2020

The role of ATGL-1 in CeTOR regulated longevity in C. elegans

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

THE ROLE OF ATGL-1 IN CeTOR REGULATED LONGEVITY IN *C. ELEGANS*

by

DRAKE HECHTER

B.Sc., University of Winnipeg, 2019

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2020

Approved by

First Reader

Konstantin Kandror, Ph.D.
Professor of Biochemistry

Second Reader

Nava Zaarur, Ph.D.
Senior Research Scientist

Third Reader

Jude T. Deeney, Ph.D.
Assistant Professor of Medicine

DEDICATION

To my parents and siblings. Thank you – this would not have been possible without you.

ACKNOWLEDGMENTS

I am thankful for the knowledge and connections I have gained throughout my time at Boston University in the Nutrition and Metabolism program. A huge thank you Dr. Moore, Dr. Deeney, Dr. Spartano and Dr. Pickering. Your contagious passion, determination and love for the field will remain with me always. Thank you to Dr. Kandror for allowing me to join the lab and for your guidance during this project. Toli, Debasish and, of course, Nava, thank you so much for your patience and expertise. This would not have been possible without you and I am incredibly thankful.

THE ROLE OF ATGL-1 IN CeTOR REGULATED LONGEVITY IN C. *ELEGANS*

DRAKE HECHTER

ABSTRACT

Aging is a major risk factor for many chronic diseases and a complex biological phenomenon. The most well studied and characterized pathways involved in metabolism and known to regulate longevity include sirtuins, AMP-activated protein kinase, insulin-like growth factor (IGF) and the mechanistic target of rapamycin (mTOR).[1] These signaling pathways and related transcriptional factors are evolutionarily conserved from yeast to primates.

Evidence suggests adipose tissue plays an important role in the regulation of lifespan particularly through energy homeostasis during times of scarcity and excess. Our laboratory has shown adipose triglyceride lipase (ATGL), the rate-limiting enzyme within the lipolytic pathway, is the target of dietary restriction and insulin/IGF-1 signaling pathways, both of which regulate lifespan.[22] Given the convergence and necessity of ATGL-1 in the longevity response of dietary restriction and reduced insulin/IGF1 signaling pathways and the uncertainty of the downstream effects TOR has on longevity, we hypothesize that ATGL-1 plays an important role in CeTOR regulated longevity in C. *elegans.*

This investigation was carried out by (a) determining whether levels of ATGL-1 are influenced by TOR inhibition via rapamycin and TOR specific RNA interference (RNAi) and (b) examining the role of ATGL-1 in CeTOR regulated longevity in C.

elegans. We have found that rapamycin treatment does not increase expression of ATGL-1::GFP in C. *elegans*, however, continued research with CeTOR inhibition using rapamycin and RNAi treatment is necessary. The RNAi and longevity experiments need to be conducted.

Tissue specific regulation of ATGL expression has been shown to be implicated in chronic disease and in longevity. However, there are still many insights to be discovered and understood about its role in longevity pathways, including feedback mechanisms and second messengers lipolytic products play. Elucidating the downstream effects of ATGL within model organisms will impact future chronic disease research and longevity studies. Given that these pathways are widely evolutionarily conserved, future findings will aid in understanding longevity regulatory mechanisms in humans.

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LIST OF ABBREVIATIONS

AC ..... Adenylyl Cyclase

AMP ..... Adenosine Monophosphate

ATGL ..... Adipose Triglyceride Lipase

C. *elegans* ..... Caenorhabditis *elegans*

cAMP ..... Cyclic Adenosine Monophosphate

DR ..... Dietary Restriction

dsDNA ..... double stranded deoxyribonucleic acid

dsRNA ..... double stranded ribonucleic acid

E. coli ..... Escherichia coli

Egr-1 ..... Early growth response transcription factor

FoxO ..... Forkhead Box Protein O1

FUdR ..... 5'-fluorodeoxyuridine

GAP ..... GTPase-activating protein

GTP ..... Guanosine-5'-triphosphate

HSL ..... Hormone Sensitive Lipase

IGF ..... Insulin-like Growth Factor

IIS ..... Insulin/Insulin-like Growth Factor 1 Signaling Pathway

IPTG ..... Isopropyl β-D-1thiogalactopyranoside

IRS-1 ..... Insulin Receptor Substrate-1

JNK ..... c-Jun N-terminal kinase

LB ..... Lysogeny broth

MGL ..... Monoacylglycerol Lipase

Nrf1/2/3 ..... Nuclear respiratory factors

PDK-1 ..... Phosphoinositide-dependent kinase-1

PI3K ..... Phosphoinositide 3-kinase

PIP3 ..... Phosphatidylinositol Phosphate 3

PKA ..... Protein Kinase A

PKB ..... Protein Kinase B

PPAR ..... Peroxisome Proliferator-Activated Receptor

Raptor ..... Regulatory-associated protein of mTOR

RHEB ..... Ras homolog enriched in brain

Rictor ..... Rapamycin-insensitive companion of mTOR

RNAi ..... Ribonucleic acid interference

RNase ..... Ribonuclease

SREBP ..... Sterol Regulatory Element-Binding Protein

Tet ..... Tetracycline

TOR ..... Target of Rapamycin

TSC ..... Tuberous Sclerosis Complex

VLDL ..... Very low-density lipoprotein

INTRODUCTION

I. Longevity/Aging Regulation

Aging is a major risk factor for many chronic diseases and a complex biological phenomenon. Many genes and pathways have been discovered to modulate aging and longevity, however, the complete mechanism by which these pathways affect longevity requires further research. Aging is subject to regulation by signaling pathways and transcriptional factors that are evolutionarily conserved from yeast to primates. The most well studied and characterized pathways known to regulate longevity with regard to metabolism include sirtuins, AMP-activated protein kinase, Insulin-like growth factor (IGF) and the mechanistic target of rapamycin (mTOR).[1] Mediators of lifespan and health-span extension are found within nutrient and stress sensors,[2] thus, energy homeostasis plays a vital role in the regulation of longevity. For example, dietary restriction is the best-known signal to extend lifespan across organisms.[2,8]

In the mammalian organism, adipose tissue is largely responsible for energy homeostasis.[3] Given the storage capacity and elasticity of adipose tissue, control of lipolysis, the process by which triglycerides are broken down, is crucial for energy partitioning and balance.[4,5] The accumulation of excess adipose tissue is associated with aging and other diseases such as diabetes, hypertension, cancer and neurodegenerative diseases. There is a negative correlation between fat and longevity. Decreasing and/or surgical removal of adipose tissue stores have been shown to increase longevity. Previous studies have shown that mutations affecting fat mass and/or removal of visceral fat increases lifespan.[6] Energy homeostasis is a complex process that requires multiple

signaling cascade networks which detect and respond to nutrient availability. These networks play an essential role for life and have been shown to mediate lifespan.[7]

II. Adipose Triglyceride Lipase (ATGL)

Under readily available nutrient conditions, insulin stimulates the uptake of glucose and free fatty acids, inhibits lipolysis and stimulates de novo fatty acid synthesis within adipose tissue. In addition, through control of gene expression of fat-specific transcription factors such as SREBP-1c and PPARγ, insulin regulates growth and differentiation of adipose tissue.[9] During lipolysis, free fatty acids are released from adipocytes and are transported to other tissues where they can be used as fuel via fatty acid oxidation. Complete hydrolysis of triglycerides to glycerol and free fatty acids is accomplished by tri, di- and mono-acylglyceride lipases. These lipases are adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL), which act on triglycerides, diacylglycerides and mono-acylglycerides, respectively, and release a free fatty acid during each step. ATGL has been found to be the rate-limiting enzyme within in the lipolytic pathway.[4] The expression of ATGL is regulated by upstream targets such as FoxO1 and mTOR[22], the same metabolic pathways known to modulate longevity (**Figure 1**).

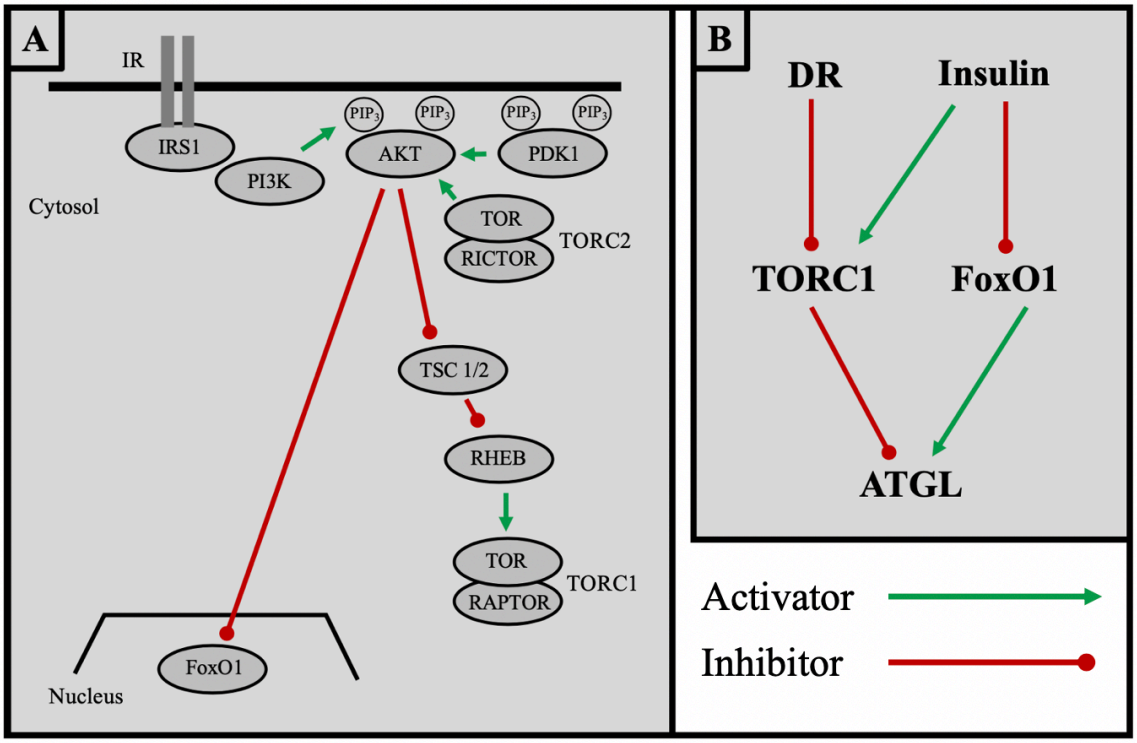

Figure 1. (A) Simplified diagram of the Insulin/Insulin-like Growth Factor 1 signaling (IIS) pathway. (B) TORC1 and FoxO1 regulation of ATGL expression. DR, Dietary Restriction.

Inhibition of lipolysis and promotion of triglyceride storage within adipose tissue is largely controlled by insulin. Impaired insulin response within individuals who are insulin resistant and/or type 2 diabetic result in high levels of circulating free fatty acids. These free fatty acids are picked up and stored within non-adipose peripheral tissues.[10] Accumulation of lipids within non-adipose tissue can lead to lipotoxicity which can have a number of adverse effects including endothelial dysfunction, insulin resistance and pancreatic β-cell death.[10,11] Therefore, adipose tissue and control of the lipolytic pathway, specifically through the rate-limiting enzyme, ATGL, is important for energy homeostasis and longevity.

The "critical nodes"[14] within the insulin/insulin-like growth factor 1 signaling (IIS) pathway that are responsible for the longevity response seen in model organisms are FoxO1 and TORC1, both of which have been shown to control the expression of ATGL.[3,4,18,24,40] Given the convergence and necessity of ATGL-1 in the longevity response of dietary restriction and reduced insulin/IGF1 signaling pathways, as well as the uncertainty regarding the downstream effects of TOR on longevity, we hypothesize that ATGL-1 plays an important role in CeTOR regulated longevity in C. *elegans.*

III. Insulin/Insulin-like Growth Factor 1 Signaling (IIS) Pathway

The IIS pathway plays a major role in the longevity response and is regulated by nutrient availability. During nutrient scarce conditions, catecholamines are released by the sympathetic nervous system to activate lipolysis. Stimulation of the β-adrenergic receptor on adipocytes activate adenylyl cyclase (AC) which elevates the levels of cAMP and increases protein kinase A (PKA) activity. PKA initiates lipolysis by direct phosphorylation of hormone-sensitive lipase (HSL) and perilipin as well as through indirect activation of ATGL.[4,15]

During nutrient rich conditions, the IIS receptor acts through insulin receptor substrate 1 (IRS-1), which recruits and activates PI3K. The increase in phosphatidylinositol phosphate 3 (PIP3) near the plasma membrane by PI3K activates the serine/threonine kinases PDK-1 and protein kinase B (PKB/Akt), which phosphorylate and inhibit tuberous sclerosis complex (TSC) made up of TSC1 and TSC2. TSC acts as a GTPase-activating protein (GAP) on Rheb (Ras homolog enriched in brain). Rheb is a small key GTPase

upstream activator of TORC1 which directly binds with TORC1 and, in the GTP-bound form, stimulates its kinase activity. Therefore, phosphorylation and inactivation of TSC allows Rheb to activate TORC1 signaling (**Figure 1A)**.[13,14,16,50]

Critical Nodes within the IIS Pathway

3.1 FoxO

Transcription factors are also regulated by the IIS pathway. The Forkhead box O (FoxO) family of transcription factors alter gene expression and have been shown to have effects on longevity. These FoxO transcription factors are conserved from C. *elegans* to mammals. Within mammalians, they have a number of functions such as tumor suppression, stress resistance, DNA damage repair, cell cycle regulation and energy homeostasis. FoxO proteins promote gluconeogenesis and enhance food intake, thus are critical in energy metabolism. FoxO1 has proven to be an important regulator of lipid homeostasis and triglyceride partitioning between different tissues. FoxO1 stimulates VLDL production within the liver, boost lipid oxidation in skeletal muscle and increases lipolysis in adipose tissue. These actions work in concert to regulate lipid homeostasis.[4]

Changes in subcellular localization is the major mechanism by which FoxO transcription factors are regulated.[3] This is accomplished through post-translational modifications particularly through acetylation, mono- and polyubiquitination and phosphorylation. Most notably of the FoxO family, FoxO1 has been found to play a central role in the regulation of metabolism within several cell types. More specifically, FoxO1 increases the rate of lipolysis by increasing the expression of ATGL.[4]

Activation of the IIS pathway by insulin, inhibits the expression of ATGL in adipose tissue. Phosphorylation of FoxO1 by PKB/Akt renders the transcription factor unable to enter the nucleus and sequesters it within the cytoplasm, thereby preventing FoxO1 from modifying gene expression.[4,17] In contrast, when this pathway is not active, such as under low nutrient availability, FoxO1 can enter the nucleus to transcribe target genes such as ATGL (**Figure 1**).[13,14]

3.2 Target of Rapamycin (TOR)

The other major nutrient sensing pathway and critical node is TOR. TOR functions as the master regulator of cellular growth and metabolism in response to nutrient as well as hormonal cues. It has also been implicated in diseases such as diabetes, cardiac hypertrophy, cancer, neurodegenerative syndromes and aging. TOR has two complexes, complex 1 and 2 (abbreviated as TORC1 and TORC2), which function in distinct ways and regulate different downstream processes. TORC1 and TORC2 are defined by their association with Raptor and Rictor, respectively.[16]

The multi-function of TORC1 make the complex a key regulatory nexus which responds to nutrients, hormones and cellular energy status balancing anabolic and catabolic processes.[12] Amino acids, oxygen, energy and growth signals activate TORC1, which regulates lysosomal biogenesis, ribosomal biogenesis, cap-dependent translation, thermogenesis, protein and lipid synthesis and autophagy.[18,22] Previous research in C. *elegans* and mice has demonstrated that post-developmental inhibition of TORC1 results in adult life extension. Consistent with the role of TOR, knockdown of TORC1 also enhances environmental stress tolerance.[16] Within C. *elegans*, TORC1 inhibition results in

reduced mRNA translation as well as has a positive effect on lifespan partially through DAF-16/FoxO and SKN-1 (ortholog of mammalian Nrf1/2/3 proteins). These transcription factors regulate genes that protect against environmental, metabolic and proteotoxic stress as well as promote longevity.[18,19] TORC1 suppresses lipolysis within adipocytes through the immediate-early response transcription factor (Egr-1), which directly inhibits ATGL gene expression.[20]

The biological functions of TORC2 are not well understood, although evidence indicates that it is also important for growth.[19] TORC2 signaling is responsive to growth factors mediated by PI3K but is insensitive to nutrients.[15,21] Glucose uptake in response to insulin involves TORC2 phosphorylation of Akt at Serine position 473, which is required for Akt activity on AS160.[15] 3

IV. C. *elegans* as a Model Organism

The functionality of adipose tissue and lipid droplets is conserved between many species including drosophila, mammals and C. *elegans*. Complete genome sequencing of the nematode reveals not only the evolutionarily conservation of the genes involved in lipolysis and fatty acid synthesis but also the nutrient sensing and energy homeostasis signaling pathways such as the IIS and CeTOR. C. *elegans* express the insulin receptor (DAF-2) and have evolutionarily homologues of FoxO (DAF-16) and TOR kinase (LET-363) (**Figure 2)**. The worms lack defined adipose tissue, however, they store fat within their intestine which appear to function and store lipid droplets efficiently.[27,34] The nematodes have proven to be excellent model organisms for energy homeostasis, specifically within lipid droplets, as well as longevity studies. The frequently used wildtype

Bristol strain N2 have an average lifespan of approximately 20 days. This short lifespan contributes to the convenience of using these nematodes as a model organism for longevity experiments.

Fertilization to hatching at room temperature occurs in approximately 12 hours and the resulting first-stage larva contain about 550 cells. Adulthood is reached in ~3 days after the newly hatched larvae progress through four larval molts. The pharynx and intestine make up the inner tube which is surrounded by a fluid-filled hydrostatic body cavity and the outer tube is made up of the musculature, hypodermis and collagenous cuticle. This simple anatomy of C. *elegans* contributes to its use as a model organism.[28]

The lifespan of C. *elegans* can be extended by a number of treatments or mutations demonstrating that it is an important model organism for longevity research. These manipulations shift cells from states favouring growth to states of maintenance and stress resistance. TOR signaling, dietary restriction, FoxO/DAF-16, SKN-1/Nrf, heat-shock factor, sirtuins and c-Jun N-terminal kinase (JNK) have been shown to influence the lifespan of C. *elegans*.[26,53] In addition, the ability to utilize pharmacological agents such as rapamycin as well as regulate gene expression, make C. *elegans* excellent models for longevity studies.

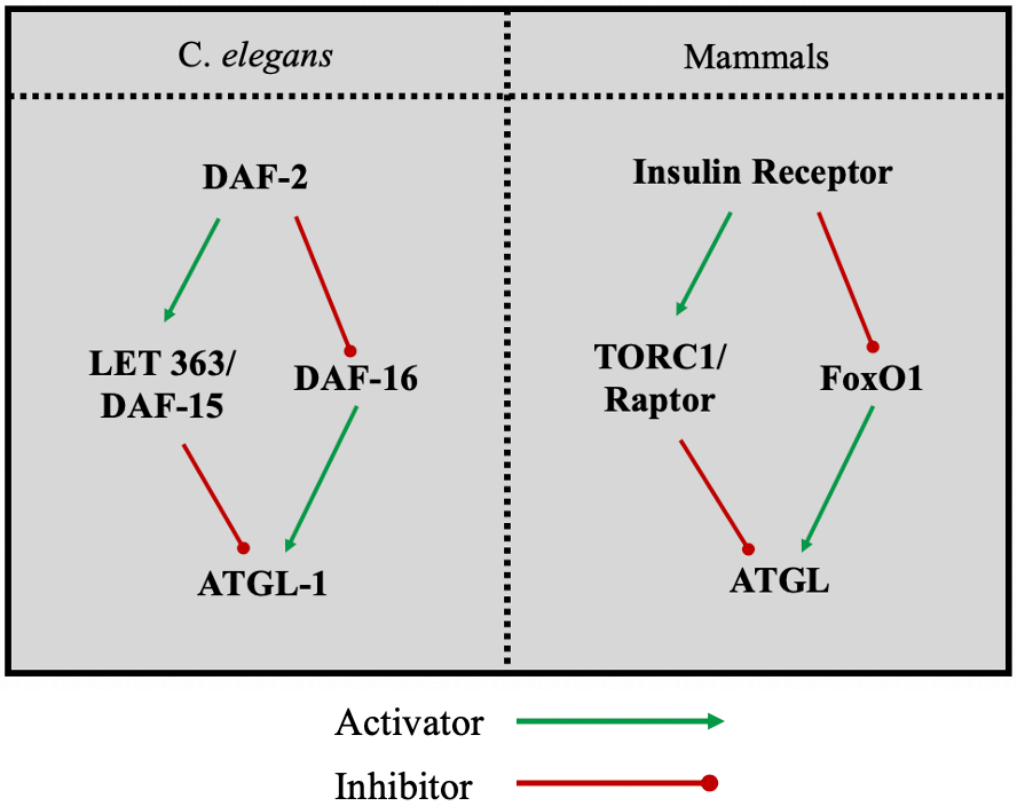

Figure 2. Regulation of ATGL expression in mammals and C. *elegans*.

Studies using fluorescence microscopy have indicated that ATGL-1::GFP are localized on the surface of lipid droplets in overexpressed ATGL-1 (orthologue of mammalian ATGL) mutant and wildtype C. *elegans*, which are distinct from the natural auto-fluorescent lysosomal organelles the worms contain.[29,30] ATGL-1::GFP which contain overexpressed levels of ATGL-1, have an average lifespan of about 28 days, a 40% lifespan increase when compared to wildtype. It was also shown that ATGL-1 is required for the longevity effects of both daf-2-induced (ortholog of mammalian insulin receptor) and dietary restriction, and that ATGL-1 is regulated by the DAF-2/DAF-16 (Insulin

receptor/FoxO) axis (**Figure 3**). These results suggest that the life extension response of reduced insulin/insulin-like growth factor 1 signaling (IIS) and dietary restriction pathways not only converge on, but also require the activity of ATGL-1.[22]

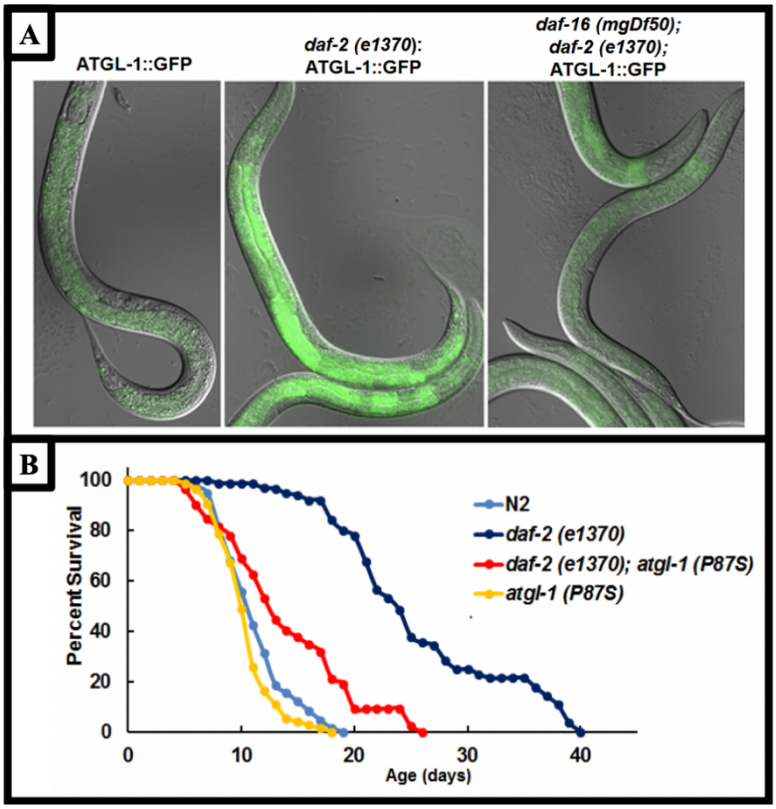

Figure 3. Expression of ATGL-1 is regulated by the *daf-2/daf-16* pathway (A). The lifespan of different C. *elegans* strains. The ATGL-1 loss-of-function mutant suppresses the longevity response of *daf-2* mutant (B). Taken from (Zaarur et al., 2019).[22]

V. TOR inhibition on Longevity

Rapamycin, dietary restriction and TOR inhibition via drug treatment and/or genetic modification appear to promote longevity at least partially through reduced mRNA translation.[18] Rapamycin treatment has been shown to extend lifespan in yeast, worms, flies and mice. Though it was first thought that the longevity effects of rapamycin were through inhibition of TORC1, evidence indicates sustained rapamycin treatment also reduces TORC2 activity, thus some of its effects on longevity may involve both TOR complexes.[18,19] Data has illustrated that the IIS and TOR pathways influence longevity via regulation of SKN-1 and DAF-16. It is largely accepted that DAF-16/FoxO is not required for lifespan extension by reduced TOR activity or most dietary restriction interventions.[18] Inhibition of TOR via RNAi has been shown to extend lifespan independently of daf-16, suggesting that TOR may act in a pathway distinct from the IIS. However, it has been postulated that these two pathways converge downstream of DAF-16 as TOR RNAi does not further extend lifespan of daf-2 mutants.[18]

Rapamycin upregulated genes activated by genetic TORC1 inhibition as well as genes that encode TORC1 pathway components in C. *elegans*. Similar to results from mammalian cells lines, it is suggested that rapamycin promotes longevity in C. *elegans* by interfering with both TOR complexes.[26] However, the downstream effects of TOR that promote longevity are still largely unknown.

VI. RNAi

RNA interference also called Post-Transcriptional Gene Silencing (PTGS) is a conserved biological response to double-stranded RNA that mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids and regulates gene expression.[31] RNAi is a specific, rapid and simple method for determining loss-of-function phenotypes of genes in C. *elegans* and genetic interactions can also be examined.[28]

Microinjection, feeding and soaking to deliver dsRNA are three methods used to induce RNAi in C. *elegans*. The feeding technique is simple and requires no microinjection system. Briefly, a cDNA that corresponds to the gene of interest is cloned into a bacterial expression vector between opposing phage T7 polymerase promoter sites. This feeding vector is then transformed into the *E. coli* HT115 strain which carries the DE3 lysogen. The HT115(DE3) strain contains the gene that encodes for the T7 RNA polymerase but lacks RNase III, thus, deficient in degrading dsRNA. The RNAi is prepared and seeded onto nematode growth medium (NGM) which contain the inducible factor, IPTG. IPTG is required to induce the expression of genes cloned downstream of the T7 promoter.[28] This study will utilize RNAi to inhibit the entire TOR kinase complex (let-363 in C. *elegans*).

VII. ATGL-1 in CeTOR Regulated Longevity in C. *elegans*

FoxO1 and TOR are "critical nodes"[14] within the IIS pathway, are opposingly regulated by Akt phosphorylation and convergence of these pathways has been shown to be an important mediator of longevity.[14] These nutrient sensing pathways converge on the regulation of ATGL expression. Research has shown that ATGL is required for lifespan

extension,[22] and FoxO1 and TORC1 regulate the rate of lipolysis by controlling expression of ATGL.[4,23,24,25] Thus, the purpose of this investigation is to (a) determine whether levels of ATGL-1 is influenced by TOR inhibition using rapamycin and TOR specific RNAi and (b) to examine the role of ATGL-1 in CeTOR regulated longevity in C. *elegans*.

It is clear adipose tissue plays an important role in the control of lifespan particularly through energy homeostasis during times of nutrient scarcity and excess. Combining the evidence that dietary restriction and IIS mediated longevity not only converge on ATGL activity but also require ATGL, and that TOR controls the expression of ATGL, further understanding of the role of ATGL in TOR regulated longevity within model organisms is needed. Given that these pathways are widely evolutionarily conserved, future findings will aid in understanding regulatory mechanisms of longevity in humans.

METHODS

I. C. *elegans* Strains

All C. *elegans* strains were maintained at 20ºC following the standard methods.[32] The commonly used Bristol strain N2 was used as the wild-type. The two other strains utilized were the VS20 *hjIs67 [atgl-1p::atgl-1::gfp + mec-7::rfp]*, and VC20458 containing *atgl-1 (gk176565) [P87S] III* strains, which were obtained from the Caenorhabditis Genetics Centre (CGC). The VS20 strain contains chromosome-integrated transgenic array *hjIs67 [atgl- 1p::atgl-1::GFP+mec-7p::RFP]*[33]) and was used to track expression of the ATGL-1 protein. This strain will be referred to as *atgl-1::gfp*. In order to get rid of the other mutations and specifically select the *atgl-1* mutant, the *atgl-1(gk176565)* mutation was outcrossed four times to generate AGK785 *atgl-1(gk176565)*. By doing so, these strains can be considered pure from other potential mutations they may carry from the nature of the mutagenic process by which they were generated.[22]

II. C. *elegans* Maintenance

The worms were cultured on 60mm plates. The plates contained the standard nematode growth medium (NGM) recipe (3 g of NaCl, 2.5 g of peptone, and 20 g of agar and bring to 1 L with H2O; liquid autoclave for 1hr and let cool; 1 mL of cholesterol [5 mg/mL in ethanol], 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, and 25 mL of 1 M [pH 6.0] KPO_4). The food source used was the standard streptomycin-resistance *E. coli* strain OP50, prepared as described by (Admasu et al., 2018). The worms were transferred to fresh

prepared plates weekly. The 150mm plates utilized contained the standard NGM recipe with the OP50-1 food source 10x concentrated.

III. Worm Synchronization

Using the protocol previously described by (Admasu et al., 2018), cultured worms from the 60mm plates were chunked onto 150mm pre-seeded NGM plates and left to grow until a large number of eggs and gravid adults were present on the plates (2-3 days at 20ºC). 10-13 mL of M9 buffer (0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.5% NaCl, 1 mM $MgSO_4$) was pipetted onto the plate to dislodge and collect the C. *elegans* by gently swirling. The collected nematodes were transferred to a 15 mL tube and treated with 20% alkaline hypochlorite solution (8.25mL ddH_2O, 3.75 mL 1M NaOH & 3.0 mL bleach). After being washed four times with M9 buffer, the embryos were left to hatch in the M9 buffer overnight. Hatched worms were harvested and seeded onto fresh 150mm NGM plates and left to grow to the adult L4 stage.[1,22]

IV. Rapamycin and Control Plate Preparation

The rapamycin treated plates were prepared on 35mm sized dishes using the standard NGM recipe, (as described earlier) with the addition of 100µL 5'-fluorodeoxyuridine (FUdR) 100 mg/mL (a final concentration of 250µM) and rapamycin, dissolved in DMSO at 50 mg/mL and added to a final concentration of 100µM. The FUdR was added to the medium to prevent synchronized worms from egg hatching.[1] The control plates contained FUdR and DMSO instead of rapamycin.

V. Rapamycin & Control Plate Seeding

Approximately 200 synchronized L4 stage N2 and *atgl-1::gfp* worms were harvested and collected from the 150mm NGM plates using M9 buffer and transferred to rapamycin or control plates. N2 worms were seeded on one rapamycin and one control plate and *atgl-1::gfp* worms seeded the same in both the 24- and 48-hour treatment groups. These plates were cultured at 20ºC for 24- and 48-hours before quantification of ATGL-1.

Quantification of ATGL-1

VI. RT-qPCR

Following a similar workflow described by (Zaarur et al., 2019), quantification of ATGL-1 RNA levels was accomplished using RT-qPCR. Synchronized L4 stage worms (roughly 200 worms) were washed by PBS, re-suspended in 1 mL of cold TRIzol (Ambion, Austin, TX), incubated at -80ºC and RNA was isolated following the manufacturer's instructions. Reverse transcription (RT) was carried out using a RETROscript kit (Ambion) and quantitative RT-PCR was performed using iTag Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) following the protocol of manufacturer with the following primers. *atgl-1*: forward 5' GATCGACCGATGATTTATCGAG 3', reverse 5' GAGCCAATCCACATTTGGTC 3'; a*ctin-1/3*: forward, 5' CACGAGACTTCTTACAACTCC 3', reverse, 5'GCATACGATCAGCAATTCCT 3'. Relative expression levels of all mRNAs were normalized to *actin-1/3* mRNA.

VII. Fluorescence Microscopy

Approximately 10-15 worms were hand-picked and mounted on 2% agarose pads which was positioned on glass microscope slides. The worms were immobilized with a drop of sodium azide to aid in accurate and consistent images. 10-14 photographs were taken using Zeiss Axiolmager Z1 (Carl Zeiss, Germany) at 20 and 40X magnification. All images had identical exposure times when GFP intensity was compared between the different conditions. Images of N2 worms were taken in the same fashion to determine the level of auto-fluorescence. The calculated mean of the N2 was subtracted from the *atgl-1::gfp* in order to quantify the fluorescence of ATGL-1 only. The level of fluorescence within the intestine was assessed using ImageJ software. The background of each image was accounted for by subtraction. The calculated mean of the N2 worms seeded on DMSO and FudR control plates was subtracted from the *atgl-1::gfp* worms to quantify the fluorescence of ATGL-1. In 10 randomly selected images from each group an unpaired two-tailed t-test was used to calculate the difference in means between treated and non-treated worms.

VIII. Western Blot

The western blot was completed as previously described.[34,49] Roughly 200 L4 synchronized N2 and *atgl-1::gfp* worms were sonicated in 1 M TSE solution at 20% output, 30s on 30s off, for eight rounds using the Branson 500 Sonic Dismembrator. The samples were then centrifuged at 12 000 rpm and 4ºC (Eppendorf AG 5424) and total protein content in the supernatant was quantified using Bradford Dye Reagent (Bio-Rad). Equal

amounts of total lysate from each sample was resolved using SDS-PAGE on a 10% gel (Invitrogen NuPAGE Bis-Tris) and transferred onto a 2-µm nitrocellulose membrane (Thermo Scientific) at 100 mA for 60 mins. The membrane was blocked with 5% (wt/vol) skim milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 (TBST; 25 mM Tris-HCl [pH 8.0], 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween 20) at room temperature for 30 min, then overnight incubation at 4ºC with anti-GFP antibody and anti-actin (Millipore MAB1501R) diluted 1:2000 in PBST-3% BSA. Following incubation, the membrane was washed with TBST 3 times and hybridized with secondary antibodies conjugated with horseradish peroxidase (Sigma-Aldrich) in 5% skim milk dissolved in TBST at room temperature for 2 hours. The membrane was then washed with TBST 3 times, incubated with enhanced chemiluminescence reagents and quantified with LuminoImager (LAS-3000) and Science Lab Image Gauge software (Fuji Photo Film). The intensity of each band was quantified using ImageJ.[34,49]

IX. RNAi Feeding

Following the RNAi feeding protocol as described by (Kamath et al., 2000) and (Li et al., 2018), synchronized L4 staged adults were mounted onto standard NGM plates which were seeded with individual dsRNA-expressing bacterial clones. HT115 bacteria transformed with either RNAi clones or empty vector pL4440 were grown over night in 12.5 µg/mL tetracycline and 50 µg/mL ampicillin. Cultures were diluted 1:10 and grown to an OD600 of 0.8-1.0 and induced with 0.7 mM IPTG, the following day. This culture was used to seed NGM plates containing tetracycline, ampicillin and 1 mM IPTG. RNAi

plasmid for *let-363*(RNAi) has been described in great detail by (Vellai et al., 2003). Given that the absence of LET-363/TOR activity causes developmental arrest at L3 larval stage, treatment with *let-363* double-stranded RNA during adulthood (L4 stage) was used. The *let-363* RNAi and empty vector control plates made up the experimental and control group respectively.

X. Oil-Red-O Staining

The 200 synchronized L4 stage N2 and *atgl-1::gfp* worms were harvested from their plate. The worms were washed with PBS and fixed in 1 mL of -20ºC methanol for 5 mins. Before centrifugation at 1000 rpm for 1 min, 2 mL of PBST (PBS with 0.01% Tween-20) was added. The supernatant was removed and the C. *elegans* were washed with PBST twice. 60% by volume water was added to Oil Red O (0.5% solution in isopropanol, Sigma) for 10 mins at room temperature. The solution was filtered using a 0.4µm syringe filter. Filtered 40% isopropanol Oil Red O solution (1 mL) for 20 mins was used to stain fixed worms. Stained worms were washed with PBST twice then mounted on slides with 2% agar. Images were taken with a colour camera using an inverted epi-fluorescent microscope (Carl Zeiss; Axio Observer D1).[22]

XI. Longevity Experiments

The longevity experiment used both the control N2 and the *atgl-1* mutant strain. Two L4 worms from each strain were picked and mounted onto standard NGM plated with OP50 bacteria. This allowed a synchronized generation to be grown. 30 worms from each

strain were picked and put onto standard NGM dishes. After 6 hrs many eggs were laid on each plate and the adult worms were removed. The hatched L1 larvae, were left for 2 days to reach late L3 stage/early L4 stage and the transfers began on that day, noted as day 1. 200 worms per strain were transferred to their respective plates, rapamycin treated, RNAi or control NGM plates. The plates (N = 20 worms per plate) were monitored and worms were scored as either live or dead every day. Once the worms stopped responding to probing by wired picks, they were considered dead and removed from the plate. Any worm(s) that disappeared, was under the agar or died unnaturally were removed and excluded from scoring. Unnatural deaths included internal larva hatching and bursting.[22]

All plates were kept at 20ºC and surviving worms were transferred to a fresh plate every second day to ensure the same worms were being included daily. The maximum lifespan was defined as the total time, in days, the worms were recorded as live starting at day 1. The time at which 50% of the worm population was alive, was defined as mean lifespan. Statistical significance of lifespan between the strains was found using a log rank analysis by Oasis software.[39]

RESULTS

I. Rapamycin Experiment

To determine whether levels of ATGL-1 are influenced by CeTOR inhibition by rapamycin, quantification of the level ATGL-1 after the 24- and 48-hour treatment was conducted. 200 synchronized L4 N2 and *atgl-1::gfp* C. *elegans* were each seeded onto two rapamycin treated plates and two control plates. This allowed two treatment groups of 24- or 48-hours to be made. The 24- and 48-hour groups contained N2 worms seeded onto one rapamycin and one control plate and *atgl-1::gfp* worms seeded on one rapamycin and control plate, thus each group contained four plates. After the 24- or 48-hour treatment was complete, RT-qPCR was conducted on each plate to determine the ATGL-1 RNA level. The design of this experiment can be seen in **Figure 4A.**

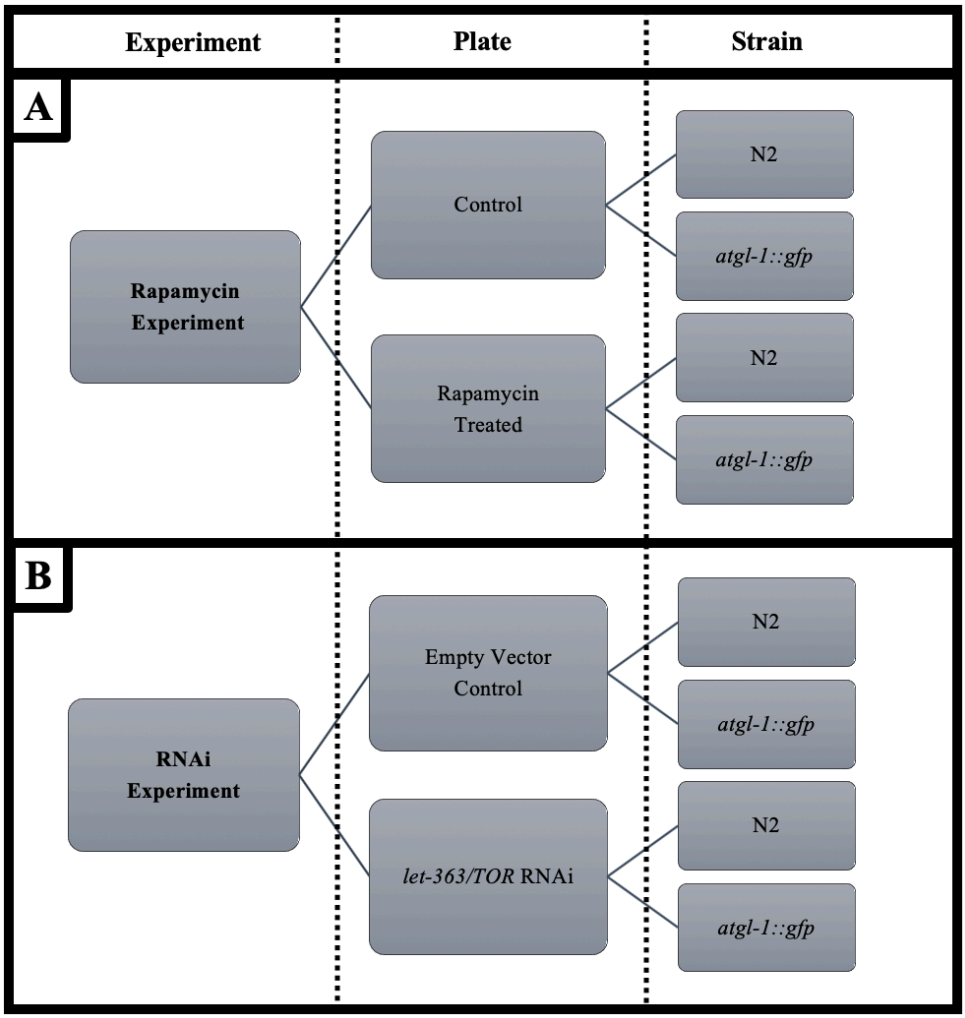

Figure 4. Design of the rapamycin and RNAi experiments.

Fluorescence microscopy was conducted after the 24- and 48-hour treatment using 10-15 hand-picked N2 and *atgl-1::gfp* C. *elegans* from each plate then mounted onto their own agarose pad positioned on microscope slides. The worms were immobilized with sodium azide and 10-14 images per slide were taken using Zeiss Axiolmager Z1 (Carl Zeiss, Germany) at 20 and 40X magnification. Images of the N2 worms were taken to determine the level of auto-fluorescence. The calculated mean of the N2 was subtracted

from the *atgl-1::gfp* in order to quantify the fluorescence of ATGL-1 only. Each image was also corrected for its background fluorescence. Fluorescent ATGL-1::GFP levels corrected for N2 auto-fluorescence from the *atgl-1::gfp* C. *elegans* after 24-and 48-hours of rapamycin or control treatment are shown in **Figure 5**. The same values collected from N2 worms seeded on DMSO and FudR control plates were used to correct for auto-fluorescence of the *atgl-1::gfp* worms regardless of the treatment time. Using an unpaired two-tailed t-test, the corrected fluorescent ATGL-1::GFP levels within the *atgl-1::gfp* rapamycin treated worms were decreased when compared to the *atgl-1::gfp* control. These results were found to be statistically significant after 24-hours but not after 48-hours **(Figure 5)**. These results reveal that rapamycin treatment decreased the protein level of ATGL-1. These findings could be attributed to small sample size and/or large inter-worm variation. Results of the Oil-Red-O staining are critical to obtain to see if lipolysis is increased. These results are expected to indicate that CeTOR inhibition by rapamycin result in an increase in lipolysis. Using the Oil-Red-O stain as confirmation, a more complete understanding of the level of ATGL-1 can be discovered.

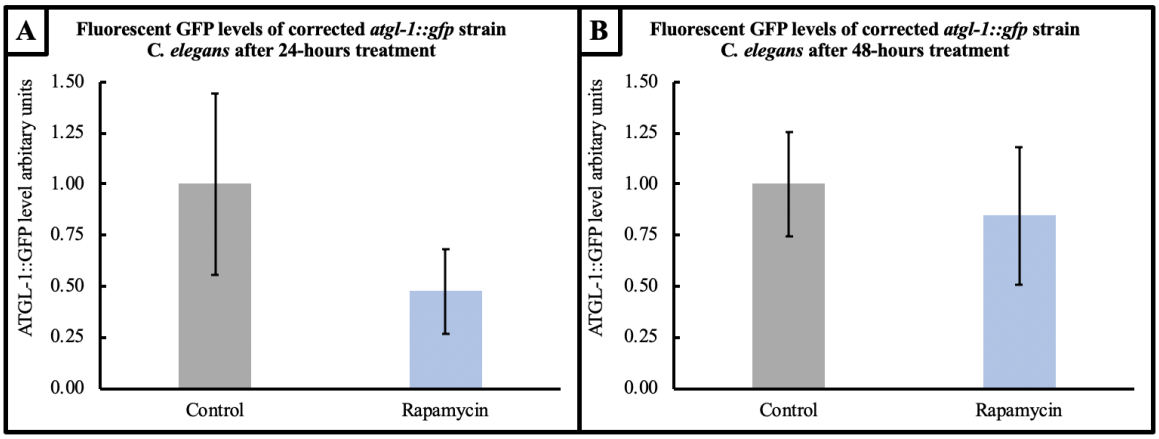

Figure 5. Rapamycin treatment does not increase expression of ATGL-1::GFP in C. *elegans*. GFP fluorescence was measured in *atgl-1::gfp* expressing C. *elegans* after treatment with rapamycin for 24-and 48-hours. Individual worms (10 per slide) were randomly selected from 10-14 images taken at 20 and 40X magnification. The intestine of each worm was captured during the ImageJ assessment of the level of fluorescence. The calculated mean of the N2 worms seeded on DMSO and FudR control plates was subtracted from the *atgl-1::gfp* worms to quantify the fluorescence of ATGL-1. Unpaired two-tailed t-test was used to calculate the difference in means between the 24- and 48-hour treatment times. The values were found to be significant after 24-hour treatment ($p < 0.05$ after 24-hours [p=0. 0.003]), and not statistically significant after 48-hour treatment ($p > 0.05$ after 48-hours [p= 0.26863113]).

200 L4 synchronized N2 and *atgl-1::gfp* plated C. *elegans* were used to conduct a western blot analysis on each plate to determine ATGL-1 protein level. Western blots were done following the 24- and 48-hour treatment of rapamycin or control plates.

The RT-qPCR, fluorescent microscopy and western blot used to quantify ATGL-1 after CeTOR inhibition via rapamycin are expected to result in an increase lipolysis via increased ATGL-1 expression and activity. In order to confirm the effectiveness of the rapamycin treatment methodology, to ensure it was performing as expected and increasing the rate of lipolysis, Oil-Red-O staining within each of the 24- and 48-hour treated plates

was conducted. This was used to detect triglyceride stores. A depletion of these reserves indicates an increase in lipolysis. Previous findings published by our laboratory have shown dietary restriction of N2 worms for 6-hours depletes triglyceride reserves and up-regulates *atgl-1* mRNA. These results can be seen in **Figure 6.**

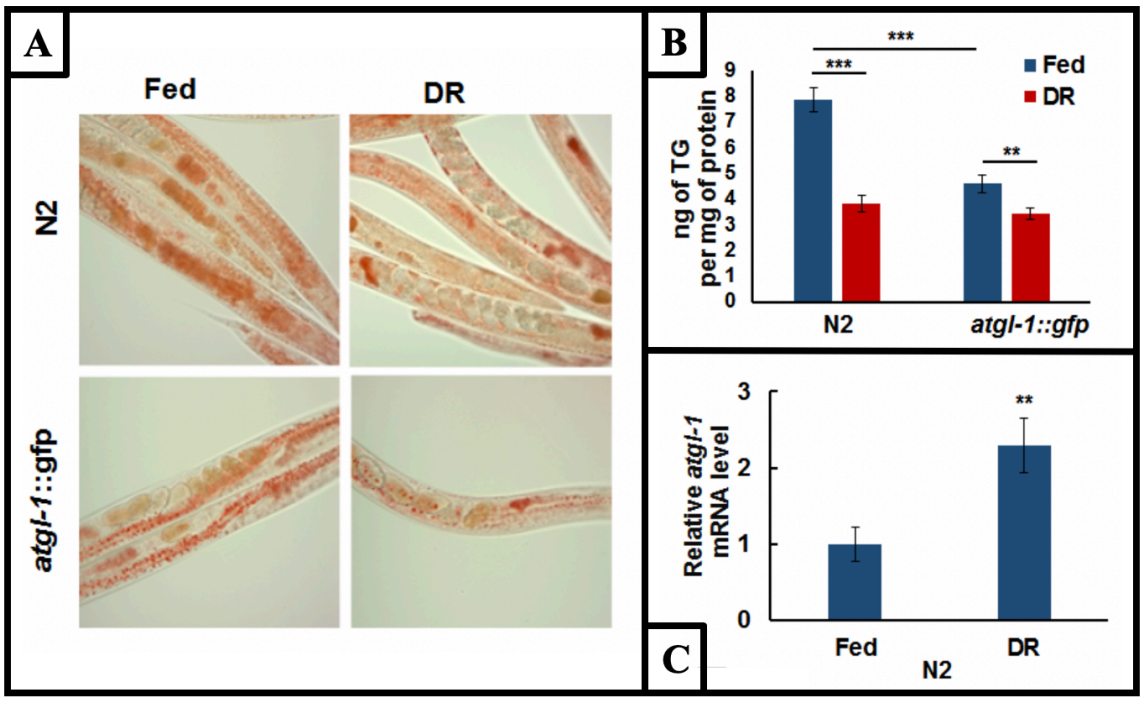

Figure 6. ATGL-1 is up-regulated by DR. Wild type (N2) and *atgl-1::gfp* worms split into control (Fed) and dietary restricted (DR) groups then stained with Oil-Red-O (A). Triglyceride content measured in control (Fed) and DR groups of N2 and *atgl-1::gfp* worms (B). RNA extracted from control (fed) and DR groups and *atgl-1* mRNA levels measured by qRT-PCR (C). *Actin-1/3* was used for normalization. Taken from (Zaarur et al., 2019).[22]

II. RNAi Experiment

Following a similar experimental design and layout as the rapamycin experiment, CeTOR specific RNAi (*let-363)* and empty vector control plates were used to make up the control and experimental plates. These plates were also divided into two treatment groups of 24- and 48-hours. The 24- and 48-hour treatment groups contained four plates, two empty vector control plates and two *let-363* RNAi plates. 200 L4 N2 and *atgl-1::gfp* worms were seeded onto one treated and one control plate each within each treatment group. After the 24- or 48-hour treatment, ATGL-1 quantification methods were performed. **Figure 4B** outlines the design of this experiment.

ATGL-1 RNA using RT-qPCR and protein quantification by fluorescent ATGL-1::GFP and western blot were conducted. Similar to the expected results of the rapamycin experiment, CeTOR specific RNAi should result in an increase in atgl-1 level and increase of lipolysis as indicated by the results of the Oil-Red-O staining. The rate of lipolysis is expected to increase as the expression and activity of ATGL-1 is also increased.

III. Longevity Experiment

Once the ATGL-1 quantification results are found from the rapamycin and RNAi experiments, the second part of this investigation, to determine the role of ATGL-1 in CeTOR regulated longevity in C. *elegans*, can be examined. Using the N2 and *atgl-1* mutant strain C. *elegans*, the longevity experiment was conducted. 20 N2 and *atgl-1* mutant strains were each seeded onto 10 rapamycin and 10 control plates as well as 10 CeTOR specific RNAi (*let-363)* and 10 empty vector control plates. The plates (N = 20 worms per

plate) were monitored and worms were scored as either live or dead every day. Once the worms stopped responding to probing by wired picks, they were considered dead. The experimental design of this experiment is illustrated in **Figure 7**.

If ATGL-1 does in fact play a role in CeTOR regulated longevity response in C. *elegans*, the life extending effect of CeTOR inhibition by both rapamycin and RNAi will be reduced by atgl inactivation. The maximum lifespan used here is defined as the total time, in days, the worms are recorded as live starting at day 1 and the time at which 50% of the worm population are alive, is defined as mean lifespan. The statistical significance of lifespan between the strains can be found using a log rank analysis by Oasis software.[40]

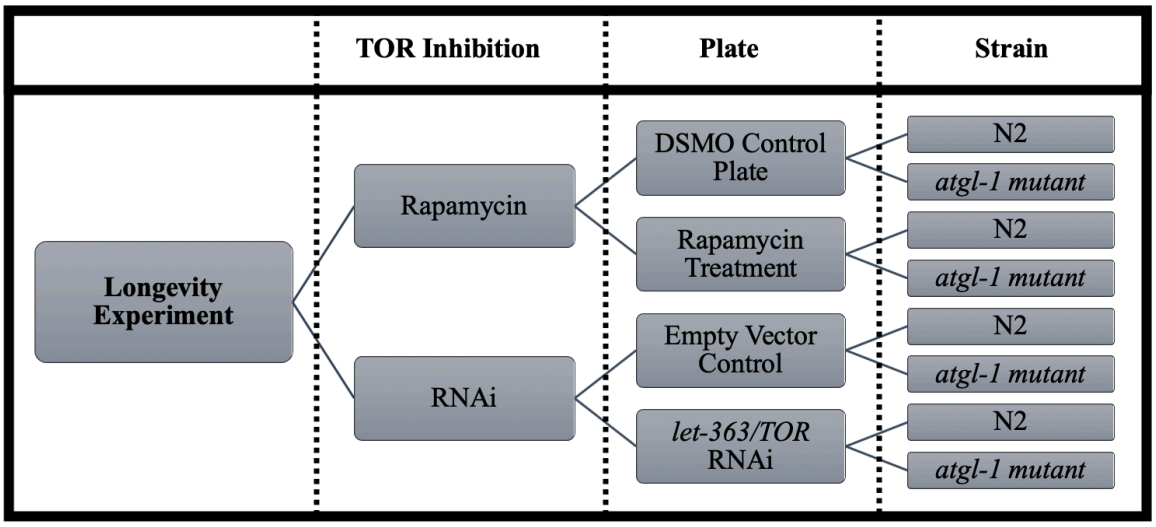

Figure 7. Design of the longevity experiment.

DISCUSSION

Traditional thinking has labelled lipids to be detrimental given their association with many age-related diseases, however, numerous studies, including work done in our laboratory, has shown that lipid metabolism regulates aging and longevity, thus continued research is crucial.[49] The purpose of this investigation was to (a) determine whether levels of ATGL-1 is increased by CeTOR inhibition using rapamycin and RNAi, and (b) to determine the role of ATGL-1 in CeTOR regulated longevity in C. *elegans.* This experiment has illustrated some important and interesting preliminary results, further research should be conducted.

The significant and nonsignificant findings found using the fluorescent ATGL-1::GFP levels in the control and rapamycin treated C. *elegans* could be attributed to small sample size and/or large inter-worm variation. Repeating this experiment with confirmation that rapamycin treatment is effective and inhibiting CeTOR, would allow more concise and potentially significant results to be found. The results of the Oil-Red-O staining to ensure the rate of lipolysis is increased may prove to be crucial for future experiments. It is possible that 24- and 48-hour treatment is not the ideal exposure time to observe an increase in ATGL-1, however, longevity experiments using C. *elegans* maintain the worms on rapamycin throughout the entire experiment. Thus, it is unlikely that the exposure time plays a large role in these unexpected results.

CeTOR inhibition through rapamycin treatment and RNAi feeding may result in increased ATGL-1 expression shown by RT-qPCR, fluorescent microscopy of GFP and western blot. These findings would indicate that CeTOR inhibition increases ATGL-1

expression. Previous studies have shown mTORC1 inhibition by rapamycin within cultured mouse adipocytes, result in increased lipolysis in response to β-adrenergic stimulation via enhanced HSL phosphorylation and by activation of Egr1-dependent ATGL transcription.[42] However, within adipose deposits from adipose specific Raptor knockout (Raptor^{aKO}) mice, there was no significant increase in ATGL protein expression.[42,44,46,46,47] The results of the Oil-Red-O staining will indicate whether or not lipolysis is increased with these two CeTOR inhibitory methods and future experiments based on these results can then be conducted. Evidence would suggest this increase in lipolysis is due to an increase in expression and activity of ATGL-1, however, the preliminary results found in this experiment do not support this theory. The rapamycin and CeTOR specific RNAi inhibition used here may be explained similarly to those explained by (Paolella et al., 2020) in the Raptor^{aKO} mice.

Suppression of PPARγ and C/EBPα transcriptional activity by TORC1 inhibition may account for the increased lipolytic rate. Germline ablation of the C/EBPα target perilipin 1 as well as Raptor^{aKO} exhibit increased basal lipolysis and decreased stimulated lipolysis.[51] In addition, mice treated with rapamycin show decreased expression of perilipin 1. Perilipin 1 coats the outside of lipid droplets and mediates the access of ATGL and HSL to lipid droplets. Therefore, decreased perilipin 1 expression within these mice could explain an increase in ATGL-dependent lipolysis without an increase in ATGL-1.[42] The lipolytic pathway in mammals and C. *elegans* with the ATGL activator ABHD5, which is controlled by PKA, is shown in **Figure 8.** C. *elegans* have close homologues of each protein within the lipolytic pathway. First identified as W01A8.1 and now *plin-1,* has been

recognized as the nematode's perilipin-related protein.[52] Though the complete function is not fully understood, further experimentation in C. *elegans* is necessary. Perhaps CeTOR inhibition by rapamycin and RNAi has similar effects on the perilipin-related protein and therefore, though an increase in lipolysis is observed, an increase in ATGL-1 is not. Repeating this experiment with quantification of the perilipin-related protein may aid in further understandings.

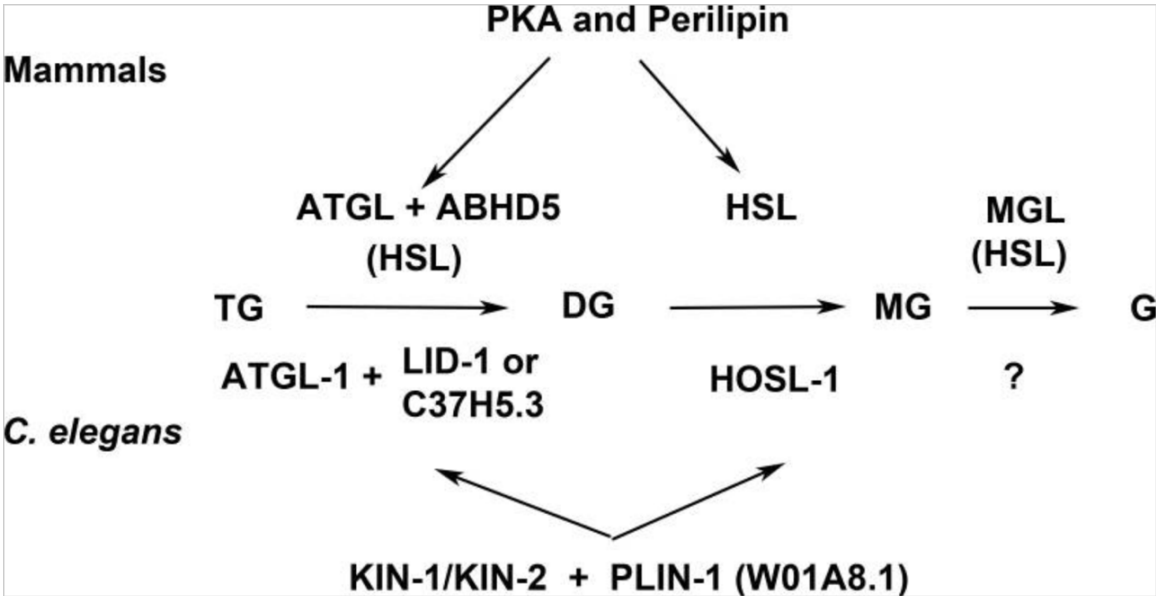

Figure 8. Lipolytic enzymes and regulatory proteins.
Mammalian proteins are shown above the arrows and their corresponding C. *elegans* orthologues are below. The lipolytic pathway includes adipose triacylglycerol lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL). These lipases act on triacylglycerol (TAG), diacylglycerol (DAG) and monoacylglycerol (MAG), respectively. LID-1 and C37H5.3 were proposed to be orthologues of ABHD5/CGI58 in C. *elegans* (Lee et al., 2014: Xie & Roy, 2015).[34,35] Under control of protein kinase A (PKA), Perilipin regulates the access of ATGL and HSL to lipid droplets. *Plin-1* is proposed to be a perilipin orthologue. Taken from (Chughtai et al., 2015).[52]

Using the results from the expression of ATGL experiments, the next question is the impact ATGL-1 has on CeTOR regulated longevity. In order for this to be assessed, CeTOR inhibition by both rapamycin and RNAi feeding methodology used here would have to prove to be effective in increasing longevity within C. *elegans* compared to nontreated worms, both of which have previously proven to be successful. Once our methodology is confirmed and working as expected, the *atgl*-1 mutant can be utilized to determine whether or not ATGL-1 is required for the longevity response of CeTOR inhibition. If these results reveal that the life extension of CeTOR inhibition is decreased within the *atgl-1* mutant, it may be feasible to conclude that ATGL-1 is required in the CeTOR regulated longevity response. These results may also highlight the important impact ATGL-1 has downstream of CeTOR regulated longevity within C. *elegans*. Together, these results would agree with our previous findings that dietary restriction and IIS pathways converge on ATGL-1 expression and that ATGL-1 is required for the life extension response of dietary restriction and both critical nodes within the insulin/IGF1 signaling pathway. Given that these pathways, as well as the enzymes involved, are evolutionarily conserved from yeast to primates, these findings may have a direct impact on future human longevity research.

Adipose tissue plays an important role in the regulation of energy homeostasis and lifespan. The balance by which this is accomplished is through opposing anabolic and catabolic processes. Caloric/dietary restriction decreases triglyceride storage within adipocytes and extends life, on the other hand, obesity, has been shown to have the opposite effect. Lipolysis is elevated by inflammatory cytokines, natriuretic peptides, growth

hormones, and cortisol, in addition to β-adrenergic stimulation.[15] The products of the lipolytic pathway are DAG, MAG, fatty acids and glycerol. These products are differentially utilized depending on the tissue. In vitro data from mice adipocytes has indicated that products or a specific lipolytic product may inhibit TOR, which suggests that they may facilitate catecholamine-induced inhibition of glucose uptake within adipocytes. In addition, it was found that ATGL was required for both TORC1 and TORC2 dissociation. Thus, the way in which the lipolytic pathway product(s) inhibit TOR was found to be through complex dissociation.[15]

ATGL is highly expressed within adipose tissue, although it is also expressed within several other tissues including skeletal muscle, liver, heart, testes, lung, retina, immune cells, pancreas, small intestine and the brain.[41] The brain has the second highest lipid content second to adipose tissue. In contrast to peripheral tissues, the brain utilizes glucose as its main energy source. Therefore, lipids play crucial roles in nervous system structure and function and various signaling processes. For example, central regulation of energy homeostasis requires FA availability within the hypothalamus. Though understanding of ATGL within the brain is incomplete, it has been found that ATGL may be required for transport of FA across the brain-CSF interface, the brain-CSF barrier and the blood brain barrier. This indicates that the products of lipolysis within the brain and their metabolites may serve as important second messengers.[43]

Tissue specific regulation of ATGL expression has already been shown to be implicated in chronic disease research and in longevity studies. However, there are still many insights to be discovered and understood about its role in longevity pathways,

including the feedback mechanism and second messengers lipolytic products play. Further research on ATGL may prove to have a major impact on future chronic disease research and longevity studies.

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