinase inhibitor desipramine and lysomotopic agent chloroquine reduced mucolipin-1 mediated calcium signaling (Más Gomez *et al.* 2018). These studies suggest that the luminal lysosomal environment could affect mucolipin-1 function, and therefore that chronic lysosomal storage may promote additional *secondary* abnormalities in lysosomes. (Shen *et al.* 2012). As these findings may have a broad influence a variety of lysosomal disorders, efforts to understand the role of accrued storage affecting mucolipin-1 function as a further mechanism of neuropathogenesis may be informative.



## Autophagy

Furthermore, along the endosomal/lysosomal pathway in the mucopolipin-1-deficient cell, there is also a large body of evidence in MLIV indicating a second pathogenic pathway: defective autophagic progression and function. LC3 is a useful marker of autophagic flux, as during the formation of autophagosomes, cytosolic LC3-I is conjugated to phosphatidylethanolamine to form LC3-II, tethering LC3 to the growing autophagosome membrane (Settembre *et al.* 2008; Tanida *et al.* 2008). LC3-II turnover levels therefore can serve as an indicator of both autophagosomal maturation (by the level of LC3-II relative to LC3-I) and degradation (turnover of LC3-II within the autolysosome) (Tanida *et al.* 2008). Studies in both human MLIV fibroblasts as well as neuronal cultures from MLIV mice indicate that LC3-II levels are significantly higher than WT, and are not responsive to challenge with rapamycin (Curcio-Morelli *et al.* 2010). This finding is suggestive of a defect in the completion of autophagosome degradation during macroautophagy (hereafter referred to as autophagy), and is consistent with findings of an accumulation of autophagosomes in MLIV disease (Wong *et al.* 2012). It has also been shown that mitochondria accumulate in MLIV disease, which is further evidence consistent with defective autophagy (in this case, mitophagy) in this disorder (Jennings *et al.*, 2006).

Another widely studied marker of defective autophagy is p62 aggregation - this too has been reported to occur in the MLIV mouse model and even in human MLIV cells (Micsenyi *et al.* 2009; Curcio-Morelli *et al.* 2010; Vergarajauregui *et al.* 2008a; Saijo *et al.* 2016). In order to better characterize aggregation of p62 in MLIV disease we carried out a series of confocal imaging studies on cells in the cerebral cortex, hippocampus and cerebellum of the *Mcoln1*<sup>-/-</sup> mouse model. We found that aggregates of p62 in the neocortex occur as large, clustered rings, as well as smaller punctae, which are particularly abundant in the cerebellum (Fig. 2). Large clusters of p62 such as those documented in the MLIV brain (and other lysosomal diseases) are not reported in WT tissues, yet smaller p62 punctae are typically observed and in fact can be enhanced in response to starvation in culture (Itakura and Mizushima 2011). Overall, the size of aggregates in MLIV brain regions we studied ranged from small (i.e., 1 micron or less in diameter), to large (reaching 12 microns in diameter), with the frequency of occurrence being relatively low in neocortex compared to the granule cell layer of the cerebellum (data not shown). The prevalence of large aggregates appeared to be less than that observed in other lysosomal diseases, e.g., in the mouse model of CLN2 disease (Micsenyi 2013). Here, p62 aggregation has been associated with lysosomal membrane permeability (LMP) rather than a defect in autophagy (Micsenyi *et al.*, 2013). Whether LMP is a phenomenon occurring in MLIV disease is presently unknown, but leakage of cathepsin B from lysosomes can induce cell death and has been suggested as a potential apoptotic mechanism in this disease (Colletti *et al.*, 2012; de Castro *et al.* 2016). Importantly, release of cathepsins from damaged lysosomes is associated with other modes of cell death as well, including caspase-independent cell death (Bröker *et al.* 2004). Thus, at present, whether the formation of p62 aggregates in the MLIV brain reflects a simple blockage in autophagy, or a more complex event occurring in response to LMP (as described in CLN2 disease), or involving mTORC1/TFEB dysregulation (see below), or a complex multifaceted autophagic defect, remains to be determined. Similar to how p62 aggregates may arise from a multifaceted defect in MLIV, it is possible that the heterogenous storage

observed in MLIV cells arises from these multiple events growing out of the loss of mucolipin-1 as described above. These include compromises in autophagy, lipid trafficking, and even a third possible pathway of neuropathogenesis - disturbances to lysosomal exocytosis.

### Lysosomal exocytosis

Mucolipin-1 is well documented to play an important role in regulating lysosomal exocytosis (Medina *et al.* 2011; LaPlante *et al.* 2006; Lima *et al.* 2012; Park *et al.* 2015). It has been shown that ionomycin-induced lysosomal exocytosis in MLIV patient fibroblasts fails to deliver LAMP1 to the plasma membrane, indicative of deficient exocytosis (LaPlante *et al.* 2006). This finding is further supported by studies inducing gain of function mutations in mucolipin-1, which cause increased rates of lysosomal exocytosis (Dong *et al.* 2009). Lysosomal exocytosis through mucolipin-1 is also reported to be required for large particle phagocytosis, wherein calcium efflux through mucolipin-1 mediates the exocytotic delivery of lysosomal membrane to neighboring phagosomes (Samie *et al.* 2013). In contrast to these studies on defective exocytosis in the absence of mucolipin-1, a recent report examining pancreatic acinar cells and neurons from the *Mcoln1* mouse model provided the surprising finding that exocytosis appeared enhanced rather than compromised (Park *et al.* 2015). Proteins necessary for synaptic exocytosis such as SNAP25 and VAMP2 also appeared increased in MLIV mouse cortex, cerebellum, and medulla, potentially implicating further exocytotic alterations in the MLIV brain (Park *et al.* 2015). Determining the exact implications of exocytotic dysfunction at the organelle level involving lysosomes and at the synaptic level in neurons clearly are important to understanding neuropathogenesis in MLIV.

### mTOR, TFEB, and a complex feedback loop with mucolipin-1

The fourth neuropathogenic pathway emerging in MLIV focuses on the complex mechanisms underlying the master cell regulator mTOR and the CLEAR (coordinated lysosomal expression and regulation) network activator, TFEB, investigation on which has caused the lysosome to become the center of a wealth of research in recent years (Jaworski and Sheng 2006; Peña-Llopis *et al.* 2011; Troca-Marín *et al.* 2012; Sharma *et al.* 2010; Settembre *et al.* 2013a; Medina *et al.* 2011; Sardiello *et al.* 2009; Martina *et al.* 2014a). These signaling hubs form an axis sensitive to cellular nutrient states to modulate cellular resources via autophagy, and in the case of mTORC1, even to dock on the lysosomal surface itself as a signaling platform (Settembre *et al.* 2013b). For lysosomal diseases, this presents the possibility that the mTORC1-mediated metabolic sensing mechanism becomes compromised, potentially contributing to alterations in this, and its sister signaling pathway, TFEB, and the myriad pathways they control. The mTOR complex, comprised of mTORC1 and the more enigmatic mTORC2, plays many roles, influencing broad cellular processes that include starvation/nutrient responses, autophagy, lysosomal biogenesis and exocytosis, dendritogenesis and synaptic modulation, and lipid metabolism (Troca-Marín *et al.* 2012; Hay and Sonenberg 2004; Settembre *et al.* 2012; Urbanska *et al.* 2012; Sarbassov *et al.* 2005; Jaworski and Sheng 2006). A delicate ensemble of proteins modulates mTORC1's cellular location and activity to orchestrate these many events. In response to high amino acid availability mTORC1 translocates from the cytosol to the surface of late endosomes/lysosomes, where it is activated and initiates downstream signaling to inhibit autophagy and



promote cellular growth (Sancak *et al.* 2010). This translocation to the lysosomal surface involves a concerted effort among numerous proteins, including Rag GTPases, the Ragulator protein complex, and p62 (Duran *et al.* 2011; Linares *et al.* 2013; Sancak *et al.* 2010). A partial summary of these complex interrelationships in relation to the known functions of mucolipin-1 is provided in Fig. 3.

In MLIV, it has been shown that *Trpml*-deficient flies exhibit reductions in downstream targets of mTOR, such as phosphorylated S6 kinase, suggestive of reduced TORC1 activation (Wong *et al.* 2012). Interestingly, administering a high protein diet to *trpml*-deficient flies restores activation of mTOR and reduces mutant phenotypes such as pupal lethality and improves overall survival (Wong *et al.* 2012). Cell culture and fly studies have gone on to show an interaction of mucolipin-1 with AMPK (Onyenwoke *et al.* 2015), and demonstrate mTORC1-mediated JNK activation is diminished in *trpml*-deficient flies and is the underlying cause behind defects in NMJ synaptic development (Wong *et al.* 2015). These studies provided the first insights into potential perturbations to the mTOR signaling axis in MLIV. As flies express one homolog of mucolipin, it is possible that *Drosophila trpml* acts in pathways different from other mammalian systems. For example, studies from MLIV patient fibroblasts report no detectable changes in phosphorylated levels of mTOR and downstream member p70S6 kinase (Curcio-Morelli *et al.* 2010). Recent evidence in cell culture suggests that mTORC1 itself influences mucolipin-1 activity by phosphorylating the protein on its C-terminus, at sites Ser<sup>572</sup> and Ser<sup>576</sup> (Fig. 3) (Onyenwoke *et al.* 2015). Further, recent studies using cell culture suggest that during a prolonged starvation state, mucolipin-1 is necessary for the reactivation of mTORC1, consistent with mucolipin-1 and mTORC1 being linked via a complex negative feedback loop sensitive to cellular nutrient levels (Sun *et al.* 2018).

In addition to the role of mucolipin-1 during prolonged starvation in the mTORC1 pathway as described above, this TRP channel also plays a role in the TFEB-mediated response to acute low nutrient availability. In a state of low cellular resources with reduced mTORC1 activity, TFEB is not phosphorylated by mTORC1 and is further susceptible to dephosphorylation by the phosphatase calcineurin (Medina *et al.* 2015). As a result, TFEB is translocated to the nucleus where it would initiate lysosomal biogenesis and trigger autophagy upregulation, thus utilizing the lysosomal system to restore nutrient caches (Medina *et al.* 2015). Activation of calcineurin requires calcium from local lysosomal calcium stores specifically through mucolipin-1 (Fig. 3) (Medina *et al.* 2015; Wang *et al.* 2015). Similarly, TFE3, another member of the TFE transcription factor family, appears to mimic the starvation response activity of TFEB, and would therefore be an important marker to explore in MLIV given the high expression of TFE3 in brain relative to the other TFEBs (Martina *et al.* 2014b; Martina *et al.* 2014a). In addition to the regulatory influence of mucolipin-1 on TFEB signaling, it has been reported in immortalized cell culture studies that mucolipin-1 activity is required for fission of new lysosomes from parental lysosomes (Miller *et al.* 2015; Wang *et al.* 2015). This is additional evidence that mucolipin-1 plays an intimate role in the regulation of lysosomal biogenesis, both in its initiation (via TFEB-network activation) and completion (via proper fission of nascent lysosomes).

Collectively, these findings are consistent with mucolipin-1 being part of an important feedback loop between mTOR and TFEB, these being connected by the recent finding of mTORC1-mediated phosphorylation of mucolipin-1 discussed above (Onyenwoke *et al.* 2015). When phosphorylated, mucolipin-1 is inhibited (Vergarajauregui *et al.* 2008b), which in turn reduces its channel activity leading to other downstream effects such as reduced activation of TFEB as a transcription factor (Onyenwoke *et al.* 2015). Considering these seminal findings implicating mucolipin-1's role in this fourth neuropathogenic pathway, the mTOR/TFEB signaling axis, a number of cellular consequences can be predicted in the mucolipin-1-deficient cell. For example, as TFEB regulates expression of *MCOLN1* just as it does many genes coding for lysosomal proteins, this reduced transcription would be expected to lead to less mucolipin-1 expression amongst deficiencies and alterations to autophagy and lysosomal biogenesis (Palmieri *et al.* 2011; Sardiello *et al.* 2009; Medina *et al.* 2011) (Fig. 3, 4). Meanwhile, irregularities in the mTORC1 pathway may lead to aberrations in cell growth, structural changes to dendritic complexity and cell size, and alterations in synaptic function (Settembre *et al.* 2013a; Medina *et al.* 2015; Sardiello *et al.* 2009; Urbanska *et al.* 2012; Chen *et al.* 2014b; Takei and Nawa 2014; Bowling and Klann 2014). In specialized post-mitotic cells like neurons, perturbations to the mTOR/TFEB signaling axis in response to varying nutrient availability may culminate in subtle differences across cell types, and perhaps a subset of cells becoming locked in a futile metabolic cycling state (Wang *et al.* 2015). Such changes may also help explain neurological disease progression as these defects would be expected to progressively accumulate cellular damage over time.

### Heavy metal regulation and oxidative stress

Lastly, a fifth pathway of neuropathogenesis has emerged in MLIV – that of regulating lysosomal heavy metal homeostasis, particularly of iron and zinc (Dong *et al.* 2008; Silva *et al.* 2014; Eichelsdoerfer *et al.* 2010; Kiselyov *et al.* 2011). It has been shown that mucolipin-1 normally plays a role in iron handling, and its loss therefore would explain iron dysregulation noted in mucolipin-1-deficient cells and iron deficiency in patients (Grishchuk *et al.* 2015; Coblenz *et al.* 2013; Dong *et al.* 2008; Schiffmann *et al.* 1998). Notably, iron catalyzes the production of reactive oxygen species (ROS), and it is suggested that this buildup of iron in mucolipin-1-deficient cells is a contributing mechanism to degeneration and mitochondrial deterioration (Coblenz *et al.* 2013). Further, mucolipin-1's activity regulating lysosomal zinc levels has been shown to contribute to zinc elevation in lysosomes and in MLIV brain tissues (Silva *et al.* 2014; Eichelsdoerfer *et al.* 2010; Chen *et al.* 2014a; Cuajungco *et al.* 2014). Zinc dyshomeostasis in MLIV neurons may therefore represent another key neuropathogenic pathway given the relationship between zinc, mitochondrial toxicity, and zinc-associated accumulation of ROS (Eichelsdoerfer *et al.* 2010; Cuajungco and Lees 1997; Choi *et al.* 1988; Sensi *et al.* 2003; Bishop *et al.* 2007; Dineley *et al.* 2005). Iron buildup and heavy metal overload has even been suggested to play a role in the onset of axonal dystrophy (Chiueh 2001), another documented feature of Purkinje cells in MLIV disease (Micsenyi *et al.* 2009). In fact, iron and heavy metal dysregulation has long been associated with neurodegenerative diseases such as Alzheimer's, Huntington's, Parkinson's, and progressive palsy disease (a historical review available by Gerlach *et al.* 2002), and associations between metal dysregulation in the brain and neurodegenerative disease

continue to be identified (Salazar *et al.* 2008). Enriching our understanding of heavy metal regulation in the context of MLIV may uncover additional mechanisms of cell death in specific neuronal populations.

In MLIV disease, the two cell types best documented to undergo degeneration and death are cerebellar Purkinje cells and retinal photoreceptors (Grishchuk *et al.* 2016; Micsenyi *et al.* 2009; Venugopal *et al.* 2007; Boudewyn *et al.* 2017). However, the link between loss of this protein in brain cells and subsequent neuronal dysfunction and degeneration remain enigmatic. Oxidative stress has long been implicated in cell death mechanisms in more common neurodegenerative diseases – and is an appropriate candidate for cell degeneration in MLIV that unifies many reported pathologies in this disease. As described above, the formation of ROS and mitochondrial aberrations have been well documented to occur in MLIV models, and further, it is known that mitochondria play a central role in the production of ROS (Naziro lu *et al.* 2012; Jennings *et al.* 2006; Coblenz *et al.* 2013; Coblenz *et al.* 2014; Venkatachalam *et al.* 2008). The role of ROS in neuropathogenesis therefore may represent a significant pathway in MLIV as it has recently been shown that mucolipin-1 may also act as a ROS sensor, during which these reactive moieties stimulate calcium release through mucolipin-1 and activate TFEB, promoting clearance of damaged organelles (Zhang *et al.* 2016). This finding revealed not only that mucolipin-1's loss can lead to mitochondrial damage and a cellular failure to degrade dysfunctional mitochondria, but is further evidence supporting mucolipin-1's link to TFEB and regulation of autophagy (Medina *et al.* 2015; Zhang *et al.* 2016; Peña and Kiselyov 2015). Much like the loss of mucolipin-1 in the TFEB-mediated starvation response, it is possible TFEB activation in response to ROS may similarly be impacted and fail to clear out damaged mitochondria producing these toxic ROS in MLIV.

### Connecting mucolipin-1 to neuropathogenesis

The above-described changes – in endosomal/lysosomal trafficking, autophagy, lysosomal exocytosis dysregulation, mTORC1/TFEB signaling axis aberrations, and heavy metal dyshomeostasis with ROS formation - are all likely contributing factors to the kaleidoscopic pathologies canonical to MLIV (Fig. 5). Defects along each of these steps may help explain the heterogenous storage phenotype and organelle dysfunction observed in MLIV brain tissues (Fig. 5). Considering the mounting evidence of mucolipin-1's multipronged role in lysosomal signaling and function, the question arises as to what the consequences are for neurons and other brain cells in the partial absence of this protein, that is, would partial expression of the protein as would occur in heterozygotes lead to neuronal dysfunction?

### The heterozygous state of Mcoln1

In 1974, one of the first clinical studies of an MLIV patient included analysis of skin fibroblasts from the individual's healthy mother, aged 27 (Tellez-Nagel *et al.* 1976). This pioneering analysis revealed ultrastructural storage material identical in morphology to that seen in her son's cultures, yet occurring to a lesser degree and with lower frequency (Tellez-Nagel *et al.* 1976). Interestingly, no specific study has been carried out to date investigating any possible dosage effects of heterozygosity in individuals with mutations in *MCOLN1*. Studies of carriers of MLIV mutations probing for any potential effects of haploinsufficiency

may further our understanding of the disease as well as mucolipin-1 protein function and may prove informative for families affected by MLIV. In addition to the major MLIV-causing mutations, independent mutations causing MLIV continue to be identified (Slaugenhaupt 2002; Shiihara *et al.* 2018; Casteels *et al.* 1992; Bargal *et al.* 2001; Saijo *et al.* 2016). As the array of mutations to mucolipin-1 existing in the population continue to be unveiled, understanding the potential impact of these mutations on carriers will become increasingly important. One means of doing this would be to evaluate family members of MLIV patients with known mutations for health-related issues incurred as a consequence of *MCOLN1* heterozygosity. The importance of this issue is underscored by findings in other lysosomal disorders in which heterozygotes of lysosomal disease-causing mutations are documented to manifest clinical symptoms, as described below.

Lysosomal diseases stem from defects ranging from lysosomal enzyme loss to the absence of lysosomal transmembrane proteins vital to proper organelle function (Platt and Walkley 2004). Storage disorders resulting from the loss of lysosomal enzymes are not typically thought to result in clinical phenotypes within the heterozygote, as the partial expression of lysosomal hydrolases that would occur is believed sufficient to provide enzymatic activity to prevent any metabolic dysfunction (Brown *et al.* 1996). Yet conditions like Late Onset Tay-Sachs (LOTS), caused by reduced function of lysosomal  $\beta$ -hexosaminidase and characterized by late life onset of neurodegeneration, have been documented (Deik and Saunders-Pullman 2014; Neudorfer *et al.* 2005). A large body of evidence also has shown that individuals carrying single mutations in the *GBA* gene encoding the lysosomal enzyme, glucocerebrosidase, responsible for the complex, recessive disorder Gaucher disease have also been documented to exhibit clinical symptoms, such as impaired cognition and hyposmia (McNeill *et al.* 2012). Being a carrier of a *GBA* mutation has also been identified as a major risk factor for Parkinson disease and parkinsonian syndrome (Kluenemann *et al.* 2013; Sidransky and Lopez 2012; Goker-Alpan *et al.* 2004; Bras *et al.* 2008; Halperin *et al.* 2006). In light of emerging evidence of lysosomal proteins such as the NPC1 protein and now even mucolipin-1 in the neuropathogenesis of such age-related disorders as Parkinson disease, and more recently, Alzheimer disease, the relationship between impaired lysosomal function and increased risk for age-related brain disorders is becoming increasingly tightened (Kresojevi *et al.* 2018; Zhang *et al.* 2017a).

In contrast to lysosomal disorders caused by absence of lysosomal enzymes, those caused by loss or malfunction of an endosomal/lysosomal transmembrane protein offer a greater possibility of a “dosage effect” in carriers. NPC disease, for example, results from mutations in either the lysosomal transmembrane protein NPC1 or its soluble protein counterpart NPC2, which together function as cholesterol transporters (Kruth *et al.* 1986). Canonical NPC disease results in severe and progressive neurological symptoms, cognitive decline, profound lipid storage (most notably of unesterified cholesterol), hepatosplenomegaly, and premature death (Platt and Walkley 2004; Ikonen 2004; Kruth *et al.* 1986). Much like for carriers of *GBA* mutations, adult heterozygous carriers for NPC1 mutations have exhibited multiple cases of parkinsonian syndrome indicated by resting tremor, motor difficulties, and bradykinesia (Kluenemann *et al.* 2013; Halperin *et al.* 2006; Sidransky and Lopez 2012; Josephs 2004). Further, cultured fibroblasts and blood samples from NPC-mutation carriers and healthy controls revealed that heterozygotes exhibited storage of cholesterol esters and a

metabolic profile intermediate between NPC patients and healthy controls (Kruth *et al.* 1986; Kresojevi *et al.* 2018; Probert *et al.* 2017). Foam cells, or storage-laden cells which are given their name due to their “foamy” appearance when viewed by an electron microscope, are a characteristic feature of NPC tissues and have even been noted in the bone marrow of parents and siblings of NPC patients (Harzer *et al.* 2014). Storage accumulation of unesterified cholesterol, vacuolization, and GM2 accumulation have also been documented by Walkley and colleagues in the feline model of NPC1 within obligate heterozygotes (Brown *et al.* 1996). As in aged adults, *Npc1* heterozygous mice also exhibit reduced motor coordination as well as Purkinje cell loss and increased tau protein (Yu *et al.* 2005). Such findings clearly show that the consequences of heterozygosity can be replicated in animal models of NPC1 disease (Yu *et al.* 2005; Hung *et al.* 2016). Similar findings also can be found outside of NPC disease as well. Asymptomatic siblings and parents of individuals with juvenile Batten (CLN3) disease (which also result from the loss of a lysosomal transmembrane protein) have been documented to possess cytoplasmic inclusions within lymphocytes (Markesbery *et al.* 1976).

These cases – Gaucher, Niemann-Pick C1, and juvenile Batten diseases – highlight the vital role of proper lysosomal function in maintaining neuronal health and raise the possibility that heterozygosity for mutations in the global gene network of lysosomal transmembrane proteins, such as mucolipin-1, may contribute to perturbed cellular function. Understanding if MLIV mutations in heterozygous individuals cause similar metabolic defects or a predisposition to neurological symptoms during aging would be informative to MLIV carriers and potentially improve care for identified affected individuals. Further, understanding neurological health in the heterozygous state may shed light on the role of mucolipin-1 in normal brain health and maintenance.

In order to begin addressing these questions and in the absence of comprehensive data from human MLIV carriers, we turned to the *Mcoln1* mouse model to evaluate the consequences of heterozygosity for mucolipin-1 expression. In other lysosomal disorders exhibiting haploinsufficiency such as NPC1 disease, animal models and even human patients often do not display overt neuronal features until later in life (Yu *et al.* 2005). If heterozygosity for mucolipin-1 expression impacts neuronal function, lysosomal storage-related features may similarly only appear during senescence. We predicted that if mucolipin-1 heterozygosity leads to insufficient lysosomal function, aged mice might develop a milder form of the canonical neurological features detectable in the *Mcoln1* homozygous mutant, such as lysosomal storage, glial activation, behavioral deficits, or ultrastructural abnormalities. Such an investigation would also determine if the MLIV mouse is an appropriate model for assessing milder forms of the disease. As no phenotypes have thus far been observed in heterozygotes at 6-9 months of age from previously published work, this study was directed at assessing aged mice from 15-25 months old to correspond to murine “old age” (Flurkey *et al.*, 2007). A particular focus was given to mice aged 20-25 months for exacerbated signs of aging or delayed neurological manifestations intermediate between the aged WT state and *Mcoln1*<sup>-/-</sup> tissues.

## Methods

### Mouse generation and collection

Heterozygous (*Mcoln1<sup>+/-</sup>*) mice were crossed to obtain littermate wild type (*Mcoln1<sup>+/+</sup>*), heterozygous mice (*Mcoln1<sup>+/-</sup>*) and mutant (*Mcoln1<sup>-/-</sup>*) mice genotyped per previously described methods (Venugopal *et al.* 2007) and included both males and females. *Mcoln1<sup>-/-</sup>* mice (C57/BL6 background) were generously provided by the laboratories of Drs. Susan Slaugenhaupt at Harvard Medical School and James Pickel at the National Institutes of Health. Mice were housed in standard IACUC-approved cages with corn-cob and cotton cloth-square bedding, with access to food and water ad libitum. Mice were housed 2-5 animals per cage, with mixed genotypes housed together, and animal husbandry performed routinely to monitor the welfare of all mice. For p62 analyses, mice were collected at end-stage in the MLIV mouse model at 6-9 months of age alongside WT controls, with a total of 32 mice used (16 *Mcoln1<sup>-/-</sup>*, 16 WT). For aged heterozygous studies, mice were aged to 15 months through 25 months of age corresponding to murine old age (Flurkey *et al.*, 2007). A total of 47 male and female mice were used for this study (24 heterozygotes and 23 WT). Prior to tissue collection, mice were anesthetized using an overdose of ketamine/xylazine delivered via intraperitoneal injection to minimize pain and discomfort, and once insensate euthanized transcardially perfused with normal saline and tissues collected and frozen on liquid nitrogen, then perfused and fixed with 4% paraformaldehyde per previously described protocols (Micsenyi *et al.* 2009). The use of an overdose of the xylazine/ketamine mixture used in these studies is an accepted and IACUC-approved procedure for reducing pain and suffering in rodents. This study was not pre-registered. All procedures using animals were approved by the Institutional Animal Care and Use Committee (IACUC, approval numbers 20160903 and 20160913) at the Albert Einstein College of Medicine.

### Behavioral analyses

Motor assessment was performed using a balance beam task as previously described (Boudewyn *et al.* 2017). In brief, following an equilibration period to the testing room within their home cages, training on a 3-inch wide-set flat beam was performed to promote passage across the balance beam. During testing, three round wooden beams of increasing diameter (18 mm - “narrow”, 24 mm - “medium”, 30 mm - “wide”) were elevated using tripods, with cushion-covered bins located underneath as an added safety precaution. Hides were located at the end of each beam, with a light gradient cast over the beams to stimulate beam crossing (ie, beginning of the beam was bright; end of the beam was dark). Mice were run through the narrow, medium, and then wide beams and the number of slips were counted manually. Prior to testing, mice were assigned a random number in order to blind the tester to genotype and age. Random numbers were allocated via a simple randomization method of assigning mice sequential numbers within a cage in the random order in which they were selected – ie, cage 1, mice 1 through 5 were determined at random (#1 ID being randomly assigned to first mouse picked up, #5 being last (5<sup>th</sup>) mouse picked up, cage 2, #6 ID for first mouse picked up, #11 for last mouse (5<sup>th</sup> in a cage) picked up, etc), with these randomly assigned numbers retained for any subsequent behavioral testing to preserve blinded coding. Tails were color-coded for mice housed within a cage to represent the assigned blinded number (ie, repeating patterns of red-blue-green-white-black tail codes to correspond to sequential ID numbers).



No pain or discomfort was anticipated or observed during this task. Differing sample sizes for each beam were due to varying ability of aged mice to complete all three beams during testing, and only mice capable of completing a beam were included for each test. All work was performed in accordance with the Albert Einstein College of Medicine Animal Phenotyping Core Facility protocols and IACUC approved protocols.

### **Immunofluorescent staining and confocal imaging**

For immunofluorescent staining, fixed tissues were sectioned at 35 micron thickness in coronal orientation for cerebrum and sagittal orientation for cerebellum using a Leica V100S vibratome and stained per previously established protocols (Boudewyn *et al.* 2017; Micsenyi *et al.* 2013). Primary antibody(ies) included anti-CD68 (1:500, rat monoclonal antibody, AbD Serotec MCA1957, RRID:AB\_322219), anti-p62 C terminal specific (1:500, guinea pig polyclonal antibody, American Research Products 03-GP62-C, RRID:AB\_1542690), anti-GFAP (1:1000, rabbit polyclonal antibody, Sigma G9269, RRID:AB\_477035), and anti-NeuN (1:1000, mouse monoclonal antibody, Chemicon MAB377, RRID:AB\_2298772). For those sections stained in conjunction with anti-guinea pig p62 (such as in Fig. 2), pre-adsorbed secondaries were purchased to provide minimal cross reactivity (Alexa dyes 647 and Cy3, diluted as above). 35-micron sections were mounted without antibody staining and imaged under the same conditions as stained sections to control for auto-fluorescence. Confocal microscopy was performed with a Zeiss Meta 510 Duo V2 laser scanning microscope as previously described (Boudewyn *et al.* 2017). Slides were blinded to genotype by being assigned a coded record number and unveiled following processing. Image analysis and processing was performed using ImageJ software and MetaMorph software (Molecular Devices).

### **Western blot analysis**

Cerebellar tissues were homogenized and prepared for western blot analysis per the full protocol described in (Boudewyn *et al.* 2017). In brief, tissues were homogenized with a Dounce homogenizer in freshly made ice cold lysis buffer containing 50nM TrisHCL, 150 mM NaCl, 1% Igepal, 1% Sodium Deoxycholate, 1% Sodium Dodecyl Sulfate, 1% Triton  $\times$  100, and Complete Mini Protease Inhibitor Cocktail (Roche, 11-836-153-001), then homogenate passed through a 27-gauge needle. Following protein quantification, samples were run on an SDS-PAGE 12% Tris-Glycine gel. Blots were imaged using an Odyssey CLx imaging station equipped with Image Lite Studio Software and quantified with Image Lite Studio Software. The intensity of bands for calbindin were normalized to those of beta-actin to correct for loading differences, with antibodies including: anti-calbindin 1:1000, rabbit polyclonal antibody, Millipore AB1778 and anti-beta actin 1:3000, rabbit polyclonal antibody, Sigma A2066.

### **Tissue processing and imaging via transmission electron microscopy (TEM)**

Tissues were prepared for electron microscopy by previously established methods (Micsenyi *et al.* 2009). In brief, tissues were micro-dissected into <1mm blocks of neocortical, hippocampal, and cerebellar sections. Cerebellar sections were further dissected into anterior, central, and posterior + nodular zones (Sillitoe and Joyner 2007) to allow for regional analysis. Tissue blocks were rinsed in 0.1M cacodylate buffer (Fisher Scientific

50-980-232/Electron Microscopy Sciences (EMS) #11652), fixed using Trump's fixative (Fisher Scientific 50-980-239/EMS #11750), then placed in 2% osmium (Fisher Scientific 50-332-20/EMS #19150) and dehydrated in graded ethanol steps. Tissues were then rinsed in Acetonitrile (Fisher Scientific 50-980-146/EMS #10020) and incubated in a 1:1 solution of Acetonitrile:Epon at room temperature overnight, then placed into fresh Epon solution, embedded in Epon, and cut on an ultramicrotome. Blocks were mounted on grids and stained with uranyl acetate and lead citrate, and then they were imaged using a Phillips CM10 transmission electron microscope for ultrastructural analysis. Images were processed using ImageJ and Fiji software.

## Statistical Analyses

All data were compiled using GraphPad Prism Software Version 7.0a and statistically analyzed using JMP Software from SAS Version 13.0.0. To determine the statistical significance of behavioral data, ANOVA was performed. If ANOVA detected a difference, post-hoc parametric Tukey-Kramer HSD comparisons were then performed and a difference was deemed as significant where  $p < 0.05$ . Tests for normalcy and outliers on these pilot data were not performed. Sample size calculations were not performed prior to testing, and sample sizes were obtained based on literature standards (Bailey *et al.* 2008). Post-hoc sample calculations were performed via power analysis of pilot data. For example, for the hard balance beam, any future studies would require group samples of 45 to achieve 80.6% power to reject the null hypothesis of equal means when the population mean difference is 5.6 with a standard deviation of 11.7 for heterozygous mice, and 5.8 for WT mice with a significance level (alpha) of 0.05 using a two sided two-sample unequal-variance t-test. For statistical analyses of western blots, band measurements were analyzed using an unpaired t-test with Welch's correction. For all graphs, mean  $\pm$  standard error is plotted.

## Results

Upon gross assessment, end stage (6-9 months) homozygous affected (*Mcoln1*<sup>-/-</sup>) mice exhibit features such as bladder enlargement, hunched posture, and hindlimb clasp behavior (Venugopal *et al.* 2007). These characteristics were not observed at any point in heterozygous mice or WTs from 15 months of age out to 25 months of age (20-25 months). The mutant MLIV mouse model exhibits poor coordination on a balance beam that is accompanied by Purkinje cell loss as measured by calbindin D western blot analysis (Boudewyn *et al.* 2017). MLIV mice also exhibit widespread storage of gangliosides like GM2 in the neocortex and other parts of the CNS (Boudewyn *et al.* 2017; Micsenyi *et al.* 2009). To evaluate if our cohort of *Mcoln1* heterozygotes displayed similar abnormalities, we carried out balance beam assessments, western blots for calbindin D, and stained tissues for GM2 ganglioside. Mice were tested on three balance beams of decreasing difficulty (i.e., increasing width) and the number of slips counted to assess cerebellar-mediated motor coordination (Fig. 6). No significant differences were detected when comparing heterozygote performance to that of age-matched WTs (Fig. 6A-C). Given the high variability in balance beam performance in both heterozygote and WT mice (likely due to effects of old age), as a further test of cerebellar integrity, we also examined this brain region for evidence of loss of Purkinje cells based on an analysis of calbindin-D levels. Calbindin is

a calcium buffering protein highly expressed in Purkinje cells, and therefore is a useful and widely used marker of Purkinje cell loss (Celio 1990; Barski *et al.* 2003; Sarna *et al.* 2003). Calbindin density was probed via western blot but no significant decrease in levels compared to age-matched WT mice was observed (aged 20-25 months, Fig. 6D). We also examined heterozygotes for evidence of GM2 storage in neurons in cerebrum and cerebellum (which is characteristic of mutant *Mcoln1* mice) (Micsenyi *et al.*, 2009), but again no differences from WT mice were observed (data not shown). While the above studies were not able to detect any significant behavioral or gross cellular phenotypes in aged heterozygote *Mcoln1* mice, it is possible that aged heterozygotes could still manifest changes at the organelle level. To test for this, ultrastructural studies were performed to probe for abnormal MLIV-related subcellular features.

Previous electron microscopy studies of patient biopsy tissues and published data in the MLIV mouse illustrate the presence of highly characteristic storage bodies – so-called compound bodies - in the MLIV brain (Tellez-Nagel *et al.* 1976; Micsenyi *et al.* 2009). The current study delineated here extends this earlier work and reveals MLIV neocortical cells with compound bodies along with multilamellar bodies congesting the cytoplasm of both neurons and support cells (representative examples in Fig. 7). Cytoplasmic structure of neurons was found to be disorganized and accompanied by enlarged mitochondria as previously reported (Micsenyi *et al.* 2009; Jennings *et al.* 2006).

As the above-described features are readily evident in the mutant MLIV mouse even at early ages, we predicted that if partial expression of mucolipin-1 is insufficient to maintain neuronal health the heterozygote MLIV mouse would likely undergo at least an intermediate level of storage detectable as compound storage bodies. Sections of neocortex, hippocampus, and cerebellum were examined, with the latter organized into anterior, central, and posterior + nodular zones (due to the progressive lobular pathology noted in the MLIV mutant cerebellum). No compound bodies, lamellar bodies with electron dense material, or similar distinctive features were detected in heterozygote or WT mice aged 15-25 months (Fig. 7). Indeed, heterozygote tissues were not observed to display any ultrastructural characteristics differing from WT in any brain region analyzed. Lipofuscin granules could be detected readily in all regions in both WT and heterozygous mice but did not appear to occur at a higher rate in either genotype (Fig. 7). Lipofuscin was identified morphologically per established electron microscopy guidelines (Peters *et al.* 1991) and is consistent with lipofuscin-associated autofluorescence detected via immunofluorescent staining.

## Discussion

The lack of an overt cellular or behavioral phenotype in aged *Mcoln1* heterozygotes suggests either that partial expression of mucolipin-1 is sufficient for proper cellular function, or that mucolipin-1 channel function may be partially compensated for by other proteins. Mucolipin-1 is ubiquitously expressed and the only mucolipin channel believed to be expressed in the brain (Cheng *et al.* 2010). It is possible that CNS compensation for mucolipin-1's partial loss of function could arise from transmembrane proteins with similar pore activity, such as the so-called two pore channels (TPCs). The TPCs are endolysosomal ion channels with similar calcium release activity to mucolipin-1 (Wang *et al.* 2017; Grimm

*et al.* 2012). Much like mucolipin-1, these proteins are also permeable to sodium and are similarly activated by PI(3,5)P<sub>2</sub>, suggesting the possibility that these proteins may share activation mechanisms and potentially even similar functions (Dong *et al.* 2010; Wang *et al.* 2017). It is thought that the permeability to cations and response to PI(3,5)P<sub>2</sub> exhibited by both mucolipin-1 and TPCs may facilitate membrane fusion events by modulating these gradients, such that increases in the levels of PI(3,5)P<sub>2</sub> promote lysosomal calcium (documented by mucolipin-1 activity) or sodium (documented by TPC activity) efflux into the cytosolic space (Wang *et al.* 2017; Dong *et al.* 2010). It is possible that TPCs located within the endosomal/lysosomal system which exhibit similar channel conductance may compensate for such activity in the face of reduced expression of mucolipin-1 in the heterozygous state. Still other channels may compensate for mucolipin-1's loss, perhaps even from the greater TRP family – TRPM (melastatin) family members for example also exhibit similar channel conductance, and some TRPM members are expressed in the brain (Fonfria *et al.* 2006). Alternatively, the mouse model may be limited in replicating any phenotypes that could develop in aged human populations. In the *Mcoln1* mutant, the timeline of disease onset and progression differs from human disease manifestations. In human MLIV, children present early in life and experience a relatively stable adolescence, while MLIV mice are relatively stable early in life and develop overt symptoms in adulthood at 6 months of age (Amir *et al.* 1987; Venugopal *et al.* 2007). It is possible that a heterozygous timeline could similarly differ between mammalian species, or not develop in the mouse model compared to human populations. Future survey of human carriers of *MCOLN1* mutations may be informative to monitor the health of individuals over time.

## Conclusions and Pathogenic Crosstalk in the Absence of Mucolipin-1

In this review, we have summarized how the loss of mucolipin-1 leads to a complex, multifaceted pathogenic cascade that contributes to cellular damage culminating in neuron and brain dysfunction. Specifically, we described how primary defects in calcium and heavy metal regulation following loss of mucolipin-1 are believed to impair a host of critical processes in cells, including endosomal/lysosomal fission and fusion events, mTORC1/TFEB signaling and autophagy regulation, and the cellular response to ROS formation (Fig. 5) (Venugopal *et al.* 2009; Zhang *et al.* 2016; Curcio-Morelli *et al.* 2010; LaPlante *et al.* 2006; Coblentz *et al.* 2013). While these consequences of the loss of mucolipin-1 are all likely contributors to the neuropathogenesis of MLIV disease, how they interrelate and influence each other remains largely unexplored and deserving of additional studies. It is also important to recognize that while this review provides a discussion of how multifaceted defects in the absence of mucolipin-1 may relate specifically to neuronal dysfunction; it is not a full narrative of how such defects may impact systemic MLIV disease. Reviews of how mucolipin-1's loss can impact immunity and even cancer have been presented elsewhere (Grimm *et al.* 2018). Other important areas of research, including mucolipin-1's role in renal disease, remain largely unaddressed. Despite the broad implications of the importance of mucolipin-1 function, our studies in the aged *Mcoln1* heterozygote did not reveal abnormalities indicative of loss of mucolipin-1 function, suggesting that partial expression of the protein is adequate to maintain normal function of these intricate pathways, at least in the mouse.

The field of mucolipin-1 and MLIV research to date has unveiled new avenues in our understanding of how the loss of this critical protein leads to the complex neurodevelopmental and neurodegenerative symptoms experienced by patients. Understanding the manner in which these cascades exacerbate and interplay with one another is a crucial step toward understanding not only MLIV pathogenesis but also new approaches to therapy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

### TFEB

transcription factor EB

### mTOR

mechanistic/mammalian target of rapamycin

### MLIV

mucopolipidosis type IV

**NPC**

Niemann-pick type C

**MLI**

mucopolipidosis type 1

**MLII**

mucopolipidosis type II

**MLIII**

mucopolipidosis type III

**CLN2**

neuronal ceroid lipofuscinosis type 2

**LOTS**

late-onset Tay Sachs

**GlcNAc-phosphotransferase**

N-acetylglucosamine-1-phosphotransferase

**TRP**

transient receptor potential

**TRPML**

transient receptor potential channel mucolipin

**TRPC**

“TRP” canonical family

**TRPV**

“TRP” vanilloid family

**TRPM**

“TRP” melastatin family

**TRPN**

“TRP” NOMPC-like family

**TRPA**

“TRP” ankyrin family

**TRPP**

“TRP” polycystin family

**TPC**

two-pore channels

**AP1-3**

clathrin adaptor protein complexes 1-3

**TMD**

transmembrane domain

**BMT**

bone marrow transplant

**CHO**

Chinese hamster ovary

**ARPE**

immortalized human retinal epithelial cell line

**LC3**

microtubule associated protein 1A/1B-light chain 3

**LMP**

lysosomal membrane permeability

**CLEAR**

coordinated lysosomal expression and regulation

**ROS**

reactive oxygen species

**GBA**

glucocerebrosidase

**GBA2**

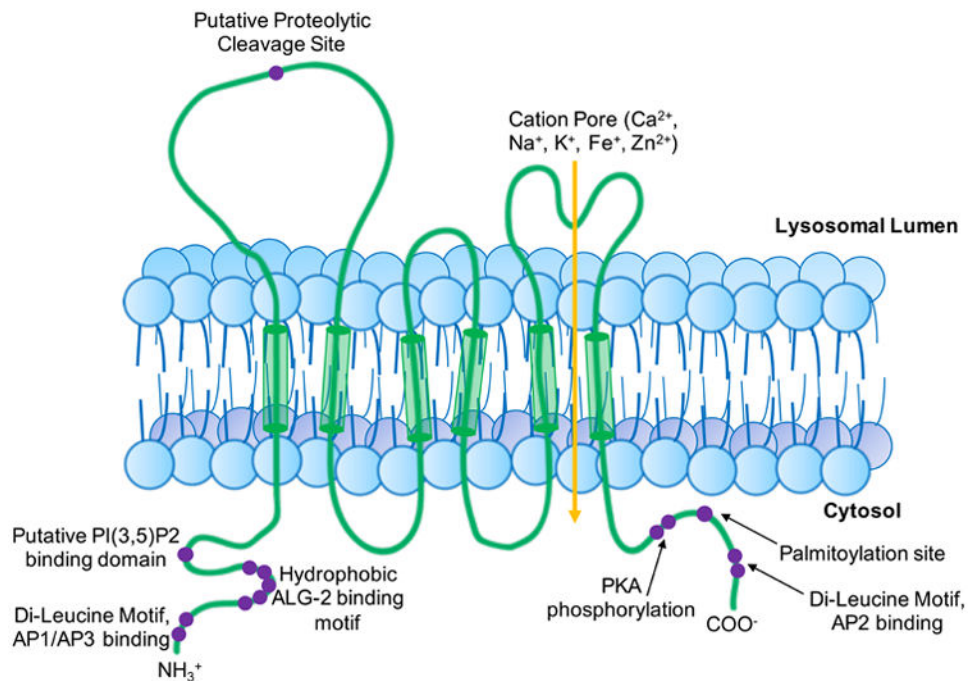
non-lysosomal glucocerebrosidase

**CNS**

central nervous system

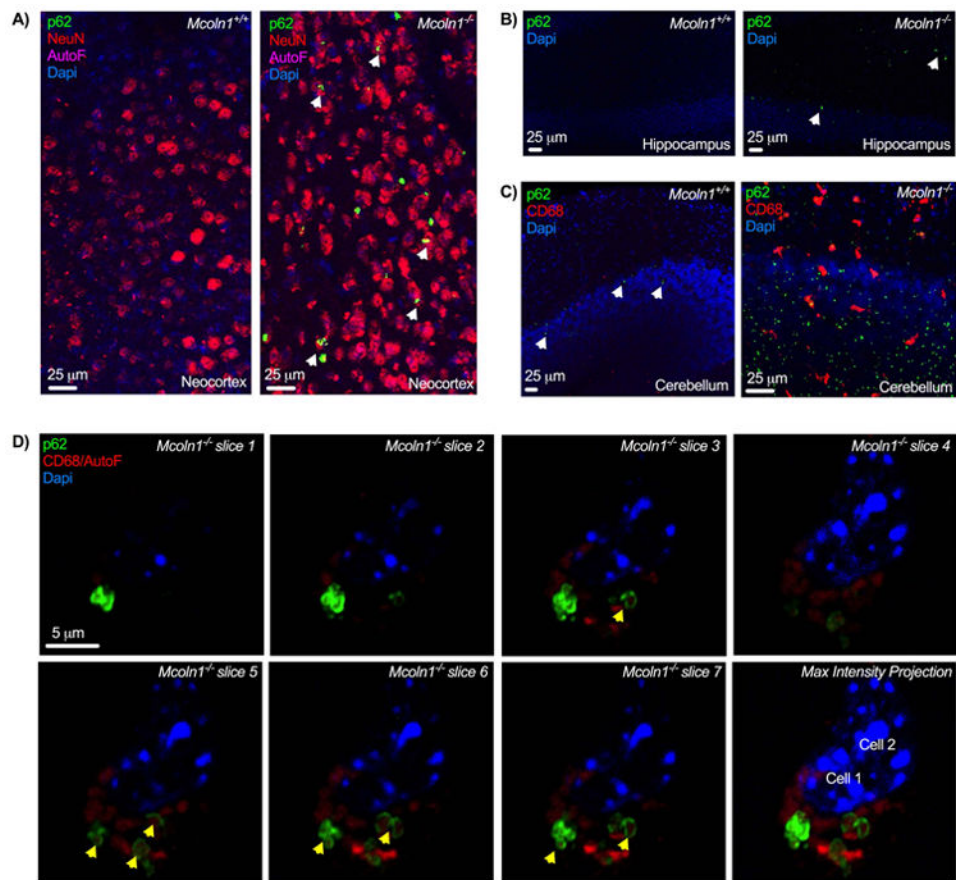
**EM**

electron microscopy



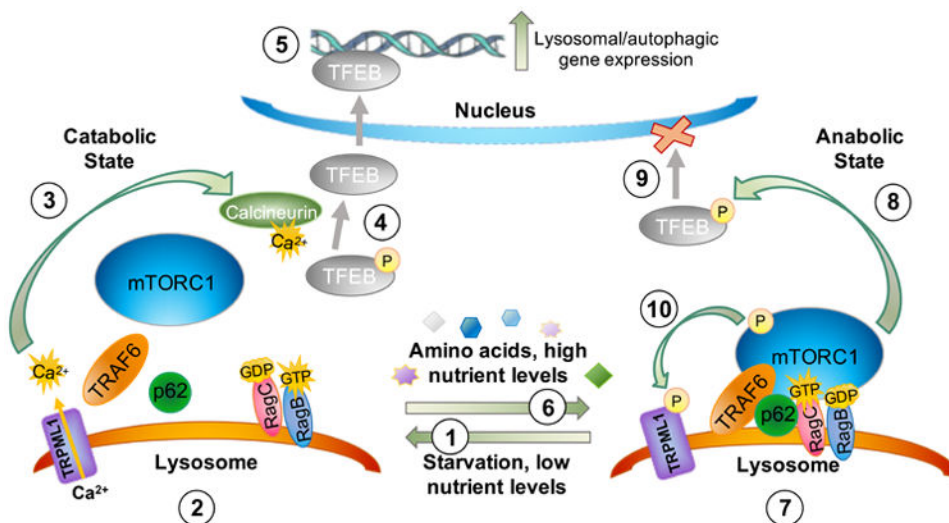
**Figure 1. Structure and functional motifs of mucolipin-1**

Schematic depicting mucolipin-1's structure, including 6 transmembrane domains (TMD), a proteolytic cleavage site between TMDs 1 and 2, and a pore between TMDs 5 and 6. Protein interacting motifs are noted in purple. N terminal domains contain binding sites for AP1/AP3 proteins, PI(3,5)P2, ALG-2 binding motifs, and di-leucine lysosomal targeting domains. The pore is permeable to calcium, iron, zinc, manganese, magnesium, potassium, sodium, and hydrogen ions. The C terminus contains binding sites for PKA phosphorylation, palmitoylation for membrane targeting, AP2 binding sites, and another lysosomal targeting di-leucine sequence. (Kiselyov *et al.* 2005; Clapham *et al.* 2001; Zeevi *et al.* 2007; Li *et al.* 2017; Zhang *et al.* 2017b; Vergarajauregui *et al.* 2008b; Abe and Puertollano 2011; Dong *et al.* 2010; Vergarajauregui *et al.* 2009; Vergarajauregui and Puertollano 2006; LaPlante *et al.* 2002)



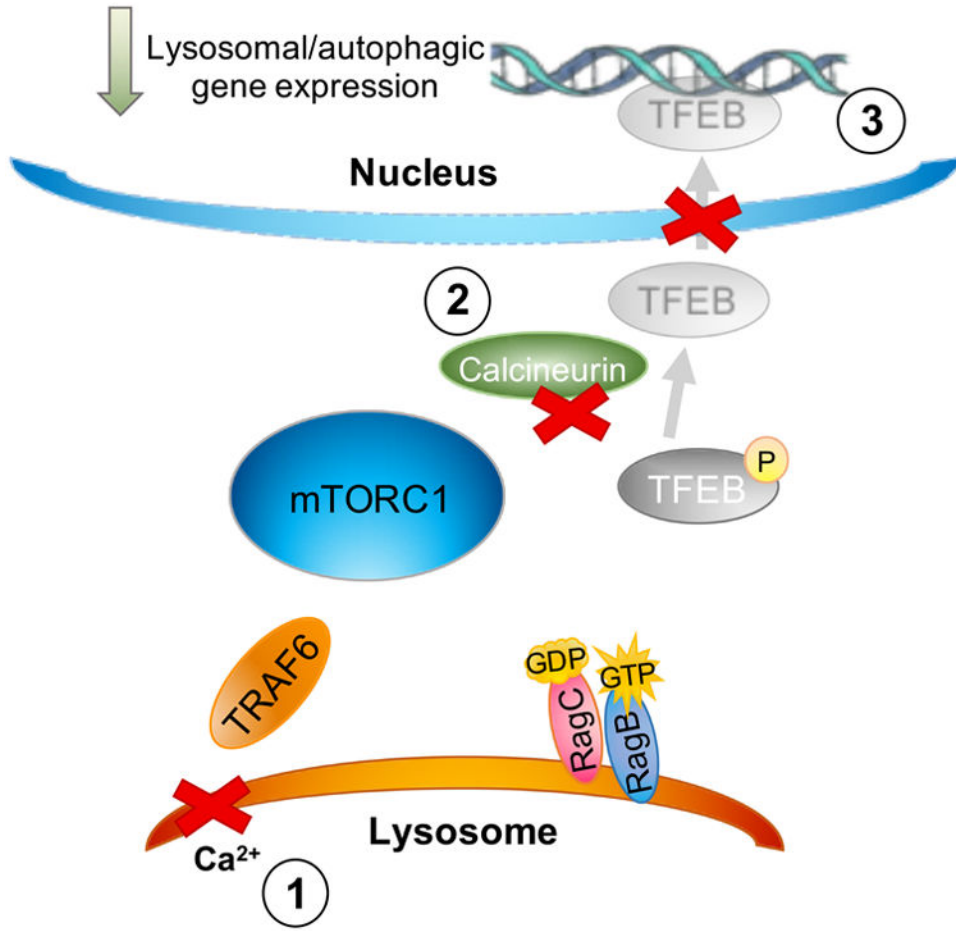
### Figure 2. Aggregations of p62 in the *Mcoln1*<sup>-/-</sup> mouse brain

A) p62 aggregation in *Mcoln1*<sup>-/-</sup> mouse neocortex. p62 aggregates were not observed in WT (left panel), but aggregations positive for p62 can be detected in scattered neurons (NeuN+, p62 in neurons denoted by arrows) from all *Mcoln1*<sup>-/-</sup> mouse neocortical layers, shown here in cortical layer VI *Mcoln1*<sup>-/-</sup> (right panel). B) p62 aggregation in *Mcoln1*<sup>-/-</sup> hippocampus. Hippocampal sections from the CA1 region of WT (left panel) and *Mcoln1*<sup>-/-</sup> (right panel) mouse tissues. P62 positive structures were again not readily detected in WT hippocampus, while scattered punctate aggregates could be found in *Mcoln1*<sup>-/-</sup>, as indicated by white arrows. C) p62 aggregation in *Mcoln1*<sup>-/-</sup> mouse cerebellum. Image of WT cerebellum (left panel) demonstrating occasional p62-positive puncta (noted by arrows), and from *Mcoln1*<sup>-/-</sup> mice (right panel), showing an abundant presence of small inclusions positive for p62. D) Example of typical neocortical p62+aggregate morphology. High magnification (63x) single plane images of p62 aggregates through a dorsal neocortical cell exhibiting clusters of aggregates, forming rings around diffuse, autofluorescent material. Yellow arrows denote distinguishable “rings” of p62. CD68 signal was tagged using Alexa Fluor 546 secondaries, and as autofluorescent material in MLIV fluoresces within near red wavelengths, CD68 signal overlapped with autofluorescent storage (labeled here as CD68/AutoF). Last image is a maximum intensity projection of previous slices in panel. Unless otherwise noted, all images are maximum intensity projections of acquired optical slices and collected from 6-9 month old mice. 9-11 *Mcoln1*<sup>-/-</sup> mice per panel were probed for p62 aggregation with matching WT counterparts.

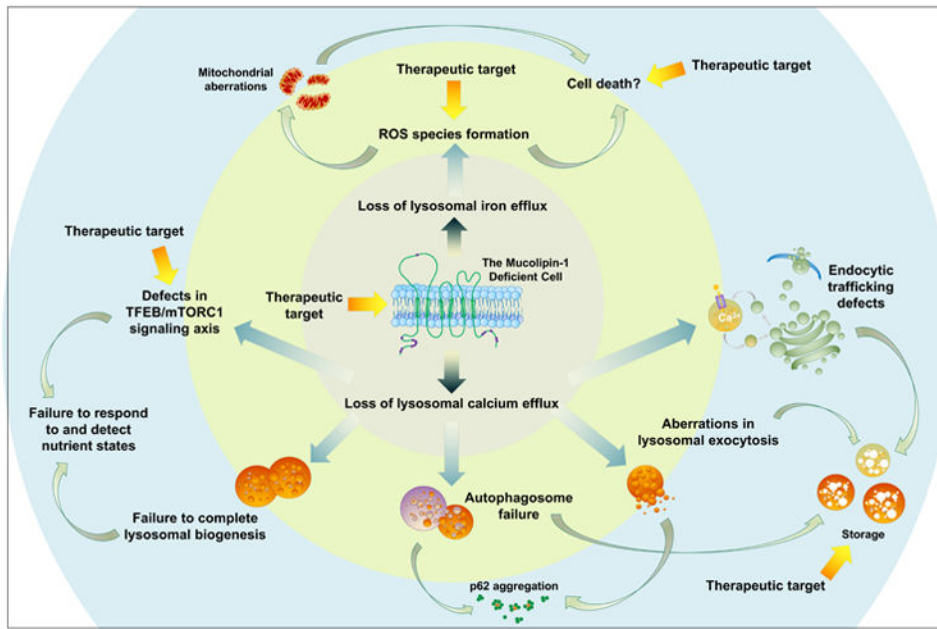


**Figure 3.** Role of mucolipin-1 (TRPML1) during mTOR and TFEB signaling. As described in the text, in response to a low nutrient state (1) mTOR dissociates from the lysosome and lysosomal calcium efflux is triggered through mucolipin-1 (2), allowing for activation of the phosphatase calcineurin (3). Calcineurin in turn dephosphorylates TFEB (4), which promotes its translocation across the nuclear envelope to activate lysosomal biogenesis and autophagy gene expression (5). In response to a high nutrient state (6), mTORC1 scaffolding proteins are recruited to the lysosomal surface and mTORC1 docks on this lysosomal platform (7). Activated mTORC1 then phosphorylates TFEB (8), which promotes its association with 14-3-3 proteins sequestering TFEB within its cytosolic location and thereby downregulates lysosomal biogenesis/autophagy gene expression (9). In response to nutrient availability, mTORC1 also phosphorylates mucolipin-1 directly (10), which reduces its channel activity and would further promote cytosolic localization of TFEB in a phosphorylated state. Mucolipin-1's ability to respond to low nutrient states by releasing calcium (see 2, above) has been suggested to be due to relief of mTOR-mediated phosphorylation of mucolipin-1. These key roles of mucolipin-1 in the nutrient sensing pathway governed by mTORC1 have been previously highlighted in other reviews (Venkatachalam *et al.* 2013). (Medina *et al.* 2015; Wang *et al.* 2015; Onyenwoke *et al.* 2015; Rocznik-Ferguson *et al.* 2012; Settembre *et al.* 2013a).





**Figure 4. TFEB signaling in the mucolipin-1-deficient cell**  
 As described in the text and Fig. 3, mucolipin-1 plays an integral role in the mTORC1/TFEB nutrient response pathway – with the reported consequences of mucolipin-1's absence reviewed here. As described in the text, loss of mucolipin-1 removes the primary calcium source required to activate the phosphatase calcineurin, thus impairing TFEB dephosphorylation and its nuclear translocation (1-3). This may contribute to the mucolipin-1-deficient cell failing to properly respond to low nutrient availability via TFEB-mediated activation of the lysosomal network. (Medina *et al.* 2011; Medina *et al.* 2015; Wang *et al.* 2015). A full review of the role of TFEB in regulating the lysosomal network has been recently presented elsewhere (Sardiello 2016).



**Figure 5. A proposed complex network of disease pathogenesis and potential therapeutic targets surrounding the mucolipin-1-deficient cell**

This schematic summarizes the multifaceted defects that may ensue in the absence of mucolipin-1. Loss of this cation-permeable TRP channel leads to defective lysosomal cation flux (inner circle), including loss of iron and calcium efflux (Dong *et al.* 2008; Dong *et al.* 2009). This loss subsequently leads to secondary defects (yellow middle circle). The absence of mucolipin-1-mediated calcium is followed by a failure to complete fusion of organelles within the endosomal/lysosomal network and a failure to balance the mTOR/TFEB signaling axis in response to cellular nutrient states (Medina *et al.* 2015; Medina *et al.* 2011; Curcio-Morelli *et al.* 2010; Miedel *et al.* 2008; Pryor *et al.* 2006). These perturbations would be expected to lead to a compromise in autophagy, aberrations in endocytic trafficking, and possibly defects in mTORC1 signaling. These secondary defects may cause further dysfunction in the mucolipin-1-deficient neuron and lead to the pathologies documented within the brain (outer blue circle), including p62 aggregation, heterogenous storage, and changes to the lipid composition of cells (Micsenyi *et al.* 2009; Boudewyn *et al.* 2017; Grishchuk *et al.* 2014). Such chronic changes beginning early in life could contribute to developmental abnormalities, synaptic dysfunction, and even perhaps structural changes to neurons or cell death. Further still, loss of lysosomal iron efflux through mucolipin-1 (inner circle) is reported to contribute to the formation of reactive oxygen species (ROS), which leads to mitochondrial aberrations (middle yellow circle) (Zhang *et al.* 2016; Colletti *et al.* 2012; Grishchuk *et al.* 2015). Many of these defective pathways would be anticipated to intersect and exacerbate one another. Defects in autophagy for example may contribute to a failure to degrade damaged lysosomes or mitochondria, the buildup of which may contribute to cell death mechanisms in MLIV through release of apoptotic-triggering cathepsins (Colletti *et al.* 2012). Understanding the interplay between this deeply intertwined network, including in different cell types, will be important to understanding the full picture of MLIV neuropathogenesis and help us better understand how to treat this disorder. Therapeutic targets are also highlighted with yellow arrows denoting potential intervention points,

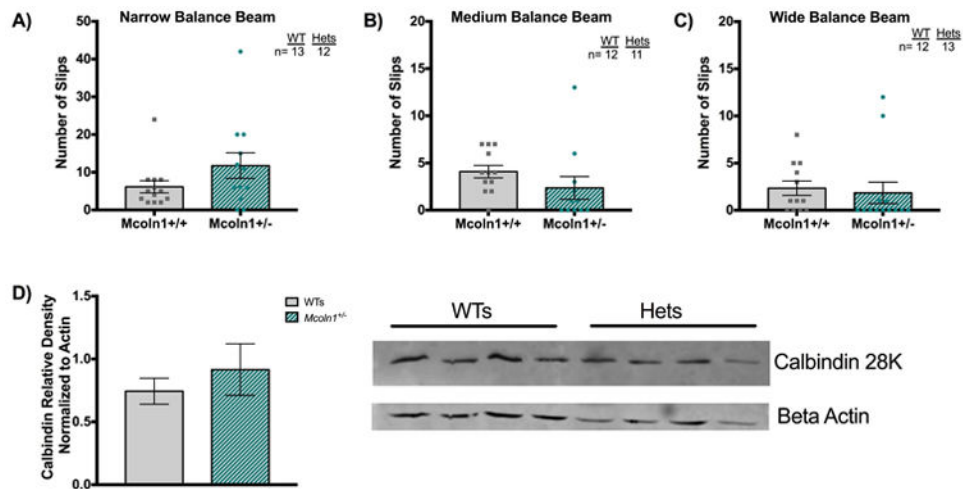
including compensation for mucolipin-1's loss (inner circle, perhaps with gene therapy), targeting reactive oxygen species for example with antioxidants (middle circle), or targeting mTOR/TFEB signaling (perhaps with mTOR and TFEB modulators), storage (perhaps with substrate reduction therapy), or cell death (perhaps with miglustat or other therapeutics when cell death mechanisms are better identified in this disease). This does not include all potential therapeutics, and additional broader therapies such as anti-inflammatories or other small molecule-based treatments remain to be explored in the context of MLIV.

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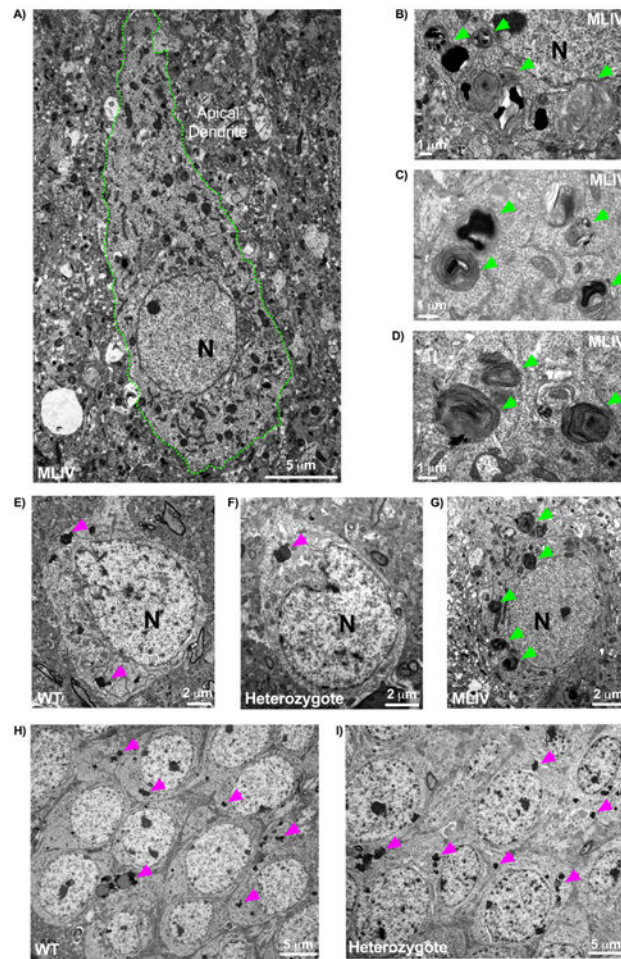
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**Figure 6. Preliminary data on behavioral performance and calbindin density in senescent *Mcoln1* heterozygous mice**

A) Average number of slips on the narrow (i.e., most difficult), medium (intermediate difficulty, B), and widest (easiest, C) beam are plotted. While heterozygotes on the hard beam appears to have slightly more slips than WT, no significant differences were detected for this or other beams analyzed by ANOVA (A-C). N=number of mice tested on each plot. D) Calbindin density detected via western blot of heterozygous versus WT tissues (n=4 mice aged 20-25 months per genotype, average density plotted  $\pm$  SEM,  $p=0.4914$ ) suggested Purkinje cell health is comparable between the two genotypes. Statistical analyses performed using unpaired t-test with Welch's correction.



**Figure 7. Canonical ultrastructural features of *Mcoln1*<sup>-/-</sup> mice compared to aged heterozygous and WT mice**

A) Characteristic pathology in *Mcoln1*<sup>-/-</sup> 3-month old tissues revealed by electron microscopy. Shown here is a neocortical neuron with the neuronal cytoplasm found congested with multiple compound, electron dense storage bodies as well as granulomembranous storage bodies and an enlarged apical dendrite (cell membrane outlined in green). B-C) Electron dense, compound storage bodies typically found in 3-month-old mutant mice, taken here from neocortex and highlighted by green arrows. E-G) Neocortical neurons from (E) WT, (F) heterozygote, and (G) mutant. E-F) WT and heterozygote neocortex exhibited neurons possessing lipofuscin accumulation (pink arrowheads) but otherwise devoid of pathological, compound storage bodies readily detectable in the mutant (G, green arrowheads). Neither heterozygous or WT neurons (both shown here at 18+ months) were found to exhibit abnormal structures, such as the swollen apical dendrites captured in 3-month old mutant neocortex (A). H-I) Hippocampal sections from WT (H) and heterozygous (I) mice. Similar to findings in cerebral cortex, hippocampal neurons displayed comparable features between WT (H) and heterozygotes (I) such as lipofuscin granulation storage with no evidence of pathological storage. Lipofuscin was not found to be quantitatively increased in the heterozygote in either cerebral cortex or hippocampus (data not shown). All tissues shown here are representative images from mice aged 18+ months,

with the exception of the mutant at 3 months. 2 heterozygotes, 4 WT, and 4 *Mcoln1*<sup>-/-</sup> mice were probed for pilot EM data.

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