Evolutionarily conserved roles of *Caenorhabditis elegans* Perilipin in lipolysis

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Neutral lipids and namely triacyl-glycerols (TAGs) are the prevalent excess energy storage molecules in all eukaryotic organisms. They are universally organized in active cytoplasmic organelles called lipid droplets (LDs) and their breakdown is performed and regulated in an evolutionarily conserved manner. In mammals, two distinct but inter-connected pathways are believed to mediate this catabolism: conventional cytoplasmic lipolysis with effector neutral lipases; and lipophagy, a specific kind of autophagy exploiting lysosomal acidic lipases. Central molecules in this regulation are LD-resident proteins, perilipins (PLINs). Our recent discovery of a sole PLIN orthologue in *C. elegans* offers a unique opportunity to study these regulatory pathways, provided that the interactive mechanisms are orthologous. To determine this, we employed classical genetics with genome editing tools and *in vivo* microscopy to provide three lines of evidence demonstrating the conserved role of the *C. elegans* perilipin. Firstly, we proved the common presence of a standard lipolytic apparatus on LDs. Next, we ascertained a functional connection between nematode PLIN-1 and the effector enzyme, hormone-sensitive lipase (HOSL-1). Finally, we identified lipophagy as a secondary lipolytic pathway, which is consistent with the mammalian model. Our data provide not only a proof of concept but also suggests interesting implications by questioning the physiological role of lipophagy in lipolysis.
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Abstract
Neutral lipids and namely triacyl-glycerols (TAGs) are the prevalent excess energy storage molecules in all eukaryotic organisms. They are universally organized in active cytoplasmic organelles called lipid droplets (LDs) and their breakdown is performed and regulated in an evolutionarily conserved manner. In mammals, two distinct but inter-connected pathways are believed to mediate this catabolism: conventional cytoplasmic lipolysis with effector neutral lipases; and lipophagy, a specific kind of autophagy exploiting lysosomal acidic lipases. Central molecules in this regulation are LD-resident proteins, perilipins (PLINs). Our recent discovery of a sole PLIN orthologue in *C. elegans* offers a unique opportunity to study these regulatory pathways, provided that the interactive mechanisms are orthologous. To determine this, we employed classical genetics with genome editing tools and *in vivo* microscopy to provide three lines of evidence demonstrating the conserved role of the *C. elegans* perilipin. Firstly, we proved the common presence of a standard lipolytic apparatus on LDs. Next, we ascertained a functional connection between nematode PLIN-1 and the effector enzyme, hormone-sensitive lipase (HOSL-1). Finally, we identified lipophagy as a secondary lipolytic pathway, which is consistent with the mammalian model. Our data provide not only a proof of concept but also suggests interesting implications by questioning the physiological role of lipophagy in lipolysis.

Introduction
Virtually all eukaryotic organisms use neutral lipids for excess energy storage. These predominantly reside in lipid droplets (LDs), dynamic cytoplasmic structures with central neutral lipids surrounded by a single layer of phospholipids and a coat of LD-associated proteins. Perversely, in conditions of nutritional deprivation or other environmental stress, neutral lipids...
are catabolized in a closely regulated and a remarkably well evolutionarily conserved way. It is widely accepted that two distinct lipolytic pathways cater for this regulated degradation of neutral lipids throughout phylogenetic tree (Sztalryd & Brasaemle 2017).

The standard lipolytic enzymatic pathway consists of gradual degradation of triacyl-glycerol (TAG) to diacylglycerol (DAG) and monoacylglycerol (MAG) and ultimately to free glycerol, always removing one non-esterified fatty acid (NEFA). At least two principal neutral cytosolic lipases mediate this catabolism in human and Caenorhabditis elegans alike: adipocyte triglyceride lipase (ATGL); and hormone-sensitive lipase (HSL) with their respective nematode orthologues ATGL-1 and HOSL-1 (Lass et al. 2011). Both of these are regulated by extrinsic signaling, mainly via protein kinase A (PKA) and Perilipins (Sztalryd & Brasaemle 2017).

Perilipin proteins (PLINs) are a group of LD-associated proteins regulating the rate of lipolysis that show a remarkable degree of evolutionary conservation. In human, at least five different PLINs have been identified to date, all sharing certain homology with the rest. Individual PLINs display a differential pattern of tissue expression and distinct functions, PLIN-1 being the dominant lipolysis regulator in white adipose tissue (Sztalryd & Brasaemle 2017). Phylogenetically, the presence of PLIN orthologues reaches as far away as insects (Teixeira et al. 2003) and fungi (Wang & St. Leger 2007), but until recently, no such protein was known in one of science’s most powerful model systems, C. elegans. We have identified C. elegans locus W01A8.1 (previously annotated as mdt-28) as the sole PLIN gene orthologue in this nematode, henceforth labeled plin-1 (Chughtai et al. 2015). Concurrently with our work, other groups have also independently identified this locus as a major LD protein influencing lipid metabolism (Na et al., 2015; Vrablik et al., 2015).

In mammals, PLINs are shown to regulate the cytoplasmic lipolysis in a PKA-dependent manner, both positively and negatively. When activated, PLIN-dependent promotion of cytoplasmic lipolysis is two-fold: it recruits HSL to LDs and stimulates its access the resident lipids (Sztalryd et al. 2003; Tansey et al. 2001); and activated PLINs allows the co-activator of ATGL, CGI-58, to activate this lipase (Subramanian et al. 2004; Yamaguchi 2010). Contrarily, in the state of energetic abundance, de-phosphorylated PLINs are known to hinder the access of HSL to LD lipids and prevent CGI-58 from efficiently binding ATGL on the surface of LDs (Tansey et al. 2001; Martinez-Botas et al. 2000). Accordingly, the nematode orthologue of CGI-58, LID-1, is required for fasting-induced activation of ATGL-1 (Lee et al. 2014).

The alternative pathway to break down neutral lipids is lipophagy, a type of selective macroautophagy and possibly chaperone-mediated autophagy (CMA) of LD-resident neutral lipids followed by their lysosomal degradation by acidic lipases (Cingolani & Czaja 2016). Lipophagy was first identified as a lipolysis-mediating pathway in mouse hepatocytes (Singh et al. 2009) and has since been found to procure neutral lipid breakdown in a variety of organisms and tissues (Sztalryd & Brasaemle 2017). Despite of being seemingly closely interconnected with the cytosolic lipolytic pathway, the in-depth mechanistic understanding of lipophagy remains a mystery (Singh et al. 2009; Lapierre et al. 2011).
Similarly, the exact regulatory pathways of lipophagy are still somewhat ambiguous. The differential LD trafficking by small GTP-ases of the Rab family, mainly Rab-7, have been shown to play an important role (Lizaso et al. 2013). Lipophagy, despite being dispensable for lipolysis \textit{in vitro}, is certainly of considerable importance in lipid catabolism \textit{in vivo} even when the conventional cytosolic pathway is intact and may be preferentially exploited in some specific situations (Singh et al. 2009; Demine et al. 2018) and various tissues (Singh et al. 2009; Sztalryd & Brasaemle 2017).

Conventional lipolysis and lipophagy might work together in the PKA-dependent metabolic activation of lipolysis (Lizaso et al. 2013) and there is considerable cross-talk of both pathways known in mammals (Cingolani & Czaja 2016). PLIN-2 and PLIN-3 have been found to undergo CAM, promoting LD degradation by the conventional pathway (Kaushik & Cuervo 2015). An autophagy-associated membrane curvature protein Bif-1 has been implicated in PLIN-1 degradation leading to lipophagic breakdown of LDs at basal conditions, but likely does not participate in stimulated lipolysis (Liu et al. 2016). Perversely, cytosolic lipolysis may contribute to autophagic initiation, as is in the case of PNLPA2 (different orthologue of nematode ATGL-1) that has been associated with providing lipids needed for seeding of autophagosome membranes (Dupont et al. 2014).

Previously conducted studies in \textit{C. elegans} lipid biology completely precluded the existence of a perilipin orthologue and thus steered research into identification of distinct mechanisms of direct PKA-dependent regulation of lipolysis (Lee et al. 2014; Zhang et al. 2010; Liu et al. 2017), which might be interpreted as redundancy of PLIN-1 in nematode lipid metabolism. All the players in PLIN-dependent cascade now being identified, one might begin to challenge the conservation of individual mechanisms of interactions involved. Here we provide evidence supporting the validity of the basic principles of the vertebrate model in \textit{C. elegans}, we describe the specific mechanisms of action in this cascade and thus affirm the robustness of this model system in the crucial pursuit of knowledge in the field of lipid research. Moreover, we provide evidence that autophagy is a second lipolytic pathway, that is constitutively silenced by PLIN-1, which could have interesting biological implications.

\section*{Materials and methods}

\subsection*{Strains, transgenic lines and genome editing}

Wild type animals, N2 (var. Bristol), were used as the original pre-edited strain and all strains were maintained as described (Brenner 1974). Several locus-modified and other transgenic lines were obtained from their respective authors, from the Caenorhabditis Genetic Center (CGC) of the University of Minnesota (Minneapolis, Minnesota, USA) and from the National BioResource Project (NBRP) (https://shigen.nig.ac.jp/c.elegans/top.xhtml), specifically: VS20 [atgl-1p::atgl-1::GFP + mec-7::RFP], tm2369 [hosl-/-] and RD204 [Ppie-1-lgg-1::gfp] (Manil-Ségalen et al.
The strain KV1 [plin-1⁻/⁻] was prepared previously by Cas9/CRISPR-induced gene deletion (Chughtai et al. 2015). The strain KV-5 [plin-1::mKate2] was prepared with the employment of a CAS9/CRISPR-induced sequence insertion with a self-excising drug selection cassette (SEC) as described in (Dickinson et al. 2015). A Cas9 target, 8 base pairs upstream of the last nucleotide of PLIN-1 isoform b, was identified using the MIT CRISPR design tool (http://crispr.mit.edu). The effector CRISPR/Cas9 plasmid was prepared by insertion of target sequence into the sgRNA of pJW1219 plasmid (Addgene plasmid # 61250) (Ward 2014) using site-directed mutagenesis by PCR with primers 8047 and 8049 (Table S1). The repair plasmid was prepared by four-fragment Gibbson Assembly (Gibson Assembly® Mix, New England Biolabs, Ipswich, Massachusetts, USA) of a 579bp-long upstream and a 585bp-long downstream homologous flanking regions PCR-amplified with the primers 8342 and 8343; and 8344 and 8345 respectively, which had 5’-overhangs homologous to remaining fragments. The mKate2 fluorescent protein with the SEC as well as the vector backbone were obtained from pDD285 plasmid (Addgene plasmids # 66826) (Dickinson et al. 2015) by PCR amplification with the use of primers 8334, 8335, 8336 and 8337 (Table S1, Fig. S2). Both plasmids were microinjected into gonads of young adult N2 hermaphrodites as described (Tabara et al. 2017; Vohanka et al. 2010) using an Olympus IX70 microscope equipped with a Narishige microinjection system (Olympus, Tokyo, Japan) along with red fluorescent co-injection markers pCFJ104 [Pmyo-3::mCherry::unc-54] and pGH8 [pRAB-3::mCherry::unc-54utr] (Addgene plasmids # 19328 and # 19359 respectively) (Frøkjær-Jensen et al. 2008). Drug-selected animals were rid of these markers as well as of the SEC, all as described by (Dickinson et al. 2015), and the final sequence was controlled by PCR and sequencing. The eventual line was named KV5.

The pre-created externally acquired lines VS20, tm2369 and RD204 were crossed with KV1 to produce KV7, KV10 and KV2 respectively and VS20 was crossed with the newly obtained KV5 to produce KV6. Successful progenies of the crossed animals were screened by single-worm PCR (lines with no or weak fluorescence) as described previously (Chughtai et al. 2015) or by fluorescent microscopy (strongly-fluorescent lines) and the final line was sequenced on target non-fluorescent locus to confirm genotype before further use. Primers used in single-worm PCR-based screening are listed in (Table S1). For practical reasons, VS20 was used as a control line in experiments where atgl-1 locus was not concerned.

Growth rate and fecundity assays

Four lines were utilized in counting: VS20, tm2369, KV7 and KV10. Young laying adult hermaphrodites for each line were placed on an NGM plate and were incubated to lay eggs, after which they were removed from plates. The progeny was then grown at 22°C and counted and staged at 48 hours, each time with three recounts. The experiment was repeated eight times under various conditions listed in (Table S3). We averaged all percentage results from the total of eight plates. The brood size was determined as a percentage relative to VS20 (100%) and the growth
rate at the various time points as an average larval stage with 0 being embryo, 1-4 being L1-L4 and 5 being adult animal. Two-sided paired t-test was used with all combinations to determine statistical significance.

Cold resistance experiment

Adult hermaphrodites from embryo-rich plates were collected and bleached as described in (Stiernagle 2006). The hatched L1 larvae were synchronized by gently shaking in 1x Phosphate Buffer Saline (PBS) solution at 22°C during 18 hours then washed by fresh PBS and about 20-50 animals per line were seeded onto NGM plates with OP50 and grown to young adults. At this point, the plates were placed to an empty refrigerator with a stable temperature (fluctuating between 0 and 0.5°C) and left without interference for 16 hours. Thereafter, the plates were left for one hour at 22°C and then scored. Animals with no pharyngeal pumping and no responsiveness to touch were scored as dead, all others as live. The experiment was repeated for a total of three times and the survival percentage was summed. A z-test for proportion of two populations was used to determine the significance of the results.

Microscopy

Fluorescence microscopy and Nomarski optics microscopy were done using an Olympus BX60 microscope equipped with DP30BW CD camera (Olympus, Tokyo, Japan). In acquisition of confocal pictures, we used an inverted Leica SP8 TCS SMD FLIM system equipped with a 63 × 1.2 NA water immersion objective, a pulsed white light laser (470–670 nm), AOBS and two internal hybrid single photon counting detectors, and operated by Leica Application Suite X program (Leica Microsystems, Wetzlar, Germany) microscope was used for confocal microscopy. Manufacturer predefined excitation and absorption wavelength restrictive filtering was used for individual fluorescent proteins. Images were harvested with same or similar microscope settings in pairs control-experiment.

Image analysis and editing

8-bit images representing the prevalent phenotype were used in a 1:1 ratio, as captured. All images were edited and analyzed using ImageJ in the distribution Fiji (Schindelin et al. 2012). Fluorescent pictures as seen in Fig. 2 were used unadjusted, cropped 1:2, presented in false green. Confocal images in Fig. 1 were captured as hyperstacks of two channels, whole image brightness adjustments were used to equalize the brightness of both channels and two different focal planes were presented.

Confocal pictures of embryos in Fig. 4 were obtained from the fluorescently most prominent z-stack section, approximately 5µm in thickness, of earliest embryos of three individual representative animals per line (Fig. S4-9). These were analyzed by ImageJ using the 3D Object Counter tool, setting the background-suppression threshold as described in (Table S3). Two-folded unpaired t-test was used to determine significance of the comparison (Fig. 4E). A z-stack sum of two of these images was presented in Fig. 4 A, C using Z-study tool with Max Intensity
option for both, followed by equivalent whole-image contrast and brightness adjustment to suppress background signal. Brightfield transmission microscopy images corresponding to individual fluorescent pictures were obtained at the same position of the slide, optionally cropped and rotated to mirror the fluorescent image portrayed.

**Statistical analysis**

Numeric data were statistically analyzed using R (The R Development Core Team 2011) with the employment of individual appropriate statistical tests including the z-test of two population proportions, two sided paired and not-paired t-tests. The maximal required $p$ value for statistical significance was set to 0.05 and exact $p$ values were cited in figures. Statistical values were rounded to three decimal points, or two valid decimal points, percentages were rounded to 0.01%.

**Results**

**ATGL-1 co-localizes with PLIN-1 in *C. elegans* enterocytes**

We postulated that PLIN-1 in *C. elegans* is also mechanistically homologous to vertebrate PLINs. For this, a close spatial relationship of PLIN-1 and the LD surface-resident cytoplasmic lipase, ATGL-1, would be expected. In order to prove this, we utilized CRISPR/Cas9 mediated gene insertion to tag the *plin-1* gene at its natural locus, allowing us to visualize PLIN-1 tagged with a red fluorescent protein (mKate2). After creating PLIN-1::mKate2 (KV5) line, we crossed it with an established ATGL-1::GFP (VS20) line. As expected, in fed animals, these proteins are mostly co-localized in small dense and annular cytoplasmic foci. Some larger PLIN-1 positive foci, more prevalent in peripheral cytoplasm of enterocytes seem not to be associated with ATGL-1 (Fig. 1).

**PLIN-1 is critical for the fasting-induced lipolysis but not for ATGL-1 recruitment to lipid droplets**

Mammalian model presumes PLIN-1-independent localization of ATGL to LDs and rather the regulation of its co-activation by CGI-58 (Yamaguchi 2010). To ascertain both, the activator function exercised by PLIN-1 on ATGL-1; and the PLIN-1-independent ATGL-1 translocation to LDs (and possible up-regulation) in fasting, we crossed the VS20 [*atgl-1::atgl-1::GFP + mec-7::RFP*] line with our KV1 [*plin-1*⁻/⁻] mutant. While during the prolonged fasting, nematodes can empirically eventually overcome the lack PLIN1 and utilize all LD stored fat, we speculated that a shorter fasting period should lead to the initial activation of critical lipolysis, possibly distinguishing dynamic differences in the lipolytic rate between wild-type VS20 and *plin-1* mutants.
As expected, in fed animals, we observe the localization of GFP-tagged ATGL-1 concentrated in small cytoplasmic foci of enterocytes with similar abundance both in presence and absence of PLIN-1 (Fig. 2). GFP expression in VS20 control animals was more frequently concentrated in annular structures than in the crossed KV7 animals, which is compatible with the previously observed smaller basal LDs in PLIN-1-deficient animals (Chughtai et al. 2015). However, when fasted for five hours at room temperature, a decrease in overall GFP fluorescence in \( plin-1^{wt/wt} \) animals is marked with few remaining enlarged annular structures, likely due to completed lipolysis. Contrary to this, \( plin-1^{-/-} \) animals display a prominent increase in GFP fluorescence around large spherical cytoplasmic structures.

Cold induced lipolysis mediated by HOSL-1 is PLIN-1 dependent

ATGL is the most robust of neutral lipases and its insufficient function results in a severely impaired lipolytic cascade with appearance of prominent phenotypical changes and can be indirectly studied (Haemmerle et al. 2006; Fischer et al. 2007). Lack of HSL, however, results in more subtle phenotypical changes with DAG accumulation and is disputably harder to study (Haemmerle et al. 2002). In \( C. \) elegans, a model of lipolysis uniquely dependent on HOSL-1 has recently been discovered, which relies on its irreplaceability to procure glycerol as a substrate during cold exposure (Liu et al. 2017). When exposed to temperatures just above water’s freezing point, \( C. \) elegans activates the cAMP/PKA signaling pathway leading to the activation of the conventional lipolytic pathway, where HOSL acts as the sole lipase responsible for the liberation of the prevalent cryoprotective substance, glycerol. The concept being proven, we developed a simplified protocol of exposure to temperatures just incrementally above 0°C, with a single survival evaluation after 40 hours and compared single- and double-mutants of \( plin-1 \) and \( hosl-1 \) to control.

In keeping with the original findings, we confirm that even in these conditions, the animals lacking \( hosl-1 \) gene exhibit significantly lower survival after 40 hours than \( hosl-1 \) wildtype animals (93.07% versus 25.21%; \( p = 2.2 \times 10^{-16} \) (Fig. 3). We show that the cold tolerance of \( plin-1^{-/-} \) animals is impaired when compared to that of a wild-type animal (43.99%; \( p = 7.58 \times 10^{-12} \)), yet superior to that of \( hosl-1^{-/-} \) animals (\( p = 5.5 \times 10^{-5} \)), suggesting that PLIN-1 is an equally important player in regulation of cytoplasmic lipolysis by HOSL-1 (Fig. 3).

Strikingly, the same experiment showed that the described cold intolerance observed in \( hosl-1 \) deficient animals was partially rescued in \( plin-1 \) disrupted animals (46.49% versus above mentioned 25.74% for HOSL-1; \( p = 0.0013 \)) and almost equivalent to that of \( plin-1^{-/-} \) single-mutant animals (\( p = 0.3598 \)) (Fig. 3).

Depletion of PLIN-1 dramatically increases autophagic activity in embryonic stages

Our results in the previous experiment evoke the question of what alternative mechanism could be responsible for lipid degradation. While cold exposure is a simple, robust and consistent way...
to indirectly study certain aspects of lipolysis, direct studies of such animals are difficult in regard to cold-induced malformations and embryo accumulation (Liu et al. 2017). Activated lipolysis is important in embryonic development in a variety of organisms, from Drosophila to several species of mammals (Teixeira et al. 2003; Sastre et al. 2014; Xu et al. 2018; Tatsumi et al. 2018), promising to offer an interesting model for direct studies. Consistently, we have previously shown that in C. elegans, early embryos display distorted perinuclear hyperaccumulation of lipids, but eventually, sufficient lipolysis is accomplished and animals overcome this lack of PLIN by possibly a different mechanism, proposing it could be autophagy (Chughtai et al. 2015). In mammals, lipophagy seems to be a plin-1-independent way of TAG degradation with most concerned proteins conserved in C. elegans (Demine et al. 2018; Lizaso et al. 2013).

Purposefully, tools to directly study autophagic processes in C. elegans are well established (Zhang et al. 2015; Chen et al. 2017). Generally, a lipidated ubiquitin-like ATG-8 orthologue, LGG-1, a crucial protein responsible for targeting lipidation of organelles destined for autophagy has long been used as a subcellular autophagic marker (Meléndez et al. 2009). To assess the embryonal distribution of autophagy, RD204 line expressing GFP-tagged lgg-1 with its expression driven by a stage-specific promoter, Ppie-1 is used to overcome the general embryonic silencing of transcripts (Manil-Ségalen et al. 2014).

The RD204 embryos are known to form small early GFP-positive cytoplasmic clusters involved in paternal organelle clearance (Djeddi et al. 2015; Manil-Ségalen et al. 2014), as observed also in our case by confocal microscopy. Depletion of PLIN-1 in these embryos, however, leads to formation of cytoplasmic clusters of GFP-signal that were immensely larger than those of the RD204 line (Fig. 4). Average fluorescent object was 4.3-times more voluminous ($p = 0.050$), predominantly attributable to formation of large foci of concentrated GFP signal. We compared the six largest objects in each line that were 9.27-times larger in KV2 ($p = 0.047$). This is in concordance with our previous postulation, that lipophagy is directly inhibited by PLIN-1 (Chughtai et al. 2015).

**plin-1 and hosl-1 double mutants show accelerated growth and brood**

Next, we studied growth rate of animals lacking PLIN-1, HOSL-1 or both. Synchronously laid animals were staged 40 hours after laying of the last one in various conditions (Table S3). Single mutants of plin-1 and hosl-1 were 23.98% ($p = 0.021$) and 21.73% ($p = 0.034$) faster in growth than the control respectively. Double mutants displayed the most accelerated growth, 38.41% ($p = 0.00047$) faster than their wild-type counterparts, which is also significantly more than plin-1-/- ($p = 0.0059$) and hosl-1-/- ($p = 0.0083$) single mutants.

While larval development utilizes basal lipolysis, activated lipolysis seems to be important for early embryogenesis as discussed above and below. Accordingly, here we show that hosl-1-/- animals display a 28.7% decrease ($p = 0.013$) in total fecundity in comparison to control (Fig. 5).
Previously shown decrease in fecundity of PLIN-1-knock-down animals (Chughtai et al. 2015) was milder and not statistically significant in plin-1-/- animals in this experiment.

Strikingly, the disruption of both plin-1 and hosl-1 led to an increase in fertility, compared not only to single mutant animals ($p_{\text{plin-1}} = 0.00019$; $p_{\text{hosl-1}} = 0.0077$), but was also 26.20% higher than wild-type control animals ($p = 0.014$).

**Discussion**

Neutral lipid breakdown is clearly an important process in cellular metabolism in physiological and pathological conditions. A possibility to study lipid metabolism in a model organism as powerful as *C. elegans* could not only provide us a better understanding of evolution of fat metabolism but possibly also have therapeutic implications for clinical medicine one day. Our aforementioned results provide evidence suggesting evolutionary conservation and thus potential transferability of findings between phyla. Our recent discovery of PLIN orthologue in *C. elegans* introduced the last missing player to the canonical lipolytic pathway previously known in vertebrates. Taken together, here we supply three lines of evidence proving the concept of phylogenetic coherence of lipolysis: permissive spatial distribution, enzymatic function regulation and an existence of an alternative pathway.

First, we prove that sub-cellular localization of individual players of classical lipolytic pathway is likely identical throughout the evolutionary tree. We and others have previously shown that in the same conditions PLIN-1 (Chughtai et al. 2015) and ATGL-1 localize to LDs along with the CGI-58 homologue LID-1 (Lee et al. 2014). The only missing link was lack of evidence that in *C. elegans*, these proteins localize to the same LDs. Here we establish that PLIN-1 and ATGL-1 mostly co-localizes in enterocyte cytoplasm. This suggests the constitutive presence of PLIN-1, LID-1 and ATGL on LDs, a model equivalent to the one in vertebrates. In addition, PLIN-1 formed some circular structures that did not co-express ATGL-1. This fact only highlights the complexity and perhaps the diversity of LDs within a cell but further studies are needed to subclassify LDs.

Next, we examined dynamics of effector enzymes. In mammals, HSL is known to translocate to LDs upon lipolytic activation, but no such translocation has been observed in the case of ATGL (Tansey et al. 2001). In the classical model, ATGL is constitutionally localized on LD membrane and is activated by its cofactor, CGI-58 (Yamaguchi 2010). CGI-58 can only bind and activate ATGL when PLIN is phosphorylated at multiple sites by PKA. Interestingly, this PKA-dependent PLIN phosphorylation is accompanied by formation of micro-LDs, which are thought to be a different entity from large LDs, yet possessing the same membrane-bound lipolytic apparatus (Marcinkiewicz et al. 2006). Accordingly, the nematode homologue of CGI-58, LID-1, seems to function in a similar way (Lee et al. 2014).

In response to its phosphorylation by PKA, ATGL-1 is also known to become more resistant to proteasomal degradation resulting in its accumulation around LDs (Lee et al. 2014). Moreover, it...
is not known how micro-LDs are formed, but their formation is important for lipolysis and might be PLIN-regulated (Itabe et al., 2017; Sztalryd & Brasaemle, 2017). PLIN depletion should therefore result in insufficient lipolysis activation in fasting with LDs that do not decrease in size and have more ATGL-1 bound to their membrane. As expected, we have shown the concentration of intense GFP fluorescence in large annular structures in enterocyte cytoplasm of \textit{plin-1}^{-/-} animals. The seemingly increased size of LDs suggests ineffective lipolysis on one hand and possible deficiency of essential micro-LD formation discussed above. Hence, we conclude that while the activation of fasting-induced lipolysis is PLIN-1-dependent, recruitment of ATGL-1 to the LD membrane is not. This is in concordance with the previous evidence of a stark increase in LID-1 concentration on LDs in \textit{plin-1} locus-disrupted nematodes (Na et al., 2015).

Low temperature intolerance as an experimental model dissecting HOSL-1 function is conclusively connecting PLIN-1 with PKA-dependent activation of this lipase, which may only be interpreted as strong evidence that PLIN-1 is an upstream regulator of HOSL-1. Taken together with the reported dependence of ATGL-1 function on PLIN-1 and the relative homology of microdomains of both lipases and PLIN-1, a complete conservation of this basic model of activated lipolysis between nematodes and vertebrates is the most probable (Fig. 6B and 6D).

Finally, we have also observed a relative rescue of the pronounced cold sensitivity of \textit{hosl-1}^{-/-} animals by \textit{plin-1} knock-out. This can be most likely explained by the existence of an alternative glycerol-generating pathway, while it was shown that cAMP/PKA-dependent lipolysis is categorically required for sufficient glycerol production (Liu et al. 2017). Hence, the only possible situation in which HOSL-1-deficient animals would reestablish glycerol production would be in presence of an alternative lipolytic pathway that is suppressed by activated PLIN-1.

As discussed above, function of lipophagy as a second lipolytic pathway in mammals is now widely accepted (Singh et al. 2009; Liu et al. 2016; Dupont et al. 2014; Haemmerle et al. 2006; Lizaso et al. 2013; Tatsumi et al. 2018; Cingolani & Czaja 2016; Sztalryd & Brasaemle 2017). With most autophagy-related proteins being closely conserved in \textit{C. elegans} (Chen et al. 2017), it is evolutionarily very likely that lipophagy would be conserved and act as an alternative lipolytic pathway in \textit{C. elegans}. Utilizing embryonal model of activated lipolysis, we confirm the involvement of autophagy in neutral lipid degradation when it is uninhibited by lack of PLIN-1. This not only clarifies our results but also completes the \textit{tableau} of the entirety of conserved mechanisms of lipolysis between species as distant as \textit{C. elegans} and humans.

As mentioned above, PKA-activated PLIN-1 is inhibiting the secondary lipolytic pathway, identified here as lipophagy. By logical consequence, the activity of lipophagy under physiological conditions can only pronounce itself in conditions where PLIN-1 is inactive, i.e. in cells without lipolytic activation. In this regard, it is enticing to hypothesize a model where lipolysis in state of satiety and in intermittent inter-prandial fasting procuring energy for basal metabolism, growth and constant lipid turnover, all in single cell-centered context, would be preferentially catered for by lipophagy, at least in some species and/or tissues (Fig. 6A and 6C).
Presuming that lipophagy is indeed responsible for basal lipolysis in *C. elegans*, interference with the classical lipolysis pathway should not severely influence the physiology of nematodes in normal conditions that do not require brisk liberation of energy or substrate, i.e. normal larval development. Contrarily, lack of basal inhibitory regulation by inactivated PLIN-1 combined with basal lipophagic activity could result in accelerated lipolysis and in liberation of more free energy from food that is not sufficiently stored, consistent with smaller LDs in KV1 shown previously (Chughtai et al. 2015). This freely available energy may result in faster larval development.

As expected, fed PLIN-1-deficient animals have increased growth rates compared to *plin-1* wildtype controls. This is also true for *hosl-1* knock-out animals and this effect is additive with the *plin-1* mutation, which can attest to the independence of these two mechanisms of growth acceleration. It can be presumed that complete disruption of conventional lipolysis by depletion of the effector lipase, HOSL-1, would increase PKA activation in the context of insufficient levels of NEFAs and free energy or through other feed-back mechanisms, further accelerating lipophagic rate, while absence of PLIN-1 results in uninhibited basal lipolysis by ATGL-1, HOSL-1 and lipophagy.

In contrast to growth rate acceleration, fecundity of animals lacking HOSL-1 is clearly decreased and PLIN-1-deficient animals display strongly up-regulated autophagy and the previously shown distorted perinuclear accumulation of lipids (Chughtai et al. 2015). Taken together, this strongly indicates the relevance of the classical cytoplasmic lipolysis in early embryogenesis of *C. elegans* as is the case in many other species (Teixeira et al. 2003; Sastre et al. 2014; Xu et al. 2018). Accordingly, in lack of HOSL-1 but in the presence of PLIN-1, both conventional lipolysis is inadequate and lipophagy still inhibited by activated PLIN-1. In the *plin-1*−/− line, a certain level of classical lipolysis is preserved and lipophagy uninhibited, successfully compensating for the loss of physiological regulation. Yet, with no real activation of lipases, low levels of activated PLIN-1 in previous RNAi experiments (Chughtai et al. 2015) might suffice for lipophagy inhibition, but may not be enough for effective lipase activation, explaining the discrepancy between fecundity loss in RNAi (similar to that of *hosl*−/− animals) and relatively normal brood size in *plin-1* deleted animals.

Animals lacking PLIN-1 and HOSL-1 have disrupted normal activated lipolysis with over-activated PKA and lack the potential inhibitory effect of PLIN-1 on lipophagy. Provided that lipophagy is also directly activated by PKA, as is the case in mammals, lipophagy would be strongly stimulated, possibly increasing embryonic maturation rate and eventually total fecundity even to a supra-normal level in this line, which we have observed here.

Consistent with our findings in *C. elegans*, an aforementioned recent work found that upon knock-out of a crucial lipophagy-associated protein, *Bif-1* in mice, there was basal state lipid accumulation, but activated lipolysis was not affected (Liu et al. 2016), supporting the concept of the importance of lipophagy in basal conditions proposed here (Fig. 6A and C).
Interestingly, another crucial metabolic regulator, adenosine-monophosphate activated protein kinase (AMPK), participates in the regulation of lipolysis. In the conventional view, AMPK is directly sensing cell energy state in the form of AMP to ATP ratio, in cooperation with liver kinase B1 (LKB1) (Daval, Foufelle & Ferré, 2006; Steinberg & Kemp, 2009; Mihaylova & Shaw, 2011). Additionally, it is regulated by calmodulin-dependent protein kinase kinase (CaMKK) (Hawley et al., 2005; Woods et al., 2005), Sirtuin 1 (SIRT1) (Hou et al., 2008) and possibly other upstream regulators. The effect of AMPK on conventional lipolysis is still debated, but it is generally believed that in various species AMPK inhibits HSL action via a deactivating phosphorylation and so halts the whole conventional lipolysis despite the possible activation of ATGL in some conditions (Daval, Foufelle & Ferré, 2006; Steinberg & Kemp, 2009; Ahmadian et al., 2011).

We postulated that AMPK could be the missing coordinating pathway procuring low-intensity basal lipolysis in the basal state and during inter-prandial transient fasting based on three facts. Firstly, while other regulating mechanisms in lipolysis are transmitting extrinsic signals about the overall nutritional state of the organism i.e. β-adrenergic signaling, AMPK pathway is primarily a very dynamic tool to determine current energy state within the individual cell. As such, it would be best utilized for regulating basal state process that is individual to each cell (Daval, Foufelle & Ferré, 2006; Mihaylova & Shaw, 2011). Second, it was shown that AMPK activity is required for rationing lipid degradation in longer fasting to prevent too fast fat degradation and consecutive depletion of energy resources (Narbonne & Roy, 2009). Therefore, this pathway does provide a certain level of basal lipolysis but inhibits more pronounced active lipid breakdown. Finally, it further blocks cytoplasmic lipolysis in basal conditions via inhibition of HSL but activates various forms of autophagy (Lee et al., 2010; Mihaylova & Shaw, 2011) and specifically lipophagy as shown in case of CAM (Kaushik & Cuervo, 2016) and microautophagy (Seo et al., 2017). In conclusion, the place of AMPK-signaling in our proposed model is supported by coherence in purpose, mediation and execution (Fig 6A and 6C). Additionally, this could contribute to the apparent increase in LD size in plin-1⁻/⁻ animals upon fasting.

In addition, Insulin/Insulin-like Growth Factor-1 (IGF-1) signaling in mammals is inhibiting PLINs and conventional lipolysis in the basal state on at least three levels: PKA, PLIN and HSL (Sztalryd & Brasaemle 2017). With many of the same players shown to work in the same way, the model is probably at least partially conserved in C. elegans. Insulin signaling and its effector kinases are, however, thought to work conjointly with (mammalian) Target of Rapamycin ((m)TOR) in general silencing of autophagy, a system only permissive in cell stress and energetic deficit (Codogno & Meijer 2005; Meléndez et al. 2009). Our notion of basal state lipophagy recruitment might, therefore, seem somewhat antithetical, but lipophagy is clearly a very specific type of autophagy that would perhaps likely be regulated in a specific manner distinct to activated autophagy, as portrayed by the fact that it is active even in basal situations in many cell types, procuring up to one half of total lipolysis (Sztalryd & Brasaemle 2017).
In mammals, several Perilipin family members are exhibiting a complex tissue-specific expression pattern. Coherently, lipophagy is differentially active in various cell types (Ward et al. 2016). It is intriguing to postulate that the sum of actions of individual PLINs expressed in a specific cell directs LDs to conventional lipolysis or lipophagy. Supporting such hypothesis are published reports showing that degradation of specific PLINs are connected to lipophagy initiation (Kaushik & Cuervo 2015; Liu et al. 2016).

Conclusions

The recent discovery of PLIN-1, the sole perilipin ortholog identified to date in C. elegans, suggests the presence of molecular signaling and effector machinery orthologous to that of human and other mammals. Here we provide three lines of evidence in favor of the validity of the mammalian lipolytic model in nematodes. This promises great transferability of research done in one of biology’s most robust model organisms to human physiology and pathology in a field with great epidemiological implications. Moreover, our data provides further evidence of implication of a very specific mode of autophagy in lipid breakdown and we propose a hypothetical model where in certain tissues cytoplasmic lipolysis and lipophagy are not equally important for basal and activated lipolysis, but rather that lipophagy is employed preferentially in the former and conventional lipolysis in the latter. AMPK signaling pathway could be responsible for this preferential employment of lipophagy in basal conditions.

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Figure 1

Co-localization of PLIN-1 with ATGL-1.

(A) Confocal microscopic super-exposed montage of green and red channels, focused at the middle plane of an adult worm fed *ad libitum*, co-expressing PLIN-1::mKate2 and ATGL-1::GFP, displays the localization of the individual proteins around spherical cytoplasmic structures. These two are mostly co-localized, yet there are instances of some more voluminous objects with only PLIN-1 association, arrows point at these objects. Inlay shows a transmitted brightfield image of the corresponding region. White frame indicates the XY area of pictures (B) through (D). (B)-(D) Crop-out of a different focal plane with separated and merged channels. Arrows point to the same location of the spherical structure. (B) Green channel. (C) Red channel. (D) Montage of green and red channels showing co-localization. Whole-image brightness and contrast adjustment in individual channels was performed to normalize fluorescence levels. Bars indicate 50µm in (A) and 10µm in (B). (C) and (D) are to scale with (B).

*Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*
Figure 2

ATGL-1 recruitment to LDs in fasting.

(A)-(D) Fluorescent microscopy images of the middle gut of ATGL-1::GFP expressing lines. Inlays show corresponding transmitted brightfield images with Nomarski optics. (A) ATGL-1 expression in VS20 [Patgl-1::atgl-1::GFP] worms fed ad libitum, partly in form of solid small cytoplasmic foci and partly as spherical structures with annular GFP expression, both likely corresponding to small normal LDs. Arrows point to examples of the latter. (B) ATGL-1 in KV7 [Patgl-1::atgl-1::GFP; plin-1^+/+] line fed ad libitum is only present in small solid foci without the annular structures of plin-1^{wt/wt} line, compatible with the previously presented LD size decrease in absence of PLIN-1 in fed worms. (C) Increase in size of certain annular structures (arrows), but overall decrease in fluorescence intensity in VS20 worms grown with food to young adult stage, then fasted for five hours, suggesting completed lipolysis in case of intact lipolysis regulating pathway. (D) Stark increase in LD size (arrows) and fluorescence expression in KV7 worms after 5h of fasting suggests strong ATGL-1 expression and localization of LDs but incapability of processing the resident neutral lipids. Images representing the prevalent phenotype are presented. All pictures were captured as 8-bit images with same lamp and detector settings, cropped 1:2, unadjusted, presented in false green. Bars indicate 50µm.

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Figure 3

Lipolysis-dependent cold resistance decreases in plin-1 and hosl-1 mutants.

Upon cold exposure, lipolysis is a crucial generator of cryo-protective glycerol. VS20 plin-1\textsuperscript{wt/wt} hosl-1\textsuperscript{wt/wt} worms produce glycerol by standard HOSL-1-dependent lipolytic pathway in amounts sufficient for average survival proportion after exposure to 0-0.5°C of 93.07%. Coherently with previous findings hosl-1 knock-out worms experience significantly lower cold resistance, with an average survival of 25.21% (p = 2.2x10\textsuperscript{-16}). Equally, plin-1\textsuperscript{-/-} line, with survival of 43.99% was more susceptible to low temperature than VS20 (p = 7.59x10\textsuperscript{-12}) but less than hosl-1\textsuperscript{-/-} line (p = 5.5x10\textsuperscript{-5}). Strikingly, introduction of plin-1 locus mutation to hosl-1\textsuperscript{-/-} worms resulted in partial rescue of the phenotype, survival rate of double mutant worms being 46.03% (p = 0.0013), which is almost equivalent to that of plin-1 single mutants (p = 0.360). Error bars represent standard deviations. Significance description: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, N.S. p > 0.05.
Survival at 40h at 0-0.5°C
Figure 4

Increase in autophagic activity in PLIN-1-deficient embryos.

(A) Sum of a 5µm z-stack of most fluorescent confocal images of the first embryo of a RD204 [Ppie-1-lgg-1::gfp] worm shows modest amount of autophagy present in early normal embryo, probably participating in paternal mitochondria clearance. (B) Summed 5µm z-stack of the first embryo in a KV2 [Ppie-1-lgg-1::gfp; plin-1+] worm portrays a dramatic increase in GFP fluorescence, which can only be explained by a stark activation of autophagy in absence of PLIN-1. (C) Brightfield transmitted image of the RD204 worm. Analysis performed on the embryo in red the frame, also pictured in (A). (D) Corresponding brightfield image of the KV2 worm. Red frame depicts the embryo portrayed in (B), which was also analyzed. (E) Image-wide analysis of the most prominent 5µm-thick z-stacks of confocal images of three earliest embryos from three individual worms for each line. Average volume of a fluorescent object in KV2 line was 4.3-fold higher (p = 0.050), which was mostly due to presence of large clusters as documented by comparison of the 6 biggest objects in each line – 9.27-times larger in KV2 (p = 0.047). Images were captured with same or similar microscope setting (in pairs) and accordingly filtered to suppress background (Supplemental material ST3). Whole-image contrast and brightness adjustments were applied, same to both experiment and control. Bars indicate 10µm in (A) and (C) and 50µm in (B) and (D). Significance description: * p ≤ 0.05.
Figure 5

Growth rate and fecundity assays.

(A) Average stage of worms at 40 hours displays significant acceleration of growth in worm lacking PLIN-1 ($p = 0.021$), HOSL-1 ($p = 0.034$), or both ($p = 0.00047$). This factor is additive, as double mutants grew still significantly faster than both single mutants ($p_{x\text{plin-1}} = 0.0059$; $p_{x\text{hosl-1}} = 0.0083$). (B) Fecundity of plin-1$^{-}$ worms was not significantly reduced in this experiment, but was down 28.7% ($p = 0.013$) in hosl-1 mutants. Unexpectedly, but very consistently, plin-1 hosl-1 double mutants had significantly more progeny than both single mutants ($p_{x\text{plin-1}} = 0.00019$; $p_{x\text{hosl-1}} = 0.0077$) and 26.2% more than VS20 plin-1$^{\text{wt/wt}}$ hosl-1$^{\text{wt/wt}}$ worms ($p = 0.014$). Significance description: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, N.S. $p > 0.05$. 
Figure 6 (on next page)

Simplified diagram of proposed lipid degradation pathways in human and *C. elegans*.

(A) Hypothetical situation in the basal state with an abundance of energy in *C. elegans* enterocyte. Inactivity of PKA signaling and active insulin-like signaling (IIS) are known to suppress the cytoplasmic lipolysis with a reversion of PLIN-1 function into inhibitory. Also visualized is the proposed basal state activity of lipophagy in the absence of PLIN-1 inhibition, stimulation of lipophagy by intrinsically-activated AMPK signaling in transient halt of energy influx and possible stimulatory signals from leaky PKA-activity and IIS. (B) Upon energy requirement, mainly extrinsic signaling produces activation of PKA and downstream signal transmission via PLIN-1 to effector lipases. At the same time, PLIN-1 inhibits lipophagy in this model explaining our observations. (C) A corresponding basal state situation in a hypothetical human cell expressing some of PLIN1-5. In such context, differential basal activity of lipophagy would be determined by the specific PLINs present, e.g. in hepatocyte, satiety state basal lipolysis is very active, while same conditions do not activate much lipophagy in adipocyte, which could be due to other PLINs present in hepatic and adipose tissues. (D) Despite this variety of PLINs, activated lipolysis in human adipocyte likely works in a way that mirrors the functioning in *C. elegans*. 
Caenorhabditis elegans enterocyte - basal state

Homo sapiens cell X - basal state

Caenorhabditis elegans enterocyte - activated

Homo sapiens adipocyte - activated