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Article

Autophagy and Lipid Metabolism Coordinately Modulate Life Span in Germline-less *C. elegans*

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Summary

Background: The cellular recycling process of autophagy is emerging as a key player in several longevity pathways in *Caenorhabditis elegans*. Here, we identify a role for autophagy in long-lived animals lacking a germline and show that autophagy and lipid metabolism work interdependently to modulate aging in this longevity model.

Results: Germline removal extends life span in *C. elegans* via genes such as the lipase LIPL-4; however, less is known of the cellular basis for this life-span extension. Here, we show that germline loss induces autophagy gene expression via the forkhead box A (FOXA) transcription factor PHA-4 and that autophagy is required to extend longevity. We identify a novel link between autophagy and LIPL-4, because autophagy is required to maintain high lipase activity in germline-deficient animals. Reciprocally, *lipl-4* is required for autophagy induction. Coordination between autophagy and lipolysis is further supported by the finding that inhibition of TOR (target of rapamycin), a major negative regulator of autophagy, induces *lipl-4* expression, and TOR levels are reduced in germline-less animals. TOR may therefore function as a common upstream regulator of both autophagy and *lipl-4* expression in germline-less animals. Importantly, we find that the link between autophagy and LIPL-4 is relevant to longevity, because autophagy is induced in animals overexpressing LIPL-4 and autophagy is required for their long life span, recapitulating observations in germline-less animals.

Conclusions: Collectively, our data offer a novel mechanism by which autophagy and the lipase LIPL-4 interdependently modulate aging in germline-deficient *C. elegans* by maintaining lipid homeostasis to prolong life span.

Introduction

Reproductive capacity is closely linked to aging, and recent research has shown direct links between reproduction and longevity. For example, germ cell removal extends life span in the nematode *Caenorhabditis elegans*, where life span can be extended by up to 60% when the germline is ablated by a laser microbeam [1]. Interestingly, removal of the entire gonad results in a normal life span, suggesting that sterility per se is not necessary for life-span extension and that signals from the germline and the somatic gonad likely cooperate to regulate life span [1]. Importantly, the effects of the reproductive system on longevity appear to be conserved between worms and flies and are mediated via a forkhead box O (FOXO) transcription factor [2]. In germline-less *C. elegans*, DAF-16/FOXO translocates to the nucleus of intestinal cells [3, 4], suggesting the intestine as a central site of action for signals induced by the absence of germline cells. Such signals are likely to be mediated by lipophilic hormone signaling [2], but the cellular mechanisms underlying the extended longevity in germline-less animals remain relatively unexplored.

Macroautophagy (hereafter referred to as autophagy) is a major mechanism by which a cell degrades cytoplasmic components, including misfolded proteins and damaged organelles. During this multistep process, double-membrane autophagosomes engulf cytosolic material and fuse to lysosomes, and the vesicular contents are then degraded and recycled [5]. In yeast, numerous conserved genes control autophagy [5], and homologs of many of these autophagy genes have been identified in *C. elegans* [6]. Autophagy can be induced by multiple stress stimuli, including nutrient deprivation, through upstream regulators such as the nutrient sensor TOR (target of rapamycin), a key modulator of both metabolism and aging [7].

Autophagy was recently linked directly to aging in *C. elegans*, because autophagy was shown to play a critical role in several nutrient-sensing longevity processes, including the TOR and insulin-IGF-1 signaling pathways as well as in the dietary-restriction paradigm. Specifically, animals with reduced TOR or insulin/IGF-1 receptor *daf-2* activity as well as dietary-restricted *eat-2* worms show increased levels of autophagy, and autophagy-related genes are required for these animals to live long [8–12]. Although these observations have suggested that autophagy may represent a potential common link between several conserved mechanisms that lead to an extended life span, it remains unknown how the autophagy process influences organismal aging in *C. elegans*.

Recent studies have suggested direct links between autophagy and lipid metabolism. Lipid droplet breakdown occurs in hepatocytes by a process termed lipophagy [13], possibly involving lysosomal lipases [14]. Notably, the long life span of germline-less *glp-1* animals, as well as *daf-2* mutants, was recently linked to increased expression of a predicted triglyceride lipase, LIPL-4/K04A8.5 [15]. Specifically, DAF-16 regulates the expression of *lipl-4* in the intestine, a major site of fat storage in *C. elegans*. Moreover, *lipl-4* is required for *glp-1* animals to live long, and LIPL-4 overexpression in the intestine is sufficient to extend life span [15]. However, the mechanism that links LIPL-4 activity to longevity in *C. elegans* remains elusive.

Because autophagy appears to play a critical role in several longevity pathways in *C. elegans* and the predicted lipase *lipl-4* is important for life-span extension by germline removal, we hypothesized that lipid metabolism could be linked to autophagy to provide a mechanism by which germline-less animals live long. In this study, we provide evidence for such a link because we found that autophagy and LIPL-4 function interdependently in germline-less animals, possibly through the

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common upstream regulator TOR. Because germline-less animals and animals overexpressing LIPL-4 both display increased autophagy and require autophagy genes to prolong life span, we propose that this novel link plays a critical role in the extended longevity induced by germline removal.

Results

Germline-less *glp-1* Animals Have Increased Autophagy Levels

To determine whether autophagy is induced by germline removal in *C. elegans*, we used a genetic model of germline ablation, namely a temperature-sensitive (ts) *glp-1* mutant, which lacks a germline and is long-lived at the nonpermissive temperature [16]. To detect autophagy, we used electron microscopy (EM) to visualize and quantify autophagic events in several tissues of adult animals. We observed a dramatic increase in autophagosomes as well as in autolysosomes in intestinal and hypodermal seam cells in *glp-1(e2141)* mutants compared to N2 wild-type animals (Figures 1A and 1B; see also Figures S1A–S1D available online), consistent with increased autophagic flux in germline-less animals. To complement our EM studies, we also used a reporter strain expressing a GFP-tagged form of LGG-1 [7, 16], an ortholog of the mammalian LC3 protein that resides in preautophagosomal and autophagosomal membranes [8, 17]. In *C. elegans*, GFP::LGG-1 forms punctate structures or foci, reflecting LGG-1 sequestration to the membrane of nascent autophagosomes. We used this strain to quantify autophagic events in hypodermal seam cells (as described in [18]) and in the intestine. Consistent with our EM studies, we found a significant increase in GFP::LGG-1 foci in seam cells (Figure 1C), as well as in the intestine (Figure 1D; Figure S2A) in 1-day-old *glp-1(e2141)* mutants compared to wild-type animals. We also observed increased numbers of GFP-positive foci in another *glp-1* loss-of-function mutant, *glp-1(bn18)* (Figure S2B). Notably, a similar induction in GFP::LGG-1 positive foci was observed in *glp-1(e2141)* animals lacking the FOXO transcription factor DAF-16 (Figure S2C). Collectively, these observations indicate that autophagy is induced in germline-less mutants, in a *daf-16* independent fashion.

glp-1 Mutants Express Increased mRNA Levels for Autophagy Genes through the FOXA Transcription Factor PHA-4

To further investigate the mechanism by which germline removal induces autophagy, we used RT-PCR to measure mRNA levels of autophagy genes *unc-51*/ULK1, *bec-1*/Beclin1, and *lgg-1*/LC3 in *glp-1(e2141)* mutants and in wild-type animals. Expression of all three genes was significantly upregulated between 2- to 8-fold in young *glp-1(e2141)* adults compared to wild-type animals (Figure 1E). This upregulation was further increased during early adulthood (Figure S2D), suggesting that autophagy is substantially induced in adult *glp-1* animals. Notably, the same pattern of gene induction was observed in *daf-16*-deficient *glp-1(e2141)* animals (Figure S2E), consistent with our results using the GFP::LGG-1 autophagy reporter (Figure S2C). Thus, our data suggest that autophagy is transcriptionally upregulated in *glp-1* animals in a *daf-16*-independent manner. However, we found that another transcription factor, the forkhead box A (FOXA), or PHA-4, was required for the induction of autophagy genes in *glp-1* animals. As observed for autophagy genes, we found that *pha-4* mRNA levels were significantly increased in *glp-1(e2141)* mutants compared to wild-type animals (Figure 1E), and this was countered by feeding adult *glp-1(e2141)* animals with bacteria expressing double-stranded RNA (dsRNA) for *pha-4* (Figure 1E). In wild-type animals, *pha-4* RNA interference (RNAi) similarly decreased *pha-4* mRNA levels but only had a modest effect on autophagy gene expression (Figure S2F). Importantly, *pha-4* RNAi significantly decreased the number of LGG-1-positive foci in seam cells of *glp-1* animals expressing GFP::LGG-1 (Figure S2G). Taken together, these data demonstrate that PHA-4 is a bona fide functional transcriptional regulator of autophagy genes in *glp-1* animals.

pha-4 and Autophagy Genes Are Required for *glp-1* Animals to Live Long

To explore further the role of PHA-4 in germline-less animals, we next determined its contribution to life-span extension in *glp-1* animals. Knockdown of *pha-4* in adult *glp-1(e2141)* mutants significantly decreased their mean life span but only modestly affected the mean life span of wild-type animals (Figure 2A; Table S1), as reported previously [11, 19]. Thus, *pha-4* is required for *glp-1* mutants to live long, as was shown for dietary-restricted *C. elegans* [19] and animals with reduced TOR levels [20]. Because *glp-1* mutants also require *daf-16* to live long [16], these data suggest that two forkhead transcription factors, DAF-16 and PHA-4, play critical roles in the germline-deficient longevity model.

To determine whether autophagy genes were similarly required for *glp-1* longevity, we fed adult synchronized worms bacteria expressing dsRNA against genes involved in different steps of the autophagy process [6]. We found that RNAi inhibition of *unc-51*, *bec-1*, *vps-34*, *lgg-1*, and *atg-18* each significantly reduced the mean life span of *glp-1(e2141)* animals (Figures 2B and 2C; Table S1), and we observed similar results with an additional germline-deficient mutant, *mes-1(bn7)* [16] (Figures S2H and S2I). In contrast, RNAi inhibition of autophagy genes did not significantly affect the life span of adult wild-type animals (Figures 2B and 2C; Table S1), consistent with previous findings [10, 11]. Taken together, these observations suggest that autophagy genes, like *pha-4*, play critical roles in the extended life span of germline-less animals.

TOR Is Downregulated in *glp-1* Animals and Regulates LIPL-4 in Wild-Type Animals

The nutrient sensor TOR is a conserved negative regulator of autophagy and aging. We considered whether TOR activity was decreased in *glp-1* animals, providing a possible mechanism for increased autophagy in germline-less animals. We found that *tor* mRNA levels were reduced in a *daf-16*-independent manner in response to germline removal (Figure 3A), and we similarly observed reduced TOR protein levels in *glp-1(e2141)* mutants compared to wild-type animals (Figure S3A). Consistent with the reduced levels of TOR in *glp-1* animals, we found that the life span of *glp-1(e2141)* animals was not significantly affected by *tor* RNAi (Figure 3B; Table S1), whereas the life span of similarly treated wild-type worms was increased (Figure 3B; Table S1), as reported previously [21]. Collectively, these experiments indicate that germline removal extends life span, at least in part, by reducing TOR signaling. This possibility is supported by the observations that (1) *glp-1* animals require *pha-4* to live long (Figure 2A), (2) TOR inhibition induces *pha-4* expression (Figure S3B), (3) TOR inhibition induces *unc-51* mRNA levels in a *pha-4*

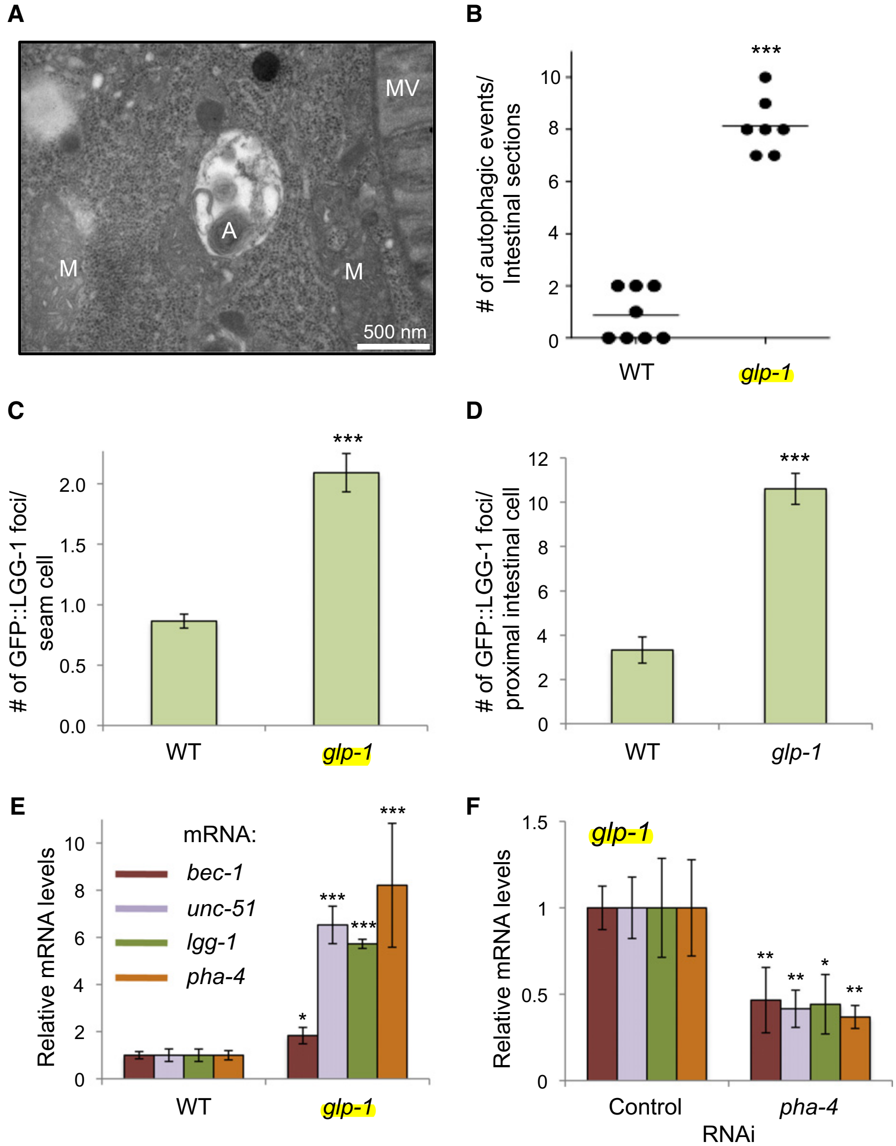

Figure 1. Autophagy Is Increased in *glp-1* Animals through a PHA-4-Dependent Mechanism

(A) Representative electron micrograph of an intestinal cell in a 1-day-old adult *glp-1(e2141)* animal. The following abbreviations are used: A, autolysosome; M, mitochondria; MV, microvilli. See Figure S1A for more information.

(B) Quantification of autophagic events detected by electron microscopy in the intestine of 1-day-old N2 wild-type (WT) and *glp-1(e2141)* animals. The observed number of events per animal is shown as dots, and the mean is indicated by vertical line (***$p < 0.0001$, t test).

(C) Quantification of GFP::LGG-1-positive foci in seam cells of 1-day-old WT and *glp-1(e2141)* animals. Mean number of foci ± standard error of the mean (SEM) is shown (n = 100–250 cells, ***$p < 0.001$, analysis of variance [ANOVA]).

(D) Quantification of GFP::LGG-1-positive foci in intestinal cells of 1-day-old adult WT and *glp-1(e2141)* animals. Cells from the proximal portion of the intestine were analyzed. Mean number of foci ± SEM is shown (n = 15 worms, ***$p < 0.001$, t test).

(E) RT-PCR analysis of mRNA levels of autophagy genes (*bec-1*, *unc-51*, and *lgg-1*) and of *pha-4* in 1-day-old adult WT and *glp-1(e2141)* animals. Relative mean expression ± standard deviation (SD) is shown (*$p < 0.05$, ***$p < 0.001$ versus WT, ANOVA).

(F) RT-PCR analysis of mRNA levels of genes in (E) in *glp-1(e2141)* animals fed control bacteria or bacteria expressing *pha-4* dsRNA from day 1 to day 3 of adulthood. Wild-type data is included in Figure S2A. Relative mean expression ± SD is shown (*$p < 0.05$, **$p < 0.01$ versus control, ANOVA). In all experiments, animals were raised at the nonpermissive temperature (25°C).

dependent manner (Figure S3B), and finally, (4) TOR inhibition extends life span via *pha-4* [20].

The predicted triglyceride lipase LIPL-4/K04A8.5 was recently shown to be upregulated in germline-less *C. elegans* in a *daf-16*-dependent mechanism [15], and we therefore asked whether TOR could modulate *lipl-4* gene expression in *C. elegans*. Interestingly, we found that RNAi inhibition of *tor* in wild-type worms significantly increased *lipl-4* mRNA levels (Figure 3C; Figure S3B), which was dependent on *daf-16* (Figure 3C) but independent of *pha-4* (Figure S3B). Consistent with this, we observed a *daf-16*-dependent increase in lipase activity in *tor* RNAi-treated animals (Figure 3D). Taken together, these data suggest that TOR may act as an upstream regulator of DAF-16 and PHA-4 to increase

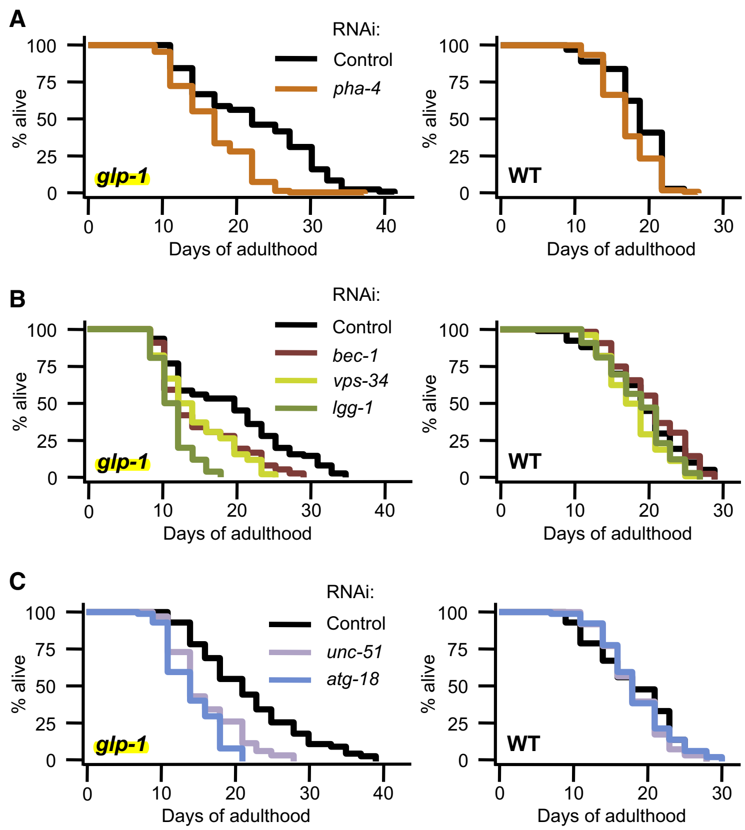

Figure 2. PHA-4 and Autophagy Genes Are Required for *glp-1* Animals to Live Long

Life-span analysis of WT N2 and *glp-1(e2141)* animals fed control bacteria (empty vector) or bacteria expressing *pha-4* dsRNA (A); bacteria expressing *bec-1*, *vps-34*, or *lgg-1* dsRNA (B); or bacteria expressing *unc-51* or *atg-18* dsRNA (C) during adulthood. See Table S1 for details and repeats. In all experiments, animals were raised at 25°C from hatching until the first day of adulthood and were then moved to 20°C for the remainder of their lives.

lipl-4 and autophagy, respectively, in germline-deficient *C. elegans*.

Autophagy and LIPL-4 Function Interdependently in *glp-1* Animals

Because inhibition of *lipl-4* and of autophagy genes both reduced the life span of *glp-1* animals, we next considered that autophagy and LIPL-4 might converge to modulate longevity. Recent studies have demonstrated a link between autophagy and lipolysis in which lipid stores can be subjected to lysosomal-related hydrolysis through lipophagy [13]. To probe such a link between autophagy, lipolysis, and longevity in *C. elegans*, we first measured lipase activity in lysates of adult *glp-1* and wild-type animals. Lipase activity was significantly increased in adult *glp-1(e2141)* mutants compared to wild-type animals (Figure 4A), consistent with the increased expression of *lipl-4* in *glp-1* animals [15]. Lipase activity was similarly increased in *glp-1(bn18)* mutants (Figure S4A). To determine whether autophagy was required for increased lipase activity, we used RNAi to inhibit expression of *vps-34*, *atg-18*, and *pha-4* and found that such reductions significantly decreased lipase activity in *glp-1(e2141)* animals (Figure 4B). The increase in lipase activity, observed in *glp-1* animals, was reduced significantly by *lipl-4* RNAi, confirming that the measured lipase activity was at least a partial readout for *lipl-4* activity (Figure 4B). Lipase activity was also dependent on *daf-16* (Figure 4B), consistent with *lipl-4* being transcriptionally regulated by DAF-16 in *glp-1* animals [15]. Notably, these RNAi treatments had only a modest effect on lipase activity in wild-type animals (Figure S4B). These data suggest that autophagy is required for *lipl-4* function in *glp-1* animals. Conversely, we found that *lipl-4* plays a role in autophagy induction, because *lipl-4* RNAi significantly reduced the number of GFP::LGG-1-positive foci in seam cells of *glp-1(e2141)* animals (Figure S5A), as observed when *pha-4* and autophagy genes were inhibited (Figures S2G and S5B). Collectively, these observations suggest that autophagy and the putative lipase LIPL-4 are linked in an interdependent fashion in germline-less *C. elegans*.

Animals that Overexpress LIPL-4 Display Increased Autophagy and Require Autophagy Genes and *pha-4* to Live Long

To better understand the link between autophagy and LIPL-4, we analyzed animals overexpressing LIPL-4 from its endogenous promoter [15]. We confirmed that LIPL-4 overexpressing animals showed a significant increase in lipase activity (Figure S4C), and we found that these animals were longer lived (Table S2), as was observed in *glp-1* mutants and in animals overexpressing LIPL-4 from an intestinal-specific promoter [15]. Interestingly, we discovered that LIPL-4 overexpression increased GFP::LGG-1 positive foci as well as autophagy gene expression (Figures S4C and S5D), suggesting that LIPL-4 overexpression was sufficient to induce autophagy. Consistent with a link between autophagy and LIPL-4, we found LIPL-4 was expressed not only in intestinal cells [15] but also in other tissues including the seam cells (Figure S5E), where an increase in autophagy was detected in *glp-1* mutants compared to wild-type animals (Figures 1A–1D). The presence of the *glp-1* mutation further increased LIPL-4 expression levels and also extended life span (data not shown), suggesting that the increased lipase activity in LIPL-4-overexpressing *glp-1* mutants might be responsible for their extended life span.

Because autophagy was increased in LIPL-4 overexpressing animals and in germline-less *glp-1* mutants that require autophagy genes to live long, we next asked whether autophagy genes were required for life-span extension in LIPL-4 overexpressing animals. Interestingly, we found that RNAi-mediated inhibition of *bec-1*, *lgg-1*, *vps-34*, or the putative autophagy inducer *pha-4* all significantly reduced the life span of LIPL-4 overexpressing animals while having negligible effects on nontransgenic siblings (Figures 5A and 5B; Table S2). These data support our hypothesis that autophagy is required for the life-span-extending effects of LIPL-4 overexpression. This is comparable to the effects of germline removal, and it is therefore possible that *lipl-4* modulates longevity via an autophagy-related mechanism in germline-less animals. Consistent with this, we found that simultaneous knockdown of *lipl-4* and *vps-34* failed to further decrease the life span of *glp-1(e2141)* animals (Figure S5F; Table S3). In summary, these experiments provide the first genetic evidence that

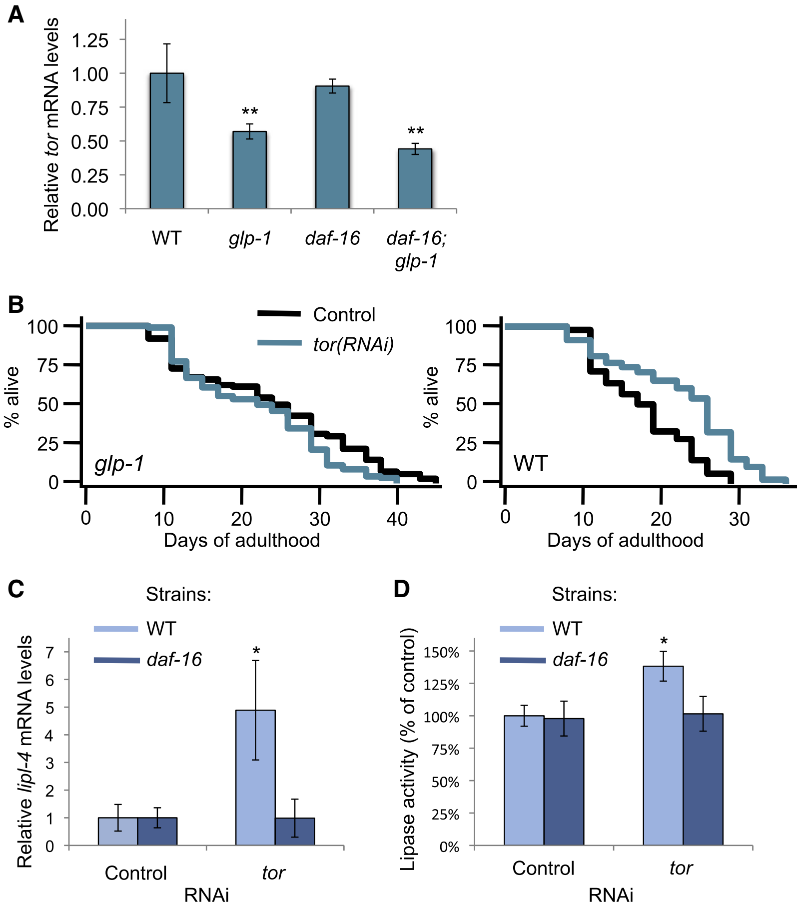

Figure 3. Germline-less *glp-1* Animals Have Reduced TOR mRNA Levels, and TOR Inhibition Results in Increased Levels of *lipl-4*

(A) RT-PCR analysis of *tor* mRNA levels in WT, *glp-1(e2141)*, *daf-16(mu86)*, and *daf-16(mu86); glp-1(e2141)* worms at day 1 of adulthood. Animals were raised at the nonpermissive temperature (25°C) from hatching, and relative mean expression ± SD is shown (**p < 0.01 versus WT, ANOVA).

(B) Life-span analysis of WT and *glp-1(e2141)* animals fed control bacteria or bacteria expressing *tor* dsRNA. Animals were raised as described in Figure 2. See Table S1 for details and repeats.

(C) RT-PCR analysis of *lipl-4* mRNA in 3-day-old adult WT and *daf-16(mu86)* worms fed control bacteria or bacteria expressing *tor* dsRNA for 2 days, starting from day 1. Animals were kept at 20°C throughout this experiment, and relative mean expression ± SD is shown (*p < 0.05 versus control, ANOVA).

(D) Lipase activity measurements in 3-day-old adult WT and *daf-16(mu86)* worms fed control bacteria or bacteria expressing *tor* dsRNA for 2 days, starting from day 1. Animals were kept at 20°C throughout this experiment, and relative mean expression ± SD is shown (*p < 0.05 versus control, ANOVA).

lipolysis and autophagy are linked to positively modulate longevity in *C. elegans*.

Discussion

We investigated the role of autophagy in the extended life span induced by germline removal in *C. elegans* and have shown using multiple complementary approaches that germline-less *glp-1* animals display increased levels of autophagic events. We also detected an increase in the expression of several autophagy genes through the activity of the FOXA transcription factor PHA-4, suggesting that autophagy is induced at the transcriptional level in response to germline removal. Accordingly, autophagy genes and *pha-4* were required for *glp-1* animals to live long. Taken together, these observations indicate that autophagy is induced in a beneficial manner in germline-less animals. Although we detected autophagy by using steady-state methods, our data strongly argue for a functional role for autophagy turnover in germline-deficient animals because we obtain the same effects on long-lived *glp-1* animals after RNAi against genes that act in multiple steps of the autophagy process. Consistent with this interpretation, we find that TOR, a known negative regulator of autophagy, is downregulated in *glp-1* animals. Because reduced TOR signaling plays an important role in other *C. elegans* longevity models that rely on autophagy genes, such as dietary restriction [10–12], these observations suggest a broader role for the TOR-regulated process of autophagy in extending life span in *C. elegans*.

The FOXO transcription factor DAF-16 is essential for life-span extension through germline removal [2], yet we find that autophagy gene expression and autophagy itself remained high in *glp-1* animals lacking *daf-16*. Thus, in contrast to life-span extension, induction of autophagy appears to be independent of *daf-16* in these animals, similar to our previous observations in long-lived *daf-2* insulin/IGF-1 receptor mutants [11]. These results suggest that *daf-16* may act downstream of, or in parallel with, autophagy function in such long-lived animals. Nevertheless, DAF-16 is required to obtain the beneficial effects of autophagy on longevity in *glp-1* animals. As discussed previously [11], we speculate that DAF-16 could play a regulatory role in the recycling of material from the autophagic process into new targets that have beneficial effects on longevity. This situation is different from overexpression of DAF-16, which is sufficient to induce autophagy in *C. elegans* [22], possibly because DAF-16 may be activated by different mechanisms in animals overexpressing DAF-16 than in *glp-1* animals. Other proteins such as PHA-4 may induce autophagy in germline-less animals in the presence or absence of *daf-16* activity. Specifically, we find that the expression of several autophagy genes (i.e., *unc-51*, *lgg-1*, and *bec-1*) is increased in *glp-1* animals compared to wild-type, and this induction requires *pha-4*. Consistent with these observations, we observed *pha-4* to be required for autophagy induction in germline-less animals. Notably, we found that overexpression of PHA-4 significantly induced *unc-51* but not *lgg-1* and *bec-1* levels (Figure S6), suggesting that PHA-4 overexpression is sufficient to recapitulate some, but not all, of the PHA-4 mediated effects observed in *glp-1* animals. These experiments not only reveal a novel mechanism by which autophagy is induced in *C. elegans* but also suggest that PHA-4 regulates the transcription of autophagy genes in metazoans. In support of this possibility, PHA-4 was recently shown to bind to the promoters of multiple autophagy genes, including *unc-51*, *bec-1*, and *lgg-1* during development [23, 24]

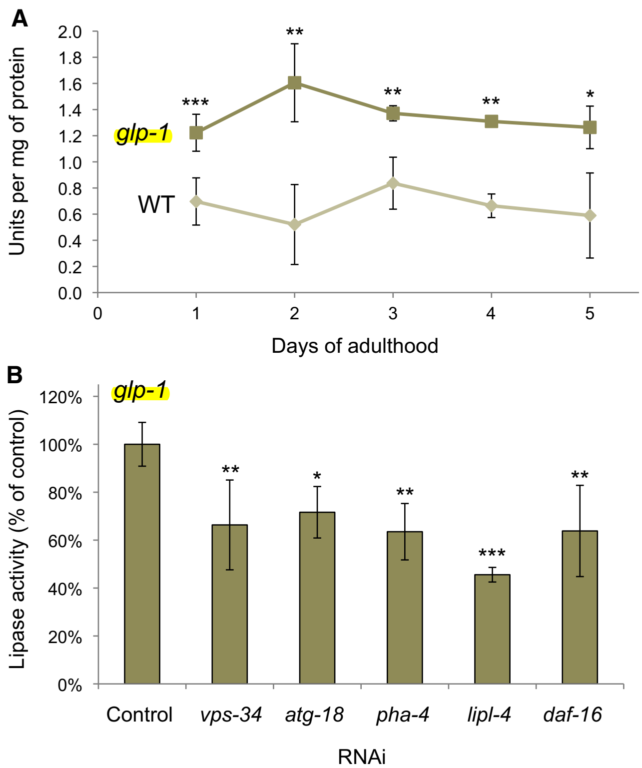

Figure 4. Increased Lipase Activity Observed in *glp-1* Animals Is Dependent on Autophagy Genes and PHA-4

(A) Lipase activity was measured daily in wild-type N2 (WT, lighter line) and *glp-1(e2141)* animals (darker line) from day 1 to day 5 of adulthood. Animals were raised as in Figure 2, and mean activity ±SD is shown (*p < 0.05, **p < 0.01, ***p < 0.001 versus WT, ANOVA).

(B) Lipase activity was measured in 4-day-old adult *glp-1(e2141)* animals fed bacteria expressing dsRNA against several autophagy genes, *pha-4*, *lipl-4*, or *daf-16*, for 3 days starting from day 1. Animals were raised as in Figure 2, and relative mean activity ± SD is shown (*p < 0.05, **p < 0.01, ***p < 0.001 versus control, ANOVA).

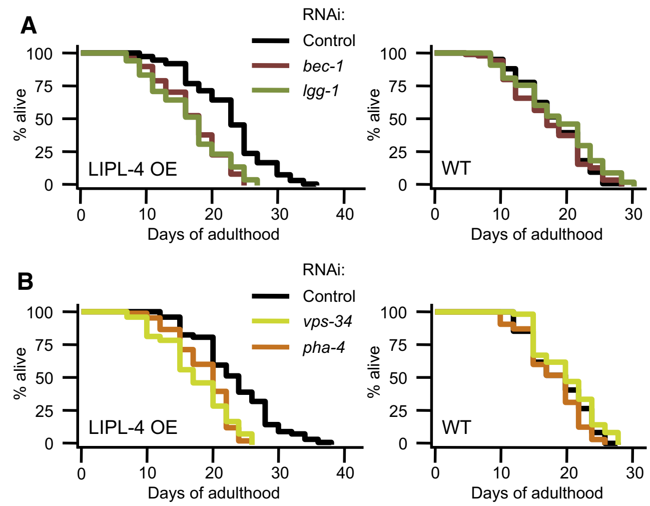

Figure 5. Autophagy Genes Are Required for the Long Life Span of Animals Overexpressing LIPL-4

Life-span analysis of animals overexpressing LIPL-4 (LIPL-4 OE) and non-transgenic siblings (WT) fed control bacteria or bacteria expressing *bec-1* or *lgg-1* dsRNA (A) or bacteria expressing *vps-34* or *pha-4* dsRNA (B) during adulthood. Animals were incubated at 20°C throughout their lives. See Table S2 for additional information and repeats.

(see Supplemental Information). Moreover, PHA-4 is required for the increase in autophagy observed in long-lived, dietary-restricted *eat-2* mutants [11]. Taken together, these observations are consistent with PHA-4 modulating life span by directly inducing autophagy gene expression, but PHA-4 may also regulate additional targets to affect longevity in germline-less animals. It will be of interest to determine how DAF-16 and PHA-4 function in the same longevity model and whether they regulate certain shared targets [19], as well as to ask whether PHA-4 also regulates autophagy in higher organisms.

In an effort to better understand how autophagy influences life span in germline-less animals, we have identified a new role for autophagy in modulating lipid metabolism in *glp-1* animals through the predicted triacylglycerol lipase LIPL-4. We determined that LIPL-4 exhibits lipase activity, at least in vitro, and significantly contributes to the elevated lipase activity observed in *glp-1* animals. Importantly, we found that autophagic activity, as well as autophagy genes and *pha-4* mRNA levels, was increased in long-lived animals overexpressing LIPL-4, and autophagy genes and *pha-4* were required for the elevated lipase activity, as was observed in *glp-1* mutants. As further support for a link between autophagy and LIPL-4, we observed that *lipl-4* is required for the increased autophagy activity observed in *glp-1* animals and thus an increase in LIPL-4 activity may promote autophagosome formation. In addition, we found that LIPL-4 was expressed in the same tissues in which we detected increased autophagy in germline-less animals, namely, hypodermal seam cells and the intestine. Finally, the autophagy regulator TOR might function as a common upstream regulator of these two processes in germline-less animals, because we discovered that inhibition of TOR was sufficient to increase *lipl-4* levels and lipase activity in a *daf-16*-dependent fashion, indicating that TOR has both *daf-16*-dependent as well as *daf-16*-independent functions [21, 25]. Collectively, these results strongly support the existence of a novel link between autophagy and LIPL-4 in germline-less *C. elegans* (see model in Figure 6).

Our results further suggest that the connection between autophagy and LIPL-4 in germline-deficient *C. elegans* may be critical for life-span extension in these animals. We found that LIPL-4 overexpressing animals are long-lived and both *pha-4* and autophagy genes are required for this extended life span, as is the case for *glp-1* animals. Although we do not yet know how LIPL-4 overexpression may induce autophagy to extend life span, it is possible that a lipase metabolite could trigger autophagy through regulation of TOR signaling (similarly to phosphatidic acid, a product of phospholipase D activity [26]) via an increase in PHA-4 activity. Such an explanation is consistent with the observation that directly reducing TOR levels by RNAi failed to extend life span in adult animals overexpressing LIPL-4 (Table S2), and such animals have increased *pha-4* mRNA levels. As further evidence for autophagy and LIPL-4 working by overlapping mechanisms, we observed that simultaneous inhibition of both lipase and autophagy functions did not further decrease the life span of *glp-1* animals, compared to inhibiting each process separately. Taken together, our genetic and biochemical analyses are consistent with a model in which LIPL-4 and autophagy work in concert to extend the life span of *glp-1* animals.

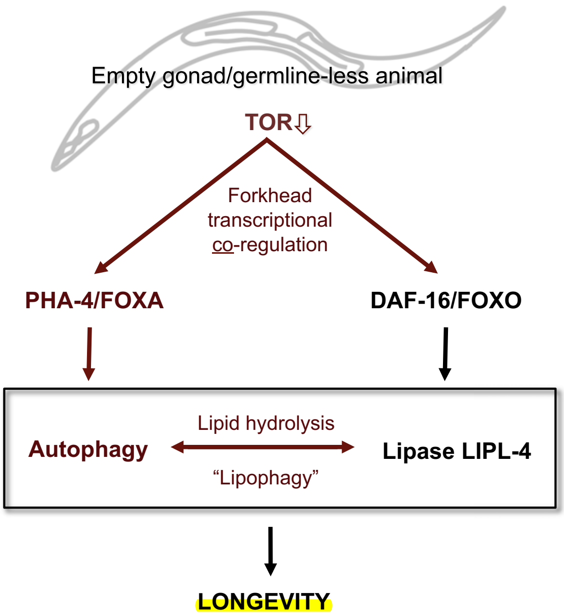

Figure 6. Model for How Autophagy and LIPL-4 Coordinately Modulate Longevity in Germline-less *C. elegans*

TOR (target of rapamycin) levels are reduced in *C. elegans* with an empty gonad (germline-less), which show enhanced activity of two forkhead transcription factors that modulate longevity and lipid metabolism. PHA-4/FOXA stimulates autophagy, and DAF-16/FOXO upregulates LIPL-4, thereby possibly inducing lipid hydrolysis. In turn, LIPL-4 requires autophagy to modulate life span, possibly through a process involving lipophagy. Novel observations made in this study are shown in red; known links are shown in black. Possible feedback and crosstalk events are not included for simplicity; see text for details.

In this model (Figure 6), the activity of the nutrient sensor TOR is reduced in response to germline removal, and this triggers the induction of two different pathways. One pathway involves activation of DAF-16 to induce *lipl-4* expression, which again may increase lipid hydrolysis. In contrast, the other pathway causes an induction of PHA-4 and subsequent autophagy gene expression to ensure increased flux through the multistep autophagy process. We note that feedback and crosstalk between components of these two pathways are possible. For example, we find that LIPL-4 overexpression causes a small (1.5- to 2-fold) increase in *daf-16* mRNA levels, and inhibition of *pha-4* by RNAi reduces *lipl-4* mRNA levels to about 50% in *glp-1* animals (data not shown). Consistent with the latter observation, PHA-4 can bind the *lipl-4* promoter [23, 24]. Taken together, these data suggest that *lipl-4* could be a common target of both DAF-16 and PHA-4. In turn, autophagy and LIPL-4 might work interdependently to ensure life-span extension in germline-less animals.

What is the nature of the link between autophagy and LIPL-4, which may possess intracellular lipolytic activity, and how could this link lead to life-span benefits? One possible mechanism may involve lipophagy, which is a large-scale hydrolysis of neutral lipid stores in the lysosome [13]. This scenario would predict lipases to be localized to the lysosome as seen for human lysosomal acid lipase, which we note shares a very high degree of sequence homology to LIPL-4 (data not shown). Alternatively, autophagy may be induced by a product of lipase activity, as is the case for autophagy induced by free fatty acids in pancreatic beta cells [27]. In this case, the lipase could be localized to the autophagosome, as has been observed for phospholipase D1 during starvation of mammalian cells [28]. It is also possible that enhanced lipolysis via autophagy prevents the accumulation of toxic byproducts or is critical for the partitioning of unused yolk, normally destined for oocytes. Lipolysis could also process phospholipids to boost membrane formation for autophagosome maturation necessary to recycle components relevant to aging. Future experiments, including cytological and biochemical profiling of lipids in *glp-1* and LIPL-4-overexpressing animals, should help clarify how autophagy is linked to lipid metabolism and LIPL-4 in germline-less animals.

Taken together, the data from this study propose a potential mechanism by which autophagy affects life span: we suggest that autophagy and LIPL-4 modulate aging in germline-deficient *C. elegans* by maintaining lipid homeostasis to prolong life span. As such, our results advance our understanding of how autophagy affects organismal aging and also offer new ideas as to how the regulation of lipid metabolism may be relevant to future treatments of metabolic disorders.

Experimental Procedures

Strains

All strains were maintained as previously described [29]. See Supplemental Experimental Procedures for details on strains used.

Life-Span Analysis and RNAi Experiments

Life-span assays were performed as described in [30] with the modification that all RNAi treatments were initiated on day 1 of adulthood. RNAi clones were obtained from the Ahringer and Vidal RNAi libraries. See Supplemental Experimental Procedures for details.

Autophagy Quantification

Autophagic events were quantified in *C. elegans* strains either by EM [31] or by use of an GFP::LGG-1 reporter [18]. See Supplemental Experimental Procedures for details.

Real-Time Quantitative PCR

RNA was extracted from biological triplicates, reverse-transcribed, and analyzed as previously described [32]. See Supplemental Experimental Procedures for details.

Lipase Activity Assay

Lipolytic activity was measured with a colorimetric assay kit (BioAssay Systems) and samples (biological triplicates) were prepared as previously described [33].

Statistical Analyses

For parametric analyses, Student's t test or one-way analysis of variance was done using GraphPad Prism 5 software. For life-span assays, Kaplan-Meier survival curves and p values were obtained by analyzing data by the log-rank (Mantel-Cox) test with Stata 8.2 software.

Supplemental Information

Supplemental Information includes six figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.07.042.

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