ATGL-1 mediates the effect of dietary restriction and the insulin/IGF-1 signaling pathway on longevity in C. elegans

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ABSTRACT

Objective: Animal lifespan is controlled through genetic pathways that are conserved from nematodes to humans. Lifespan-promoting conditions in nematodes include fasting and a reduction of insulin/IGF signaling. Here we aimed to investigate the role of ATGL-1 in accumulation of triglycerides and regulation of longevity.

Methods: We used a combination of genetic and biochemical approaches to determine the role of ATGL-1 in accumulation of triglycerides and regulation of longevity.

Results: We found that expression of ATGL is increased in the insulin receptor homologue mutant daf-2 in a FoxO/DAF-16-dependent manner. ATGL-1 is also up-regulated by fasting and in the eat-2 loss-of-function mutant strain. Overexpression of ATGL-1 increases basal and maximal oxygen consumption rate and extends lifespan in C. elegans. Reduction of ATGL-1 function suppresses longevity of the long-lived mutants eat-2 and daf-2.

Conclusion: Our results demonstrate that ATGL is required for extended lifespan downstream of both dietary restriction and reduced insulin/IGF signaling.

1. INTRODUCTION

In metazoaans, the insulin/IGF1 signaling pathway (IIS) coordinates nutrient and energy availability with growth, metabolism, and longevity [1]. Two major “signaling nodes” [2], FoxO- and TORC1-centered, are responsible for the effect of nutrients and IIS on the lifespan [3–6]. Transcription factor FoxO1 (Forkhead box O 1) that adapts mammalian organisms to starvation is negatively regulated by IIS via Akt-mediated phosphorylation and nuclear exclusion [7,8]. At the same time, oligomeric Ser/Thr protein kinase TORC1 (Target Of Rapamycin Complex 1) is activated by Akt and nutrients and promotes anabolic processes while inhibiting catabolism [9].

The downstream target(s) of FoxO and TORC1 that transmit longevity signal(s) remain largely unknown. Given that both FoxO and TORC1 are ubiquitously expressed and regulate a plethora of important biological responses, identification of the specific pathway(s) that control longevity is challenging. In fact, we do not even know whether FoxO and mTORC1 are involved in the same pathway or mediate different pathways of the longevity control.

We have recently found that FoxO1 [10,11] and mTORC1 [12–14] control the rates of lipolysis in mammalian cells by regulating expression of adipose triglyceride lipase (ATGL; a.k.a. desnutrin, PNPLA2, TTS2.2, iPLA2γ). Although complete hydrolysis of triglycerides (TG) to glycerol and fatty acids (FA) is performed jointly by tri-, di-, and monoacylglyceride lipases, ATGL represents the rate-limiting lipolytic enzyme. In other words, in every mammalian experimental system tested thus far, elevated ATGL expression increases, while attenuated ATGL expression decreases, both basal and cAMP-stimulated lipolysis [12,15–24].

Since known biochemical pathways that control longevity converge on the regulation of ATGL expression, we hypothesized that ATGL may represent the long sought after target of the nutrient and insulin/IGF1 signaling pathways that regulate life span. Here, we utilize the nematode C. elegans, a well-characterized and widely used model for longevity studies, and show that expression of the C. elegans ATGL homologue ATGL-1 (C05D11.7) is controlled by nutrients and the DAF-2/DAF-16 pathway. Moreover, we find that the partial loss-of-function ATGL-1 mutant, ATGL-1 [P87S], blocks the life-extending effects of dietary restriction (in eat-2 loss-of-function model) and DAF-2 deficiency, whereas over-expression of ATGL-1 increases C. elegans lifespan.
2. MATERIALS AND METHODS

2.1. C. elegans strains

All C. elegans strains were maintained at 20 °C using standard methods [25]. The wild-type strain used was the C. elegans Bristol strain N2. The following strains were obtained from the Caenorhabditis Genetics Center (CGC): GR1307 [daf-16(mgDf50) I], CB1370 daf-2[e1370] III, HT1980 daf-16(mgDf50) I; daf2[e1370] III, VS20 hjs67 [atgl-1p::atgl-1::gfp + mec-7::rfp], and VC20458 containing atgl-1 (gk176565) [P87S] III. The atgl-1(gk176565) mutation was outcrossed four times to generate AGK785 atgl-1(gk176565). The AVS518 eat-2(ad465) II strain was obtained from Dr. Andrew Samuelson (University of Rochester).

Other strains produced in this study include: AGK786: daf-2(e1370) III; hjIs67 AGK787: eat-2(ad465) II; atgl-1(gk176565) AGK788: daf-2(e1370) III; hjs67 AGK789: daf-16(mgDf50) I; daf-2(e1370) III; hjs67

2.2. Fasting conditions

L1 larvae were synchronized by treatment of gravid adults with 20% 2.2. Fasting conditions

AGK788: daf-2(e1370) III; hjIs67

Two other strains produced in this study include: AGK786: daf-2(e1370) III; hjIs67 AGK787: eat-2(ad465) II; atgl-1(gk176565) AGK788: daf-2(e1370) III; hjs67 AGK789: daf-16(mgDf50) I; daf-2(e1370) III; hjs67

2.3. Measurement of triglyceride content

Synchronized L4 stage worms (3000–5000) were pelleted by microcentrifugation at 13,000 rpm for 10 min, re-suspended in 50 μl of 5% Triton X-100 supplied with the protease inhibitor cocktail (Sigma, St. Louis, MO), and sonicated by 3 cycles (10 s each) using a Sonic Dismembrator (Fisher Scientific, Waltham, MA) at 30% output “3” at 4 °C. Protein concentration in lysates was determined using the Pierce BCA Protein Assay kit (Fisher Scientific) on a BioTek Synergy™ HT microplate reader (BioTek, Winooski, Vermont). The lysates underwent two cycles of heating to 90 °C for 5 min, each followed by vortexing, cooling to room temperature, and centrifugation at 8000 × g for 5 min. Triglycerides were measured using Triglyceride Quantification kit (Biovision, Milpitas, CA) according to the manufacturer’s protocol.

2.4. Oil-Red-O staining

Synchronized L4 stage worms were harvested, washed with PBS, and fixed in 1 ml of methanol at −20 °C for 5 min. Then, 2 ml of PBST (PBS with 0.01% Tween-20) was added, and worms were pelleted by centrifugation at 1000 rpm for 1 min. Supernatant was removed, and worms were washed twice with PBST in the same regime. Water (60% by volume) was added to Oil Red O (0.5% solution in isopropanol, Sigma) at room temperature, and, in 10 min, the solution was filtered through a 0.4 μm syringe filter. Fixed worms were stained with filtered 40% isopropanol Oil Red O solution (1 ml) for 20 min, washed twice with PBST, and mounted on slides with 3% agar. Images were taken using an inverted epi-fluorescent microscope (Carl Zeiss; Axio Observer D1) with a color camera.

2.5. RT-qPCR

Synchronized L4 stage worms (1000–2000) were washed with PBS, re-suspended in 1 ml of cold TRIzol (Ambion, Austin, TX), incubated at −80 °C for at least 1 h, and RNA was isolated according to 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) according to the protocol of manufacturer with the following primers. atgl-1: forward 5’ GATCGACCGATGTTATGCAG 3’, reverse 5’ GAGCCAACACATTTGTC 3’, actn-1: forward, 5’ CACGACACTCTAACTCC 3’, reverse, 5’GATCGACGACTCAGTCCT 3’. Relative expression levels of all mRNAs were normalized to actn-1/3 mRNA.

2.6. Measurement of oxygen consumption rates

OCR was measured by XF96 Extracellular Flux Analyzer - Seahorse (Agilent, Santa Clara, CA) using Seahorse’s cartridges and plates (Bioscience). All measurements have been done at 28 °C. Synchronized worms (L3-L4 stage) were washed 3 times with M9 buffer, and ca. 20 worms were placed in a well with 200 μl M9 buffer. For each strain, 10 wells were used. Basal OCR was measured every 5 min. After that, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, Sigma) was injected to each well to the final concentration of 10 μM, and maximal OCR was measured every 5 min. Finally, sodium azide (Sigma) was injected to the final concentration of 40 mM and non-mitochondrial OCR was measured every 5 min. After all measurements, worms in each well were re-counted, and results were normalized by their exact number.

2.7. Longevity experiments

Young adult worms were allowed to lay eggs for 6 h and then manually removed from the plates. The hatched L1 larvae were plated on ten 6 cm NGM Petri dishes, ca. twenty larvae per plate, and their lifespan was monitored every day. During the gravid adult stage, adult worms were separated from eggs/L1 larva and moved to new plates. The hatching day was considered Day 1. Death was scored if the animal did not move after repeated prodding with a pick. Animals disappearing from the plate or dying due to bursting or internal larva hatching were excluded from scoring, but included in the statistical analysis. Statistical significance (p value) of lifespan differences between the strains was determined by log-rank analysis for a single comparison and log-rank with Bonferroni correction for multiple comparisons using Oasis-2 software [26]. Time point when 50% of the worm population stays alive was considered mean lifespan. Each lifespan experiment was repeated three times with each group of worms. Combined p value was calculated by the Fisher’s combined probability test. Representative life span experiments are shown in the Figures, and all repeats are listed in Table 1.

2.8. Fluorescence microscopy

Live animals were mounted on 2% agarose pads and immobilized with sodium azide. Images were taken either using Axiovert 200 or Zeiss Axiolmager Z1 (Carl Zeiss, Germany) with a 20X objective. Identical exposure times were used when GFP intensity was compared between different conditions. ImageJ software was used to assess the level of fluorescence.

3. RESULTS

3.1. ATGL-1 increases oxygen consumption and longevity

Fasting of wild type (N2) worms for 6 h depletes their TG reserves (Figure 1A (top) & B (left)) and up-regulates the actn-1 mRNA (Figure 1C). To analyze expression of the ATGL-1 protein, we have utilized the VS20 strain that contains chromosome-integrated transgenic array hjs67 [atgl-1p::atgl-1::GFP + mec-7p::RFP] [27] (that will...
Mean lifespans of C. elegans strains.

| Figure | Individual Experiments | Genotypes Compared | # of Worms | Mean Lifespan ± SEM (days) | % Lifespan Change compared to N2 or as noted | p Value | Combined p Value compared to N2 or as noted |
|--------|------------------------|--------------------|------------|-----------------------------|---------------------------------------------|---------|-------------------------------------------|
| 1H     | 1                      | N2                 | 206        | 14.07 ± 0.35                |                                             |         |                                            |
|        |                        | atgl-1::gfp        | 202        | 18.62 ± 0.44                | +32.3                                       | <1.0e-10|                                            |
| 2      |                        | N2                 | 132        | 15.74 ± 0.44                |                                             |         |                                            |
|        |                        | atgl-1::gfp        | 82         | 20.20 ± 0.78                | +28.3                                       | 7.8e-8  |                                            |
| 3      |                        | N2                 | 90         | 14.78 ± 0.39                |                                             |         |                                            |
|        |                        | atgl-1::gfp        | 112        | 20.87 ± 0.67                | +41.2                                       | <1.0e-10|                                            |
| 2F     | 1                      | N2                 | 146        | 13.90 ± 0.35                |                                             |         |                                            |
|        |                        | eat-(2(ad453))     | 104        | 18.81 ± 0.80                | +35.3                                       | 1.0e-3  |                                            |
|        |                        | atgl-1(P87S)       | 156        | 13.8 ± 0.30                 | −0.7                                        | 0.5495  |                                            |
|        |                        | atgl-1(P87S)       | 166        | 12.58 ± 0.18                | −9.5                                        | 1.0e-3  |                                            |
|        |                        | eat-(2(ad453))     | 96         | 13.10 ± 0.33                | −33.1 (comp. to eat-2)                       | <1.0e-10| (comp. to eat-2)                           |
| 2      |                        | N2                 | 216        | 13.20 ± 0.33                |                                             |         |                                            |
|        |                        | eat-(2(ad453))     | 187        | 18.77 ± 0.45                | +42.2                                       | <1.0e-10|                                            |
|        |                        | atgl-1(P87S)       | 180        | 14.26 ± 0.24                | −8.0                                        | 0.0326  |                                            |
|        |                        | atgl-1(P87S)       | 206        | 12.58 ± 0.28                | −4.7                                        | 0.7903  |                                            |
|        |                        | eat-(2(ad453))     | 217        | 13.3 ± 0.34                 | −33.0 (comp. to eat-2)                       | <1.0e-10| (comp. to eat-2)                           |
| 3      |                        | N2                 | 217        | 13.3 ± 0.34                 |                                             |         |                                            |
|        |                        | eat-(2(ad453))     | 202        | 20.33 ± 0.51                | +52.9                                       | <1.0e-10|                                            |
|        |                        | atgl-1(P87S)       | 255        | 12.52 ± 0.17                | −5.9                                        | 0.85    | 0.21                                       |
|        |                        | atgl-1(P87S)       | 251        | 15.43 ± 0.41                | +16.0                                       | 4.3e-6  |                                            |
| 3D     | 1                      | N2                 | 100        | 12.0 ± 0.39                 | −24.1 (comp. to eat-2)                       | <1.0e-10| (comp. to eat-2)                           |
|        |                        | daf-2(e1370)       | 90         | 27.73 ± 1.7                 | +131.1                                      | <1.0e-10|                                            |
|        |                        | atgl-1(P87S)       | 102        | 10.92 ± 0.30                | −9.0                                        | 0.1268  |                                            |
|        |                        | daf-2(e1370)       | 110        | 14.90 ± 0.89                | +24.2                                       | 3.3e-3  |                                            |
|        |                        | atgl-1(P87S)       | 103        | 14.90 ± 0.89                | −46.3 (comp. to daf-2)                       | <1.0e-10| (comp. to daf-2)                           |
| 2      |                        | N2                 | 189        | 14.46 ± 0.29                |                                             |         |                                            |
|        |                        | daf-2(e1370)       | 171        | 28.22 ± 0.81                | +95.2                                       | <1.0e-10|                                            |
|        |                        | atgl-1(P87S)       | 174        | 14.16 ± 0.30                | −2.1                                        | 1       |                                            |
|        |                        | daf-2(e1370)       | 155        | 18.87 ± 0.63                | +16.7                                       | 3.0e-4  |                                            |
|        |                        | atgl-1(P87S)       | 156        | 18.87 ± 0.63                | −40.2 (comp. to daf-2)                       | <1.0e-10| (comp. to daf-2)                           |
| 3      |                        | N2                 | 194        | 14.07 ± 0.35                |                                             |         |                                            |
|        |                        | daf-2(e1370)       | 113        | 29.99 ± 1.65                | +113.1                                      | <1.0e-10|                                            |
|        |                        | atgl-1(P87S)       | 180        | 14.26 ± 0.24                | +1.4                                        | 1       | 0.66                                       |
|        |                        | daf-2(e1370)       | 134        | 16.84 ± 0.63                | −43.8 (comp. to daf-2)                       | <1.0e-10| (comp. to daf-2)                           |

be further referred to as atgl-1::gfp. As shown in Figure 1D,E, fasting increases ATGL-1 protein levels. Also, overexpression of atgl-1::gfp decreases TG reserves in both fed and, to a lesser extent, fasted worms (Figure 1A bottom & 1B right). In agreement with these results, it has been shown previously that down-regulation of atgl-1 by RNAi prevents TG depletion in adult worms subjected to fasting [28].

In mammals, ATGL-mediated lipolysis produces endogenous ligands for PPARs, its isotype PPARδ, and co-activator PGC-1α to control mitochondrial biogenesis and functions [29–34]. In agreement with these results, we have found that overexpression of atgl-1::gfp increases both basal and maximal oxygen consumption rate as well as reserve respiratory capacity (Figure 1F,G). As healthy mitochondria are essential for longevity in both mammals [35] and C. elegans [36], we decided to determine the effect of atgl-1::gfp on longevity. Interestingly, the life span of the VS20 strain is significantly longer compared to that of N2 (Figure 1H and Table 1).

The latter result may seem to contradict a reported negative effect of ATGL-1 on the lifespan of aak-2 (the C. elegans AMP-activated kinase α2 catalytic subunit) mutant dauer larvae [37]. However, dauer represents an arrested dormant state that nematodes convert to in the absence of food. Dauer larvae do not feed and rely on stored energy for their survival. Therefore, in this case, the lipolytic activity of ATGL-1 appears to be suppressed by AMPK, and ATGL-1 de-repression in aak-2 mutant worms leads to faster depletion of nutrient stores and lifespan reduction.

3.2. Reduction of ATGL-1 function suppresses longevity of the eat-2 loss-of-function mutant strain

As deletion of the atgl-1 gene (the tm3116 allele) is lethal [38], we used a partial loss-of-function atgl-1 mutant for further experiments. To obtain a viable atgl-1 mutant allele we searched a list of strains generated by the Million Mutation Project [39] and identified atgl-1(gk176565 [P87S]) as a potential candidate. We reasoned that substitution of a proline by a serine in the extended conserved catalytic domain of ATGL [40] should impact ATGL function, through reducing ATGL catalytic activity and/or protein stability. Importantly, this mutation did not change levels of the atgl-1 mRNA (not shown). The atgl-1(gk176565) allele was outcrossed four times prior to its use in experiments described below.

In agreement with our expectations, the atgl-1[P87S] mutant accumulated significantly more TG than N2. Furthermore, fasting of the atgl-1[P87S] strain does not decrease its TG reserves (Figure 2A,B), which supports our hypothesis that P87S is a reduction-of-function ATGL-1 mutation.

To test whether or not ATGL-1 mediates the effect of fasting on longevity, we took advantage of a long lived strain eat-2(ad465) that

Table 1 – Mean lifespans of C. elegans strains.
mimics several aspects of dietary restriction due to the defect in pharyngeal function hence insufficient food uptake [41]. As expected, eat-2 mutant worms accumulated less TG than N2 (Figure 2C,D). Interestingly, the levels of atgl-1 mRNA in fed eat-2(ad465) worms were twice as high as in fed N2 strain (Figure 2E), and similar to the fasted N2 (Figure 1C). We then combined atgl-1(P87S) with eat-

Figure 1: ATGL-1 is up-regulated by fasting and increases oxygen consumption rate as well as longevity of C. elegans. (A) Wild type (N2) and atgl-1::gfp worms were split into control (Fed) and Fasted groups and stained with Oil Red O. (B) Triglyceride content was measured in Fed and Fasted groups of N2 and atgl-1::gfp worms. (C) RNA was extracted from Fed and Fasted groups, and atgl-1 mRNA levels were measured by qRT-PCR; actin-1/3 was used for normalization. (D) Fed and Fasted L4 stage atgl-1::gfp worms were visualized by fluorescence microscopy (200X, equal exposure times). Bar = 50 μM. (E) Quantification of the results shown in panel D by ImageJ (10 randomly selected worms per group). (F) Oxygen Consumption Rate (OCR) of N2 and atgl-1::gfp L4 worms was measured by XF96 Extracellular Flux Analyzer Seahorse, as described in Experimental Procedures. Each experiment was performed with ca. 200 worms and repeated 3 times. (G) Average basal OCR was calculated as the mean of the first five measurements after subtraction of average non-mitochondrial OCR; average maximal OCR is the mean of the last (before addition of sodium azide) five measurements after subtraction of average non-mitochondrial OCR; reserve respiratory capacity is the difference between average maximal and basal OCR. (H) The lifespan of atgl-1::gfp worms is extended by 32% in comparison to N2 (p value < 1.0e-10, log-rank analysis). Panels B, C, E-G show an average result of three independent experiments ±SEM, p values were calculated by unpaired two-tailed t-test. *p < 0.05; **p < 0.01; ***p < 0.001.
2(ad465) to obtain the eat-2(ad465); atgl-1(P87S) double mutant strain and used it in longevity experiments along with N2, atgl-1(P87S), and eat-2(ad465). As shown in Figure 2F and Table 1, atgl-1(P87S) completely suppressed the extended lifespan of eat-2(ad465). Therefore, ATGL-1 is required for the life span extension in this model of dietary restriction.

3.3. ATGL-1 is regulated by the DAF-2/DAF-16 axis and is required for extended lifespan of daf-2 mutants

In mammalian adipocytes, expression of ATGL is suppressed by insulin via inhibition of FoxO1 [11]. The worm expresses homologues of the insulin receptor (DAF-2) and FoxO (DAF-16), but it is unknown whether expression of ATGL-1 in C. elegans is controlled by DAF-2 and DAF-16. Supporting this possibility, bioinformatics analysis shows a canonical DAF-16 binding site in the promoter of the atgl-1 gene (not shown). Therefore, we generated daf-2(e1370) and daf-2(e1370); daf-16(md150) strains containing the atgl-1::gfp translational fusion reporter. Indeed, we observed elevated ATGL-1::GFP protein (Figure 3A,B) and atgl-1 mRNA (Figure 3C) expression in daf-2(e1370) compared to the background transgenic strain. Importantly, this effect was suppressed by the daf-16(md150) null allele (Figure 3A–C). These results confirm the evolutionary conservation of ATGL regulation via the insulin receptor/FoxO1 axis from C. elegans to mammals.

To determine whether or not increased life span of the daf-2(e1370) mutant was dependent on atgl-1, we crossed it with the atgl-1(P87S) strain. Our longevity experiments demonstrate that the life span of daf-2(e1370); atgl-1(P87S) was significantly (by ca. 46%) shorter compared to that of daf-2(e1370) alone (Figure 3D and Table 1). Therefore, ATGL-1 function is required downstream of both dietary restriction (Figure 2F) and DAF-16/FoxO pathways to extend life.

4. DISCUSSION

Wang et al. [42] have previously identified another “life extending” lipase, LIPL-4 (K04A8.5) that shares a high degree of homology with mammalian lysosomal acid lipase and, therefore, is likely to be localized in lysosomes. Similar to cytosolic ATGL-1, LIPL-4 increases
mitochondrial oxidation and reduces lipid storage [43]. In *C. elegans*, expression of both LIPL-4 [42] and ATGL-1 (this report) is activated by DAF-16; in mammalian cells, lysosomal acid lipase and ATGL are also co-regulated by FoxO1 [44]. Thus, both lysosomal and cytoplasmic lipases are regulated by the same evolutionary conserved signaling pathway and are essential for the life span control in *C. elegans*. Adipose tissue plays a very special role in the control of the life span in mammals [45]. In particular, caloric restriction decreases TG stores in adipocytes and increases longevity, while obesity has the opposite effect [46]. Genetic ablation of the insulin receptor specifically in adipose tissue suppresses TG accumulation and significantly extends life span in mice [47]. Knock out of IRS-1 produces a similar outcome [48]. As regulatory pathways of ATGL expression are conserved from yeast [11,14], it is feasible that ATGL may have a systemic life extending effect in higher eukaryotes.

5. CONCLUSIONS

Our results suggest that (a) evolutionary conserved dietary restriction and the IIS pathways converge on ATGL-1 expression, and (b) ATGL-1 is required for the extension of life span in response to dietary restriction and reduced IIS.

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CONFLICT OF INTEREST

None.

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