INVolvEMENT OF GdAp1 IN THE REGuLaTION OF MtoRc1 ACTIVITY IN A DROSOPHILA MLIV MODEL

Kristen Clemons

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INVolVEMENT OF GDAP1 IN THE REGULATION OF Mtorc1 ACTIVITY IN
A Drosophila MLIV MODEL

A

Thesis

Presented to the Faculty of
The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
MAster of Science

by

Kristen Nicole Clemons, B.S.
Houston, Texas

December 2018
Dedication

To all who have helped along the way

With guidance, faith, a kind word to say

A smile won, a laugh shared

Knowing that someone cared

Made this thesis possible today
Acknowledgments

Many thanks and much gratitude to all who made this thesis possible.

My advisor, Dr. Kartik Venkatachalam, for his guidance, support, and patience

My lab mates, for being welcoming and supportive

   Morgan Rousseau, for being my friend

   Nick Karagas, for being helpful in all circumstances

   Yufang Chao, for showing me my first larval dissection

My advisory committee members, for having my interests at heart

My family, for being supportive no matter what

Rice Temple Baptist Church, for being the community I needed
ININVOLVEMENT OF GDAP1 IN THE REGULATION OF MTORC1 ACTIVITY IN A DROSOPHILA MLIV MODEL

Kristen Nicole Clemons, B.S
Advisory Professor: Kartik Venkatachalam, Ph.D.

The master regulator of metabolism and growth, mechanistic target of rapamycin complex 1 (mTORC1), is responsible for maintaining metabolic homeostasis by sensing nutrient and energy levels within the cell to promote or inhibit translation and autophagy accordingly. In the childhood neurodegenerative disorder Mucolipidosis type IV (MLIV), mTORC1 activity is decreased. The underlying mechanism for reduced mTORC1 signaling in MLIV is poorly understood. The gene encoding ganglioside-induced differentiation associated protein 1 (GDAP1) is transcriptionally upregulated in MLIV. This project investigated the involvement of GDAP1 in MLIV disease pathology.

Using the UAS/GAL4 system in an established MLIV Drosophila model, we knocked down expression of GDAP1. To determine the effect on mTORC1 activity, we measured phosphorylation levels of the mTORC1 downstream target S6 kinase (S6K) and quantified changes in synaptic growth at the larval neuromuscular junction (NMJ), which is a cell biological readout for mTORC1 activity. We found that knocking down GDAP1 expression can partially suppress the decreased phosphorylated S6K levels and rescue the reduced NMJ synaptic growth that occurs in MLIV Drosophila larvae. These results indicate that GDAP1 plays a role upstream of mTORC1 to affect signaling in MLIV cells. With this knowledge, we draw closer to understanding the
dysregulation of mTORC1 signaling that occurs in MLIV patients and gain insight into an incompletely understood mechanism for regulating metabolism.
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<tr>
<td>4E-BP1</td>
<td>eukaryotic translation initiation factor 4E-binding protein 1</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
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<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>AMPK</td>
<td>5’ AMP-activated protein kinase</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>aut.</td>
<td>autophagosome</td>
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<td>autolys.</td>
<td>autolysosome</td>
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<td>CMT</td>
<td>Charcot-Marie-Tooth disease</td>
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<td>DLG</td>
<td>discs large</td>
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<td>GAP</td>
<td>GTPase activating protein</td>
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<td>GDAP1</td>
<td>ganglioside-induced differentiation associated protein 1</td>
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<td>GDH</td>
<td>glutamate dehydrogenase</td>
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<td>HD1</td>
<td>hydrophobic domain 1</td>
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<td>horse radish peroxidase</td>
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<td>LSD</td>
<td>lysosomal storage disease</td>
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<tr>
<td>lys.</td>
<td>lysosome</td>
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<td>mitogen-activated protein kinase</td>
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<td>Mucolipidosis type IV</td>
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<td>mTORC1</td>
<td>mechanistic target of rapamycin complex 1</td>
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<td>NMJ</td>
<td>neuromuscular junction</td>
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<tr>
<td>OMM</td>
<td>outer mitochondrial membrane</td>
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<tr>
<td>p</td>
<td>phospho- or phosphate</td>
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<tr>
<td>Rheb</td>
<td>Ras homolog enriched in brain</td>
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<td>S6K</td>
<td>S6 kinase</td>
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<tr>
<td>SNARE</td>
<td>soluble N-ethylmaleimide sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>SSR</td>
<td>subsynaptic reticulum</td>
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<tr>
<td>TFEB</td>
<td>transcription factor EB</td>
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<tr>
<td>TMD</td>
<td>transmembrane domain</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>TRPML1</td>
<td>transient receptor potential mucolipin 1</td>
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<tr>
<td>TSC1/TSC2</td>
<td>tuberous sclerosis 1/ tuberous sclerosis 2</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activating sequence</td>
</tr>
<tr>
<td>ULK1</td>
<td>unc-51 like autophagy activating kinase 1</td>
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<tr>
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<td>Wallenda</td>
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1. Introduction

A cell’s ability to regulate its metabolism is essential to the fitness of an organism. The master regulator of metabolism and growth, mechanistic target of rapamycin complex 1 (mTORC1), coordinates various aspects of metabolism (Dibble and Manning, 2013; Laplante and Sabatini, 2012). Dysregulation of the mTORC1 pathway is implicated in the pathogenesis of many human diseases, such as cancers and metabolic disorders (Dazert and Hall, 2011; Menon and Manning, 2008; Laplante and Sabatini, 2012). One such metabolic disorder with mTORC1 signaling dysregulation is the neurodevelopmental disorder Mucolipidosis type IV (MLIV) (Wong et al., 2012). In addition to reduced mTORC1 activity, MLIV cells mishandle gangliosides (Bach et al., 1975; Zeigler et al., 1992). Gangliosides regulate the expression of the ten ganglioside-induced differentiation associated proteins (Liu et al., 1999). Preliminary data (not shown) has indicated that transcriptional upregulation of ganglioside-induced differentiation associated protein 1 (GDAP1) occurs in MLIV fibroblasts. While a connection between GDAP1 and mTORC1 activity has yet to be established, this preliminary result suggests that the two may be linked. Using the model organism Drosophila melanogaster, we investigate the involvement of GDAP1 in the metabolic regulation of MLIV cells. Probing the mechanistic underpinnings of this phenomenon will provide us with a more complete understanding of the metabolic regulation that is disrupted in MLIV. Ultimately, this insight could lead to innovative new therapies for this debilitating disease.
1.1 Mechanistic target of rapamycin complex 1

Metabolism consists of anabolism and catabolism. Anabolism consists of processes that build up the cell, such as protein and lipid synthesis. Conversely, catabolism consists of processes that break down cellular components, such as autophagy. There must be a homeostasis of these processes for the cell to function optimally. To evaluate their resources, needs, and environmental situations, cells rely on the master regulator mTORC1. As a master regulator, mTORC1 receives cues from at least four major inputs (Brugarolas et al., 2004; Dibble and Manning, 2013; Gao et al., 2002; Inoki et al., 2002; Kimura et al., 2003), integrates the information, and communicates whether the cell is equipped for anabolism or whether it should engage in catabolism.

The kinase complex mTORC1 inversely regulates protein translation and autophagy (Dibble and Manning, 2013). When active, mTORC1 phosphorylates downstream targets such as S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) in a manner that encourages anabolism by facilitating protein translation (Zoncu et al., 2011a) (Figure 1, 2C). It also phosphorylates Wallenda (WND) (Fan et al., 1996), which is the first in a MAP kinase cascade that signals for neuromuscular junction (NMJ) synaptic growth through JNK (Fan et al., 1996; Wong et al., 2015) (Figure 1, 2C). Concomitantly, mTORC1 phosphorylates downstream targets such as unc-51 like autophagy activating kinase 1 (ULK1) and transcription factor EB (TFEB) to discourage catabolism by inhibiting autophagy and biogenesis of lysosomal components (Figure 1, 2C). This coordinated activity establishes a feedback loop within the cell to maintain homeostatic amino acid levels. As such, amino acid availability is one of the main inputs regulating mTORC1 activity. Other factors that contribute to
mTORC1 regulation include ATP availability, growth factors, and stress (Brugarolas et al., 2004; Dibble and Manning, 2013; Kimura et al., 2003) (Figure 1).

Active mTORC1 is localized to the lysosomal membrane (Betz and Hall, 2013). Rag GTPase heterodimers and Ras homolog enriched in brain (Rheb) are key proteins in the localization and activation of mTORC1 on the lysosome. Rag GTPases respond to amino acid availability, and Rheb is indirectly modulated by ATP deprivation.

Rag GTPases are responsible for recruiting mTORC1 to the lysosome from the cytoplasm by responding to amino acid levels (Sancak et al., 2008). GTPases are molecular switches that interchange between GDP-bound (inactive) and GTP-bound (active) states. Rag proteins heterodimerize on the surface of the lysosome (Sancak et al., 2008). Amino acids regulate the Rag–mTORC1 interaction by promoting the active GTP-bound state of one of the Rags in the dimer (Sancak et al., 2008). Information regarding amino acid availability is derived from amino acids in the lysosomal lumen. While the amino acid sensing mechanism is not fully elucidated, it has been shown that the trimeric Ragulator complex interacts with the Rag dimer to aid in localization of mTORC1 to the lysosomal membrane (Sancak et al., 2010). Additionally, the Ragulator engages in amino-acid dependent interactions with v-ATPases, which are proton pumps that regulate lysosomal acidity (Bar-Peled and Sabatini, 2014; Zoncu et al., 2011b). In the presence of amino acids, the Rag heterodimer becomes active and promotes the translocation of mTORC1 to the lysosomal membrane (Figure 2A).

The small G protein Rheb is located on the lysosome and is an allosteric activator required for mTORC1 activity (Stocker and Rheb, 2003). The tuberous sclerosis 1/tuberous sclerosis 2 (TSC1/TSC2) complex deactivates Rheb by functioning as a
GTPase activating protein (GAP) (Inoki et al., 2003; Zhang et al., 2003) (Figure 2B). TSC1/TSC2 complex activity is positively regulated by 5′ AMP-activated protein kinase (AMPK) (Di Nardo et al., 2014). AMPK operates based on the ratio of AMP to ATP in the cell (Jeon, 2016). Through this energy sensing mechanism, AMPK inhibits mTORC1 when there are low energy levels. When there is more ATP than AMP present in a cell to indicate energy availability, mTORC1 is disinhibited (Jeon, 2016). In this manner, Rheb activates mTORC1 at the lysosome and AMPK informs mTORC1 of the energy status of the cell.
Figure 1. mTORC1 activators, inhibitors, and downstream targets

Amino acids, energy (ATP), growth factors, and stress are the major sources of mTORC1 regulation. When active, mTORC1 activates anabolic processes and inhibits catabolic processes.

Adapted from (Rabanal-Ruiz et al., 2017)
Figure 2. mTORC1 regulation and activity of mTORC1

A) Rag GTPase dimers regulate mTORC1 localization to the lysosomal membrane. The left panel indicates cytoplasmic mTORC1 localization with an inactive Rag dimer. The right panel depicts Rag activation in the presence of luminal amino acids and localization of mTORC1 to the lysosomal membrane.

B) AMPK regulates the activity of TSC1/TSC2, which modulates the activity of the mTORC1 allosteric activator Rheb. AMPK activity is regulated by the ratio of AMP to ATP in a cell. The left panel indicates that Rheb is deactivated by the AMP-driven activity of AMPK and the GAP activity of the TSC1/TSC2 complex. The right panel indicates that Rheb is active when AMPK activity is decreased due to more ATP in the cell.

C) mTORC1 is active on the lysosomal membrane in the presence of an amino acid-activated Rag dimer and the active allosteric mTORC1 activator Rheb. Activated mTORC1 phosphorylates S6K and WND, which initiates mRNA translation and NMJ synaptic growth, respectively. Activated mTORC1 phosphorylation of ULK1 inhibits the ability of ULK1 to initiate autophagy.
1.2 Mucolipidosis type IV

MLIV is an autosomal recessive lysosomal storage disease (LSD) that results from a loss-of-function mutation in MCOLN1, the gene encoding the endolysosomal ion channel transient receptor potential mucolipin 1 (TRPML1) (Altarescu et al., 2002; Sun et al., 2000). MLIV was first recognized as a disease in 1974. It was described in a male infant of Ashkenazi Jewish ancestry (Berman et al., 1974). He presented with corneal opacity and abnormal accumulation of storage bodies in the liver, conjunctiva, and cultured skin fibroblasts (Berman et al., 1974). A majority of the affected individuals are of Ashkenazi Jewish ancestry, however MLIV is a pan-ethnic disorder (Amir et al., 1987; Bargal et al., 2001). It occurs in 1 in 40,000 people, and the onset is early childhood (Altarescu et al., 2002; Amir et al., 1987; Bach, 2001). As the disease progresses, more features present themselves: severe psychomotor disability, impaired cognitive function, anemia, and achlorhydria (Altarescu et al., 2002; Amir et al., 1987; Wakabayashi et al., 2011).

There is no effective treatment currently available for MLIV patients. Physical therapy can delay loss of muscle tone, but confinement to a wheelchair occurs by the end of the second decade of life (Bach, 2001; Wakabayashi et al., 2011). No strategies have been devised to counteract the cognitive impairment, although some patients have been reported to make small, continuous progress in language and cognitive functions (Amir et al., 1987). A promising therapeutic option would be to address the defects in cell signaling. Promoting healthy cellular communication may be able to circumvent some of the downstream effects caused by having nonfunctional TRPML1 channels.
1.3 Transient receptor potential mucolipin 1

TRPML1 is a cation channel that is predominantly localized to late endosomes and lysosomes (Manzoni et al., 2004; Wong et al., 2012). It is responsible for the release of calcium that ultimately enables the fusion of endosomes and autophagosomes to lysosomes (Wong et al., 2012) (Figure 3A). Endosomes and autophagosomes bring materials to lysosomes for degradation (Fader and Colombo, 2009). As the compartments draw close to each other, fusion machinery, known as soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARES), tether the two vesicles together (Corona and Jackson, 2018). Then, TRPML1 channels release the calcium ions that enables the complete fusion of endosomes and autophagosomes to lysosomes through regulating components of the SNARE fusion machinery (Corona and Jackson, 2018) (Figure 3A). This fusion is a crucial step in delivering materials that need to be degraded to the lysosome.

Cells that do not have functional TRPML1 channels have impaired lysosomal function. Having lost their ability to complete the fusion process, lysosomes lacking TRPML1 are caught in a “fusion-clamped” state with the opposing compartment and therefore, are hindered in their ability to degrade proteins (Figure 3B) (Wong et al., 2012). This incomplete fusion results in insufficient degradation of waste materials, a consequence of which is fewer luminal amino acids available for detection by mTORC1. With a perceived lack of amino acid availability, mTORC1 ceases its suppression of autophagy. One of the main roles of the autophagy process is to raise amino acid levels by recycling current cellular components. In wild type animals, completion of autophagy leads to restored mTORC1 activity. However, in tissues from
the *Drosophila* MLIV model, autophagosomes are unable to fuse with lysosomes, resulting in an inability to complete the autophagy program and leaving the cell unable to restore mTORC1 activity (Wong et al., 2012).
Figure 3. TRPML1 function is impaired in Mucolipidosis type IV

The fusion of an autophagosome (aut.) with a lysosome (lys.) requires two steps: tethering together of compartments by the SNARE machinery and release of calcium ions from TRPML1. The fusion process is depicted in wild type (A) and MLIV (B) cells. Due to the lack of TRPML1 in MLIV cells, autophagosomes and lysosomes are unable to complete the fusion process.
1.4 Ganglioside accumulation in MLIV

Further findings from MLIV samples and models have revealed accumulations of mucopolysaccharides, phospholipids, and a 2-3-fold excess of GM3 and GD3 gangliosides (Bach et al., 1975, 1977, 1980; Bargal and Bach, 1989). Gangliosides are sialic acid-containing glycosphingolipids that primarily localize to the outer leaflets of the plasma membrane (Sonnino et al., 2007). They are integral components of the cholesterol-rich microdomains that participate in events such as cell-cell recognition and signal transduction (Lopez and Schnaar, 2009). The composition of cellular gangliosides changes from simple to more complex during the course of development due to the spatiotemporal regulation of ganglioside synthases (Ishii et al., 2007; Ngamukote et al., 2007; Vajn et al., 2013). However, in MLIV, there is an excess of the simple gangliosides GM3 and GD3. As development progresses, there is also regulation of the ten ganglioside-induced differentiation associated proteins (GDAPs). Preliminary data has shown that human retinal pigmented epithelial cells lacking functional TRPML1 have a transcriptional upregulation of GDAP1. Unsuccessful turnover of ganglioside species and the downstream results of this deviation, including GDAP1 upregulation, may have a role in the dysfunction that occurs in MLIV cells. Indeed, the mishandling of gangliosides occurring in MLIV adds a layer of complexity to our current understanding of the disease mechanism that merits investigation.

1.5 Ganglioside-induced differentiation associated protein 1

GDAP1 is an integral membrane protein of the outer mitochondrial membrane (OMM). It is a tail-anchored protein with one transmembrane domain (TMD) located at its carboxy terminal end (Wagner et al., 2009). The TMD and the basic amino acid
residues surrounding it facilitate GDAP1’s localization to the OMM (Niemann et al., 2005; Wagner et al., 2009). At the OMM, GDAP1 contributes to mitochondrial health by functioning as a fission factor (Niemann et al., 2005; Wagner et al., 2009). The actions of fission and fusion illustrate mitochondrial dynamics. This dynamic process is important for the regulation of mitochondrial morphology and overall organelle health (Youle and van der Bliek, 2012). Dysregulation of mitochondrial dynamics is associated with neurodegenerative diseases (Bertholet et al., 2016; Pareyson et al., 2015). In fact, mutations in GDAP1 results in the neuropathy Charcot-Marie-Tooth disease (CMT). Niemann et al. showed that point mutations in GDAP1 occurring among CMT patients result in reduced mitochondrial fission capabilities (Niemann et al., 2005).

According to bioinformatic analyses, GDAP1 shows structural similarity to the theta class glutathione S-transferase (GST) enzyme family (Huber et al., 2016; Shield et al., 2006). It has an N-terminal and a C-terminal GST domain, both of which are located in the cytosol. Huber et al. (2016) were able to show in vitro that these domains do possess GST ability. Although, this activity was shown in a truncated construct of GDAP1 lacking the TMD and hydrophobic domain 1 (HD1). The HD1 is amphipathic and critical for mitochondrial fission activity (Huber et al., 2016; Wagner et al., 2009).

1.6 Drosophila melanogaster as a model for investigating MLIV

With well-characterized genetics, a broad range of genetic tools available, and easily recognizable developmental stages, Drosophila melanogaster is an excellent model organism for investigating interactions in the neurodevelopmental disease MLIV. Additionally, there is an established MLIV fly model (trpml') and available assays for mTORC1 activity in flies (Venkatachalam et al., 2008; Wong et al., 2012, 2015).
The *Drosophila* larva neuromuscular system is a good genetic model for studying the excitatory synapses of the mammalian central nervous system (Menon et al., 2013). Additionally, synaptic growth at the neuromuscular junction (NMJ) can be used as a cell biological read out for mTORC1 activity (Wong et al., 2015). NMJ synaptic growth can be quantified by counting the number of boutons present at an NMJ.

Boutons are the round enlargements of an axon that form a synapse with another cell. *Drosophila* have three main types of boutons. Type 1 boutons are glutamatergic while types 2 and 3 are modulatory and use other neurotransmitters (Menon et al., 2013). Of the type 1 boutons, there are 1b and 1s boutons, for big and small respectively. The size classification is based on the amount of subsynaptic reticulum (SSR) present. At the NMJ, SSR is the post-synaptic muscle membrane that surrounds the pre-synaptic bouton (Menon et al., 2013). It consists of scaffolding proteins, neurotransmitter receptors, and post-synaptic signaling complexes. One of the scaffolding proteins of the SSR is called disc large (DLG) (Chen and Featherstone, 2005).

During neural development, boutons are frequently being generated and eliminated (Menon et al., 2013). Therefore, the ability to distinguish a functional bouton from a nonfunctional bouton is essential. Functional boutons are recognized by participation of both pre-synaptic and post-synaptic membranes in synapse formation. A reliable post-synaptic marker is the scaffolding protein DLG. An accepted pre-synaptic marker is horse radish peroxidase (HRP), which stains axons (Nässel, 1983; Romero et al., 1999). Therefore, functional boutons can be identified by containing both the pre-synaptic HRP signal and the post-synaptic DLG signal. By counting functional boutons according to these parameters, we can approximate the activity level of mTORC1.
Another way to measure mTORC1 activity in *Drosophila* is to assay the phosphorylation status of the mTORC1 downstream target S6K in larvae fat body tissue. The *Drosophila* fat body is a highly metabolic organ. It is the main site for energy storage and utilization (Arrese and Soulages, 2010; Law and Wells, 1989). In these capacities, it is similar in function to the human liver and adipose tissue. It coordinates energy utilization and biosynthesis to meet the metabolic needs of the organism (Arrese and Soulages, 2010). Being centrally located and bathed in hemolymph, the fat body is ideally positioned to integrate signals from other organs (Arrese and Soulages, 2010). With such an insight into the metabolic health of the organism, the fat body is well suited for assaying mTORC1 activity.

### 1.7 Project summary and hypothesis

With the knowledge that mTORC1 activity is essential to the well-being of cells, elucidating its regulation in disease states can give us insight into potential therapeutic targets and provide a more complete understanding of metabolism and its regulation. The aim of this project was to determine if GDAP1 plays a role in the dysregulated mTORC1 signaling present in cells lacking TRPML. We hypothesized that GDAP1 induction affects the cellular and neurological defects prevalent in MLIV patients. To investigate the relationship between GDAP1 levels and mTORC1 activity in MLIV, we knocked down expression of *GDAP1* in the *Drosophila* model of MLIV (*trpml*), and determined mTORC1 activity by measuring phosphorylation status of the downstream target S6K and quantifying synaptic growth at neuromuscular junctions. Understanding the involvement of GDAP1 in this disease process brings us one step closer to
comprehending the molecular events that occur in MLIV and draws us to a more complete understanding of metabolic regulation as a whole.
2. Materials and Methods

2.1 Drosophila strains

The Drosophila strains acquired for this study were: \textit{w^{118}; P(GD10713)v21624 (UAS-GDAP1\textsuperscript{RNAi})} (Vienna Drosophila Resource Center) (Dietzl et al., 2007), \textit{elav-GAL4} (pan-neuronal expression) (Lin and Goodman, 1994) and \textit{cg-GAL4} (hemocyte and fat body expression) (Hennig et al., 2006). The \textit{UAS-GDAP1\textsuperscript{RNAi}} line was used as the control for these studies. The \textit{trpml\textsuperscript{I}} related strains used were: \textit{trpml\textsuperscript{I}/TM6\textbeta} (Venkatachalam et al., 2008), \textit{elav-GAL4;; trpml\textsuperscript{I}/TM6\textbeta, cg-GAL4; trpml\textsuperscript{I}/TM6\textbeta}, and \textit{UAS-GDAP1\textsuperscript{RNAi}; trpml\textsuperscript{I}/TM6\textbeta}. The experimental lines were generated by crossing \textit{elav-GAL4} or \textit{cg-GAL4} or \textit{UAS-GDAP1\textsuperscript{RNAi}} with \textit{trpml\textsuperscript{I}/TM6\textbeta} or \textit{UAS-GDAP1\textsuperscript{RNAi}} accordingly.

2.2 Drosophila husbandry

All fly stocks were raised at room temperature (~22 °C) in 25 mm vials. Ingredients of the fly food were as previously described (Wong et al., 2012).

Each fly cross consisted of 5 males and 5 virgin females. These crosses were maintained at 25 °C under 12-hour light-dark cycle. After the first 2-3 days, the adults were flipped into new vials with fresh food, and the old vials were discarded. Larvae from this second set of vials were used for experiments.

2.3 NMJ dissections

2.3.1 Preparing fillets

Wandering third instar larvae were selected from vials and placed in dissection dishes containing Sylgard (Dow Corning) where they were bathed in cold 1X phosphate
buffered saline (PBS) solution. Larvae were pinned at the anterior and posterior ends. An incision was made on the dorsal surface of the body wall to expose the internal organs. The internal organs were removed, leaving only the brain inside. Four to six fillets were pinned flat on the Sylgard surface and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 30 minutes. The fillets were then washed in 0.1% Triton X in PBS for ~15 minutes.

2.3.2 Immunohistochemistry for bouton visualization

The primary antibody solution consisted of 1:100 anti-discs large-c (α-DLG) (DSHB) and 5% NDS in 200 µl 0.1% Triton X in PBS. Fillets were incubated in the primary antibody solution overnight at 4 °C. They were washed in 0.1% Triton X in PBS ~20 minutes, and then incubated with secondary antibody solution for 2 hours on a rocker at 60 rpm. Secondary antibody solution contained: 1:200 anti-horse radish peroxidase (α-HRP) (Jackson), 1:400 AlexaFluor 568nm goat anti-mouse IgG (Life Technologies), and 5% NDS in 200 µl 0.1% Triton X in PBS. Then the fillets were washed for another ~15 minutes in 0.1% Triton X in PBS. The fillets were then mounted on a glass slide using Vectashield mounting medium with DAPI (Vector Laboratories).

2.3.3 Confocal imaging of NMJs

Confocal images of the NMJs were obtained using a Nikon A1 Confocal Laser Microscope System. Using the 60X oil-immersion objective lens, a z-stack of the NMJ in segment A3 at muscle 6/7 located on either side of the midline of each animal was obtained using channel series line 1-> 4.
2.3.4 Parameters for counting boutons

NMJ synaptic growth was quantified by counting the number of type 1 boutons present at each NMJ. Boutons were counted as functional if they were positive for both the presynaptic HRP signal and the post-synaptic DLG signal.

2.3.5 Analysis of bouton numbers

GraphPad Prism 6 software was used to analyze the NMJ synaptic growth data. Welch’s t test and the Mann Whitney test were used to detect differences in conditions.

2.4 Fat body dissections

2.4.1 Larvae starvation procedure

Third instar larvae were removed from their food and placed in a vial containing cotton wet with a 1% sucrose solution. These vials were incubated at 25 °C for either 4 hours or 15 hours before being dissected.

2.4.2 Dissecting out fat bodies

Female wandering third instar larvae were selected from vials, placed in dissection dishes containing Sylgard (Dow Corning), and bathed in cold Schneider’s Insect Medium (Sigma Life Science). Larvae were pinned at their anterior and posterior ends. An incision was made on the dorsal surface of the body wall to expose the internal organs. The fat bodies were separated from the other organs and removed from the solution. They were placed in a depression well slide containing 45 µl 2X Laemmli sample buffer (Bio-Rad) with 5% β-mercaptoethanol. After pipetting briefly, the samples were heated at 100 °C for 5 minutes. Fat bodies were dissected out of animals one at a time, but the fat bodies from two animals went into each sample.
2.4.3 Western blot procedures

Fat body samples were heated at 100 °C for 2 minutes, briefly vortexed, and centrifuged for 30 seconds at 15,000 rpm. The samples were then loaded into a 4%-20% gradient gel (Bio-Rad) and run at 60 V for 2.5 hours. Proteins were transferred onto a nitrocellulose membrane at 120 mA for 3 hours at 4 °C. Before being probed with antibodies, the membrane underwent blocking in Odyssey Blocking Buffer for 40 minutes while being rocked at 60 rpm. The primary antibodies used to probe the membrane were as follows: 1:1000 rabbit P-Drosophila p70 S6 Kinase (Cell Signaling) and 1:1000 mouse anti-α-tubulin (DSHB). The secondary antibodies used were 1:15,000 IRDye 680LT goat anti-rabbit and 1:20,000 IRDye 800CW goat anti-mouse, both from LI-COR. These proteins were visualized using the LI-COR Odyssey infrared imager and Odyssey Program.

2.4.4 Analysis of pS6K quantification

Western blots were quantified using ImageJ software. Analysis of Western blot quantifications was done using unpaired t tests in GraphPad Prism 6 software.
3. Results

3.1 GDAP1 knockdown enables rescue of NMJ synaptic growth in *trpml*<sup>1</sup> mutant larvae

Neuromuscular junction (NMJ) synaptic growth is regulated by mTORC1 activity (Wong et al., 2015). In order to determine the effect of GDAP1 on NMJ synaptic growth, we used a neuronal driver in the *UAS/GAL4* system to express a *UAS-GDAP1<sup>RNAi</sup>* construct in the neurons of third instar larvae from wild type and *trpml*<sup>1</sup> mutant backgrounds (Figure 4A). Using immunohistochemistry, we visualized the NMJ by staining with HRP, a pre-synaptic marker in nervous tissue, and DLG, a post-synaptic marker in muscle tissue. Confocal images of the NMJs at muscles 6/7 of the A3 segment of these preparations were used for quantification of NMJ synaptic growth (Figure 4B).

At the molecular level, Figure 4C depicts the sequence of events that ordinarily occur to result in NMJ synaptic growth. In wild type larvae, there is no significant difference in the number of boutons in larvae expressing the *UAS-GDAP1<sup>RNAi</sup>* construct in their neurons compared with control larvae that do not express the *UAS-GDAP1<sup>RNAi</sup>* construct in their neurons (p = 0.4452) (Figure 4D-E). This indicates that knocking down expression of GDAP1 alone does not affect NMJ synaptic growth.

In homozygous *trpml*<sup>1</sup> larvae, there was a 15% decrease in the number of boutons present at the NMJ compared to *trpml*<sup>1</sup> heterozygous larvae (p = 0.0084). This deficit was rescued by expressing the *UAS-GDAP1<sup>RNAi</sup>* construct in the neurons of homozygous *trpml*<sup>1</sup> larvae (p = 0.0095) (Figure 4D-E). This recovery indicates that knocking down expression of *GDAP1* in neurons enables wild type levels of NMJ
synaptic growth in trpm1 homozygous larvae, suggesting that GDAP1 plays a role upstream of NMJ synaptic growth.
Figure 4. *GDAP1* knockdown enables rescue of NMJ synaptic growth in *trpml* mutant larvae

A) Schematic depicting the *UAS/GAL4* system used to knock down *GDAP1* expression in neuronal tissue.

Adapted from (Prüßing et al., 2013)

B) Graphic representation of a larva fillet. The red box indicates segment A3 muscle 6/7 of a larva. On either side of the midline, this is the location of the NMJs imaged for all NMJ synaptic growth quantification. The ventral nerve cord refers to the lower portion of the brain from where motor neuron axons emanate.

Adapted from (Inoshita et al., 2017)

C) The relevant molecular events that occur to yield NMJ synaptic growth. TRPML1 releases calcium ions to aid in the fusion of an autophagosome and lysosome. The lysosome degrades proteins resulting in luminal amino acids, which recruit mTORC1 to the lysosomal surface. mTORC1 phosphorylates the MAP kinase cascade that includes JNK. JNK activity stimulates NMJ synaptic growth.

D) Representative confocal images of NMJs in larvae of different genotypes. Magenta stain indicates the post-synaptic signal, DLG. Green stain indicates the pre-synaptic signal, HRP.

E) Quantification of bouton numbers in all NMJs visualized. **p<0.01

The number of NMJ images quantified for each genotype are as follows from left to right: n = 16, 28, 40, 13, 34
3.2 GDAP1 knockdown in *trpml* mutant larvae enables recovery of S6K phosphorylation levels

To evaluate mTORC1 signaling biochemically, we expressed the *UAS-GDAP1*RNAi construct in the fat body tissue of wild type and *trpml* homozygous larvae and assayed for phosphorylation of S6K, a substrate of mTORC1 (Figure 5A). Figure 5B depicts the molecular events that occur to result in S6K phosphorylation. It has previously been shown that *trpml* homozygous larvae have reduced mTORC1 signaling compared to wild type larvae (Wong et al., 2012). My results are consistent with this finding. The *trpml* homozygous larvae have decreased pS6K levels in comparison with those of wild type larvae (p = 0.0249) (Figure 5C). This decrease in pS6K level is then rescued by expressing the *UAS-GDAP1*RNAi construct in fat body tissue of *trpml* homozygous larvae (p = 0.0402). The rescued pS6K level indicates that knocking down expression of *GDAP1* in fat body tissue contributes to restoration of mTORC1 signaling in *trpml* homozygous larvae.
Figure 5. **GDAP1 knockdown in trpml' mutant larvae enables recovery of S6K phosphorylation levels**

A) Schematic depicting the UAS/GAL4 system used to knock down GDAP1 expression in fat body tissue.

Adapted from (Prüßing et al., 2013)

B) The relevant molecular events that occur to yield S6K phosphorylation. TRPML1 releases calcium ions to aid in the fusion of an autophagosome and a lysosome. The lysosome degrades proteins resulting in luminal amino acids, which recruits mTORC1 to the lysosomal surface. mTORC1 phosphorylates S6K.

C) Quantification of pS6K signal from fat body tissue normalized to tubulin. *p<0.05

The number of fat body samples quantified for each genotype are n = 8, 7, 4 for control, trpml', and GDAP1 knockdown in trpml', respectively.

D) Quantification of pS6K signal at different time points in a starvation time course.

pS6K signal is normalized to tubulin. *p<0.05

The number of fat body samples quantified for each genotype at the 4-hour starvation time point are n = 8, 7, 6 for control, trpml', and GDAP1 knockdown in trpml', respectively.

The number of fat body samples quantified for each genotype at the 15-hour starvation time point are n = 8, 6, 3 for control, trpml', and GDAP1 knockdown in trpml', respectively.
3.3 Knockdown of GDAP1 aids in recovery of S6K phosphorylation level in response to starvation in \( \text{trpml}^l \) mutant larvae

Another way to prevent mTORC1 suppression of autophagy is through starvation, which removes the dietary intake of mTORC1-activating amino acids. Through use of a starvation time course, we can evaluate the recovery process of S6K phosphorylation level in response to a stressor. During the starvation time course, the larvae had access to sucrose as a source for ATP production. This was to ensure that the mTORC1 input being affected was amino acid availability.

There is a divergent response in mTORC1 activity during starvation of wild type and \( \text{trpml}^l \) larvae. The larvae from the wild type background show a decrease in pS6K levels at 4 hours of starvation followed by a clear recovery of pS6K levels at 15 hours of starvation (Figure 5D). This indicates that the animal registered the depletion of luminal amino acids and initiated a method to restore the amino acid deficit, namely autophagy. In this manner, it was able to successfully replenish luminal amino acid levels to restore mTORC1 activity. The larvae from the \( \text{trpml}^l \) mutant background exhibit the opposite response. The \( \text{trpml}^l \) pS6K levels decrease at 4 hours of starvation and do not recover over the starvation time course. At 15 hours of starvation, there is a 50% decrease in pS6K level from wild type to \( \text{trpml}^l \) mutant larvae \((p = 0.0018)\). This suggests that the method to replenish luminal amino acids is unable to function in \( \text{trpml}^l \) mutant larvae. However, the \( \text{trpml}^l \) larvae that express the \( \text{UAS-GDAP1}^{RNAi} \) construct show pS6K levels that are 25% greater than those of \( \text{trpml}^l \) larvae not expressing the construct \((p = 0.0398)\). This indicates that knocking down expression of GDAP1 aids in the recovery of mTORC1 activity in cells that lack functional TRPML.
4. Discussion

mTORC1 plays a major role in the regulation of metabolism. Understanding how it is affected in disease states can give us insight into the complexity of metabolic regulation. Through this investigation, I have shown that knockdown of GDAP1 enables rescue of NMJ synaptic growth and recovery of pS6K levels in trpml1 mutant larvae, a cell biological and biochemical readout for mTORC1 activity, respectively. Additionally, I have shown that knockdown of GDAP1 in trpml1 mutant larvae aids in the recovery of pS6K levels in response to starvation. These results support the hypothesis that GDAP1 impacts mTORC1 activity, therefore identifying a role for GDAP1 upstream of the master kinase. This conclusion implies that the mechanism through which GDAP1 is affecting mTORC1 activity is through one of the four main mTORC1 inputs: ATP availability, amino acid availability, growth factors, or stress.

GDAP1 could be regulating mTORC1 activity through manipulating ATP availability. ATP is generated predominantly through oxidative phosphorylation in the mitochondria. Mitochondrial dynamics play an important role in regulating energy production (Youle and van der Bliek, 2012; Zemirli et al., 2018; Westermann, 2012). One role of fission is to segregate dysfunctional portions of mitochondria to be degraded eventually by mitophagy (Westermann, 2012; Youle and van der Bliek, 2012). This process reduces the amount of ATP that can be synthesized by way of eliminating some of the ATP synthesis machinery. With this understanding, we can hypothesize that by inhibiting fission, we can protect mitochondria from mitophagy.

Additionally, fusion can mitigate the loss of this machinery by causing complementation of dysfunctional mitochondria with healthy mitochondria, thereby
prolonging mitochondrial function by revitalizing the components (Westermann, 2012; Youle and van der Bliek, 2012). In this way, fusion can increase oxidative phosphorylation and ATP synthesis. Fusion during cellular stress enables the cell to meet the increasing energy demand. In this regard, a highly connected mitochondrial network correlates with increased ATP production (Westermann, 2012). It has been shown that elongated mitochondria have been spared from autophagy (Gomes et al., 2011; Rambold et al., 2011). The added connectivity of these mitochondrial networks is associated with oxidative phosphorylation efficiency. Additionally, there is some evidence to suggest that mitochondrial elongation is facilitated by knocking down GDAP1 expression (Niemann et al., 2005). In this manner, knocking down expression of the fission protein GDAP1 may cause an increase in ATP production. Increased ATP levels would garner less AMPK suppression of mTORC1 thereby enabling increased mTORC1 activity. In fact, targeting mitochondrial fission has previously been suggested to help with mitochondrial dysfunction occurring in neurodegenerative diseases (Reddy, 2014).

To investigate GDAP1 involvement in altering the ATP levels in cells lacking TRPML1, we could block fission pharmacologically and assay for AMPK activity. This would help us determine if it is the reduced mitochondrial fission capacity of the GDAP1 knockdown cells that affect mTORC1 activity.

Alternatively, knockdown of GDAP1 could be increasing mTORC1 activity through increasing amino acid availability. Loss of TRPML1 function affects amino acid availability. This is known because ectopic addition of amino acids suppresses the phenotypes caused by loss of TRPML1 function (Wong et al., 2012). Mitochondria have
the capacity to generate amino acids, such as glutamine. Being that mTORC1 is sensitive to glutamine (Jewell et al., 2015), increasing generation of glutamine in the mitochondria could aid in recovery of mTORC1 activity.

The citric acid cycle intermediate α-ketoglutarate can be transformed into glutamine in two reactions. Glutamate dehydrogenase (GDH) catalyzes the reaction from glutamate to α-ketoglutarate within the mitochondria (Prough et al., 1973). GDH is negatively regulated by ATP, meaning that high ATP levels would cause the reaction to favor glutamate generation (Prough et al., 1973). GDH is positively regulated by ADP, indicating that low energy levels would drive the reaction toward α-ketoglutarate generation (Prough et al., 1973). Glutamate can generate glutamine through a reaction catalyzed by glutamine synthase (Frigerio et al., 2008). In this model, as ATP levels increase from reduced fission, GDH is inhibited and the α-ketoglutarate in the cell is converted into glutamate which can then be converted into glutamine. Glutamine in turn can provide the amino acids to increase mTORC1 activity. In this fashion, mitochondrial activity can increase the amino acid availability of the cell.

In sum, this study has revealed a role for the fission protein GDAP1 in the regulation of mTORC1 activity in a Drosophila model of Mucolipidosis type IV. Figure 6 depicts the cross talk between lysosomes and mitochondria that our findings suggest occur to impact mTORC1 activity. Regardless of the mechanism of action, the results of this study support our hypothesis and suggest that the upregulation of GDAP1 known to occur in MLIV patients contributes to the decreased mTORC1 activity in cells that lack functional TRPML1. As this line of research continues, we may find GDAP1 to be a suitable target for MLIV therapeutics. As it stands, the research contained in this study
supplies a valuable piece of knowledge to our understanding of MLIV and metabolic regulation as a whole.
Figure 6. Cross talk between lysosomes and mitochondria impacts mTORC1 activity

Lysosomal function is known to be required for mTORC1 activation and processing gangliosides. Gangliosides regulate the transcription of GDAP1, a gene that encodes a mitochondrial protein. Our findings suggest that the upregulation of GDAP1 known to occur in MLIV patients acts to negatively regulate mTORC1 activity in cells with lysosomal dysfunction.
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Vitae

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