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Role of BEC-1/Beclin 1 and Autophagy Genes in *C. elegans* Germline Cell Proliferation

Kristina Ames

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Role of BEC-1/Beclin 1 and autophagy genes in *C. elegans* germline cell proliferation

by

KRYSTINA AMES

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2016
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Role of BEC-1/Beclin 1 and autophagy genes in *C. elegans* germline cell proliferation

By Kristina Ames

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Autophagy is an evolutionary conserved process involved in the cellular adaptation to stress and basal levels of autophagy are crucial for cellular metabolism and homeostasis. Cellular recycling by autophagy is characterized by the formation of distinctive double-membrane vesicles (autophagosomes) that engulf unnecessary cytoplasmic components, such as organelles and long–lived proteins. Failure to remove protein aggregates and/or damaged organelles, via autophagy, has been implicated in various medical conditions such as liver disease, neurodegenerative diseases and cancer.

Autophagy may suppress or promote cellular proliferation in tumors, depending on the type and metabolic state of the cell, where autophagy is generally believed to mediate these functions cell-autonomously. Here we evaluate the role of BEC-1 and autophagy gene function in cell proliferation, using the *C. elegans* germ line as an *in vivo* model. BEC-1 is the *C. elegans* ortholog of human BECN1/Beclin 1, an essential autophagy regulator and tumor suppressor protein. We show that basal levels of autophagy are required for germline proliferation and that autophagy genes are...
necessary for the timely progression of the cell cycle. Interestingly, we noticed that autophagy genes may regulate cell proliferation via several pathways. We show that BEC-1/BECN1 acts independently of the GLP-1/Notch or DAF-7/TFG-β pathways, but interacts with components of DAF-2/IIR signaling pathway, to potentiate germline proliferation during development. Moreover, BEC-1/BECN1 requires DAF-18/PTEN but not DAF-16/FOXO for this function and can both promote and inhibit germ cell proliferation depending on the genetic mutant background. Furthermore, ATG-18 and ATG-16.2 also act independently of the GLP-1/Notch and DAF-7/TFG-β pathways, however it seems that they interact with the canonical of DAF-2/IIR signaling pathway and require DAF-18/PTEN and DAF-16/FOXO for their function. Interestingly, ATG-7 functions together with the DAF-7/TFG-β and independently of the GLP-1/Notch and DAF-2/IIR signaling pathways to promote stem/progenitor cell proliferation. Thus, we conclude that autophagy regulates cellular proliferation in a multifaceted way, probably through interactions with components of at least two non-mutually exclusive signaling pathways: DAF-2/IIR and DAF-7/TFG-β. Our findings indicate that autophagy and BEC-1/BECN1 functions non-cell autonomously, to control germ line proliferation by facilitating cell cycle progression and that BEC-1/BECN1 is probably important for the G2 to M phase transition. Given the evolutionary conservation of autophagy genes from C. elegans to humans, understanding the molecular mechanisms by which autophagy genes modulate the proliferation and/or maintenance of the stem progenitor cell population in vivo may lead to novel autophagy based chemotherapeutic approaches in the future.
ACKNOWLEDGMENTS

I would like to thank all those that made this thesis and this day possible. Not one person can accomplish a great task like this without help, and I am no exception to this rule. It is said - it takes a village to raise a child, well… in my case it took help from two continents to get me where I am right now.

Especially, I want to recognize my adviser, Dr. Alicia Melendez, for sharing your wisdom and knowledge, for the support, trust, and understanding throughout these years. I am very appreciative to the members of my committee: Dr. Ana Maria Cuervo, Dr. Iva Greenwald, Dr. Cathy Savage-Dunn and Dr. Daniel Weinstein for the guidance and support throughout my graduate years. I offer my deepest gratitude for all their helpful advice, discussions, never ending questions that were, and always will be the perpetuum mobile of discoveries and learning. Thank you for all your helpful comments and insightful questions that allowed me to complete this dissertation. Many thanks to all the members of the Biology Department in Queens College and Biochemistry Program of Graduate center. Thank you to assistant program officer Judy Lee, former executive officer Dr. Lesley Davenport and current executive officer Dr. Edward Kennelly, your guidance is always indispensible.

I have been blessed to have a great company along the way. Many thanks to all my fellow students who also became my friends: Nick, Melissa, Uday, Sara, Feng, Marina, Hannah, Brenda and Lev. We have shared so many things, from our rich scientific discussions to our personal moments of happiness and struggles. Thank you for your helping hands, extra eyes when mine were going blind, extra brains to pick, and especially, for your collaborative “English 001” lessons on how to speak and write. I am very grateful to have such a great team; you all have been great partners!
And last but not least, I want to thank my parents and my family. They inspired me as a child, encourage and supported my decisions throughout my all life. Especially, I want to thank my wonderful mom, who despite the Atlantic Ocean between us, is always here for me through my “thick and thin” moments. Special thanks go to my husband Ilya, who was side by side with me on this journey and always have been a great loving support with a gentle reminder about other things existing in the world besides the lab. Enormous thanks deserve my two children Andrew and Alice, who always been a part of my dissertation and the most important part of my life.

I would like to thank every single person I mentioned and many others I did not, again and again… THANK YOU ALL for your enormous help and support!
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Chapter 1

1. Introduction

1.1. Autophagy

1.1.1. Importance of autophagy, Different types of autophagy

Eukaryotic organisms have evolved different mechanisms to resist changes in the environment to assure constant nutrient supply during organismal survival, development, and growth. Autophagy is a major catabolic pathway that generates metabolic precursors for macromolecular biosynthesis and ATP for cellular homeostasis, and is indispensable during adaptation to stress.

One of the defining features of autophagy is lysosome-dependent degradation of damaged and unnecessary cytoplasmic components, such as organelles and long-lived proteins. Based on the delivery mechanism for materials degraded by the lysosome, at least three types of autophagy are recognized. First, chaperone-mediated autophagy transports single proteins containing a known pentapeptide motif to the lysosome for degradation (Cuervo, Gomes et al. 2000, Massey, Kiffin et al. 2004, Kaushik, Singh et al. 2010, Cuervo and Wong 2014). Second, microautophagy involves membrane rearrangements and direct engulfment of cytoplasmic materials by protrusion or invagination of the lysosomal membrane (reviewed in (Li, Li et al. 2012)). Third, macroautophagy, is the dynamic reorganization of the cellular membrane and sequestration of cytoplasm and organelles into double-membrane vesicles known as autophagosomes. These autophagosomes either fuse with endosomes forming intermediate amphisomes that will fuse with lysosomes (reviewed in (Sanchez-
Wandelmer and Reggiori 2013)), or directly fuse with the lysosomes to form autolysosomes or autophagolysosome (De Duve, Pressman et al. 1955). The lysosomal hydrolases of the autophagolysosome degrade the sequestered material, which is then recycled in the cell (reviewed in (Chen and Yu 2013, Shen and Mizushima 2014)). In this work we focus on the process of macroautophagy. For an overview of autophagy genes and their functions throughout this thesis please address Table 1.

### 1.1.2. Mechanism of autophagy overview

Macroautophagy (from here on referred to as autophagy) is a catabolic pathway that degrades and recycles cellular components, such as cytoplasmic organelles and long-lived proteins (reviewed in (Dunn 1994, Klionsky and Emr 2000, Klionsky 2007, Melendez and Levine 2009, Ohsumi 2014)). Studies conducted in yeast have identified numerous autophagy-related genes (ATG genes) and the analysis of atg mutants has provided a model that describes several steps of autophagy (Mizushima, Yoshimori et al. 2011, Ohsumi 2014). This multi-step process consists of induction, cargo selection and packaging, vesicle nucleation, vesicle expansion, vesicle completion, vesicle targeting, vesicle docking and fusion with the lysosome, concluded by retrieval of recycled autophagy proteins (reviewed in (Xie and Klionsky 2007, Melendez and Levine 2009, Mizushima and Komatsu 2011) (FIG.1.1).
In yeast, induction of autophagy occurs when target of rapamycin TOR, in *C. elegans* LET-363/TOR, is inhibited. Under normal growth conditions, Atg17, Atg29, and Atg31 form a stable protein complex (Kabeya et al., 2005 and 2009; Kamada et al., 2000). During conditions that induce autophagy, such as starvation, dephosphorylation of Atg13 leads to the formation of the Atg13-Atg1-Atg17-Atg29-Atg31 pentameric complex (Klionsky 2005, Kabeya, Kawarnata et al. 2007, Suzuki and Ohsumi 2007, Kawamata, Kamada et al. 2008). This induction complex stimulates the vesicle nucleation and phagophore formation step. During vesicle nucleation, recruitment of the class III phosphotidylinositol-3-OH kinase (PI3K) complex to the vesicle (Vps34-Atg6-Vps15-Atg14) generates phosphotidylinositol-3-phosphate (PI3P) (Kihara, Noda et al. 2001). At the phagophore assembly site (PAS), autophagy core proteins expand the phagophore into an autophagosome during vesicle elongation (Legakis, Yen et al. 2007). At the PAS nucleating site, the transmembrane protein Atg9, shuttles from non-PAS to PAS structures with two additional transport factors, Atg23 and Atg27, to provide
membrane for the growing autophagosome (Legakis, Yen et al. 2007). Autophagosome expansion and completion is mediated by two enzymatic complexes: the Atg12 conjugation system (Atg12-Atg5-Atg16) and an Atg8 lipidation system (Atg8-Atg3-Atg7) (reviewed in (Geng and Klionsky 2008)). In addition, the Atg1-Atg2-Atg18 kinase complex is involved in the retrieval of Atg9 from the PAS to non-PAS structures (Reggiori, Tucker et al. 2004, Suzuki, Kubota et al. 2007). In the next section I will discuss several functional complexes that autophagy proteins form during autophagosome biogenesis.

1.1.2.1. Atg1/ULK complex

The Atg1/ULK complex is positioned at the most upstream position during autophagosome biogenesis (Suzuki, Kubota et al. 2007). Atg1 is a Ser/Thr kinase that is essential for autophagy (Matsuura, Tsukada et al. 1997). In yeast, a complex formed by Atg17-Atg31-Atg29 functions as the scaffolding unit for the Atg1 complex formation (Kabeya, Noda et al. 2009). During normal growing conditions, TOR keeps Atg13, the binding partner of Atg1, hyperphosphorylated, which prevents it from binding to Atg1 (Kamada, Funakoshi et al. 2000). Upon starvation, Atg13 is hypophosphorylated (or dephosphorylated), which leads to the binding of Atg13 to Atg1 and results in the formation of the pentameric complex with Atg17-Atg29-Atg31 (Kamada, Funakoshi et al. 2000, Kabeya, Kamada et al. 2005). In mammals, ULK1 and ULK2 were identified as orthologs of yeast Atg1, which are partially redundant in starvation-induced autophagy (Chan, Kir et al. 2007, Lee and Tournier 2011). The mammalian ortholog of Atg13, was shown to interact with FIP200 and Atg101, that are thought to be functional counterparts
of yeast Atg17-Atg29-Atg31 (Hosokawa, Sasaki et al. 2009, Jung, Jun et al. 2009). Thus, Atg17, Atg29, and Atg31 are essential components for autophagy in yeast, but are absent in mammals (Noda and Fujioka 2015). In *C. elegans*, three components of the pentameric complex were identified: UNC-51, a Ser/Thr kinase homologous to yeast Atg1, ATG-13, a divergent ortholog of the autophagic budding yeast Atg13, and more recently, EPG-9 was identified as a protein with significant homology to mammalian ATG101, that directly interacts with ATG-13/Atg13 (Tian, Wang et al. 2009, Liang, Yang et al. 2012). In mammals, Atg101 appears to stabilize Atg13 (Hosokawa, Sasaki et al. 2009, Mercer, Kaliappan et al. 2009). Taken together, the Atg1/ULK complex functions as a scaffolding for the recruitment of downstream Atg proteins.

### 1.1.2.2. The class III PI3K complex and the Atg2-Atg18/WIPI complex

Formation of the autophagosomal membranes requires presence of a phosphatidylinositol 3-phosphate (PI3P) lipid molecule, that is produced by the class III phosphatidylinositol-3-kinases (PI3K) complex (Obara, Noda et al. 2008). There are several complexes of class III PI3K known that will be discussed later. In yeast, the autophagy-specific PI3K complex is composed of four proteins: Vps34 (catalytic unit), Vps15, Atg6/Vps30, and Atg14 (Kihara, Noda et al. 2001). In mammals, Beclin 1 was identified as an ortholog of yeast Atg6/Vps30 and Barkor (Beclin 1 interacting autophagy related key regulator) as ortholog of yeast Atg14 (Liang, Jackson et al. 1999, Kihara, Kabeya et al. 2001, Itakura, Kishi et al. 2008, Sun, Fan et al. 2008, Zhong, Wang et al. 2009). The accumulation of PI3P generates a platform to recruit the PI3P-binding protein Atg18/WIPI and its binding partner Atg2, forming an Atg2-Atg18
complex, which is required for the normal distribution of Atg9 vesicles that deliver membrane to the autophagosome (Reggiori, Tucker et al. 2004, Lu, Yang et al. 2011, Orsi, Razi et al. 2012). In *C. elegans*, BEC-1 was identified as an ortholog of the yeast and mammalian autophagy proteins Atg6/Vps30/Beclin1, and EPG-8, based on physical interactions, is thought to be a functional homolog of yeast Atg14 (Melendez, Talloczy et al. 2003, Yang and Zhang 2011). Moreover, based on protein domain information, VPS-15 is predicted to have protein kinase activity and ATP binding activity and to be an ortholog of the human PIK3 regulatory subunit/yeast Vps15 (Wu, Randle et al. 2007, Lindmo, Brech et al. 2008)

### 1.1.2.3. Atg12 and Atg8/LC3 conjugation systems

In the autophagic core machinery, there are two ubiquitin-like conjugation systems involved in autophagosome formation. The Atg12 conjugation system, where Atg12 - Atg5 conjugation is catalyzed by Atg7 and Atg10, by functioning as E1-like and E2-like enzymes, respectively (Mizushima, Noda et al. 1998). The Atg12-Atg5 complex binds to Atg16, in mammals Atg16L1 (Ishibashi, Fujita et al. 2011). The Atg12-Atg5-Atg16 complex localizes to a cup-shaped double-membrane sac, called isolation membrane, and upon the completion of autophagosome formation dissociates from the membrane (Mizushima, Yamamoto et al. 2001, Kuma, Mizushima et al. 2002).

Yeast Atg8 and the mammalian Atg8 orthologs (LC3A/B/C, GABARAP and GABARAPL1/2/3; hereafter represented by LC3) are conjugated to the phosphatidylethanolamine (PE) lipid molecule (Sou, Tanida et al. 2006). To conjugate Atg8/LC3 to PE, the C-terminal of the precursor Atg8/LC3 peptide (called also LC3-I) is
truncated by Atg4 (truncated version also called LC3-II), where Atg7 and Atg3 act as the
E1-like and E2-like conjugation enzymes (Ichimura, Kirisako et al. 2000, Kirisako,
Atg5-Atg16 complex acts as the E3-like enzyme during isolation membrane formation
and autophagosome completion (Hanada, Noda et al. 2007). In C. elegans all of the
members of these conjugation systems were identified (see Table 1). It is interesting to
note that C. elegans has two homologues of yeast Atg8: LGG-2 and LGG-1, with LGG-2
more related to the Atg8/LC3 and being essential for post-translational modification and
localization to the autophagosomes (Alberti, Michelet et al. 2010).

1.1.2.4. Atg9 vesicles

Atg9, the only transmembrane protein among the core Atg proteins, is required
for the accumulation of most of the downstream autophagy proteins and plays a key
role in the delivery of membrane lipids (Suzuki, Kubota et al. 2007, Itakura, Kishi-Itakura
et al. 2012). In yeast, the majority of Atg9 is localized to mobile cytoplasmic membrane
vesicles derived from the Golgi, which participate in autophagosome formation (Geng,
Nair et al. 2010, Ohashi and Munro 2010, Yamamoto, Kakuta et al. 2012). In mammals,
starvation induces departure of Atg9 containing vesicles from the trans-Golgi network,
and results in the co-localization of Atg9 with LC3 on the autophagosome (Young, Chan
et al. 2006). Localization of Atg9 containing vesicles has been shown to be at least one
source of the membrane for autophagosome biogenesis (Yamamoto, Kakuta et al. 2012).
1.1.2.5. Origin of the autophagosomal membrane

Recent studies significantly advanced our knowledge about the origin of the autophagosomal membrane, especially in mammals. The endoplasmic reticulum (ER) is a major source of the autophagosomal membrane; additionally, mitochondria, ER-Golgi intermediate compartments (ERGIC), the Golgi apparatus, recycling endosomes and the plasma membrane were shown to contribute membrane to the autophagosome (reviewed in Shibutani and Yoshimori 2014). In response to amino acid starvation, the PI(3)P-binding protein DFCP1 (double FYVE domain-containing protein 1) localized to ER and Golgi membranes, and induced formation of LC3 positive ring like structures, named omegasomes, which serve as intermediates for the genesis of isolation membrane (Hayashi-Nishino, Fujita et al. 2009). This ER and isolation membrane connection was further confirmed by 3D tomographic analysis (Yla-Anttila, Vihinen et al. 2009). Additionally, using in vivo experiments, ERGIC was shown as a primary membrane source both necessary and sufficient to trigger LC3 lipidation (Ge, Melville et al. 2013). Furthermore, it was suggested that outer mitochondrial membrane contributes to the autophagosome biogenesis and that there is transient sharing of membranes between mitochondria and autophagosomes (Hailey, Rambold et al. 2010). The plasma membrane may also contribute to autophagosomes, as well as recycling endosomes, since Atg9 was localized on the plasma membrane and co-localized with recycling endosomes (Ravikumar, Moreau et al. 2010, Orsi, Razi et al. 2012).

Autophagosomes may either engulf bulk cytoplasm non-specifically, or target the engulfment of specific cargo molecules. Autophagy is primarily non-selective; however there are several examples of non-selective autophagy, which is defined by the type of
sequestered cargo, such as xenophagy, which sequesters bacteria and viruses (Baxt, Garza-Mayers et al. 2013), aggregaphagy, which sequesters aggregated proteins (Lamark and Johansen 2012), pexophagy which sequesters peroxisomes (Iwata, Ezaki et al. 2006), lipophagy, which sequesters lipids (Singh, Kaushik et al. 2009), and mitophagy which sequesters mitochondria (Narendra, Tanaka et al. 2008). Targeted engulfment of selected cargo is achieved, in vertebrates, by tethering cargo to the site of autophagosomal engulfment with various post-translational modifications such as phosphorylation, ubiquitylation and acetylation for substrate recognition and selectivity (Johansen and Lamark 2011, McEwan and Dikic 2011). For example, after mitochondrial damage, Parkin, an E3 ubiquitin ligase, is recruited to mitochondria and ubiquitinates mitochondrial outer membrane proteins to initiate autophagy (Narendra, Tanaka et al. 2008). In addition, ubiquitination of various substrates, such as red fluorescent protein and peroxisomes, is sufficient to induce their autophagic degradation (Kim, Hailey et al. 2008). Moreover, adaptor proteins, such as p62, NDP52, NBR1, and optineurin, can simultaneously bind ubiquitin and LC3 mediating association between the ubiquitinated substrate and the isolation membrane (Pankiv, Clausen et al. 2007, Kirkin, Lamark et al. 2009, Thurston, Ryzhakov et al. 2009, Wild, Farhan et al. 2011).

In the docking and fusion step, the autophagosomes may fuse with an endosome (forming an amphisome) before fusing with the lysosome, or fuse directly with the lysosome, via the action of the lysosomal proteins ((Rubinsztei, Gestwicki et al. 2007), reviewed in (Roy and Debnath 2010)). The resulting autolysosome or autophagolysosome is a single membrane – bound vesicle where sequestered materials are degraded, by lysosomal hydrolases, and recycled.
1.1.3. Regulation of autophagy

Organisms require a constant supply of nutrients and energy for growth, development, and survival. Under normal physiological conditions, basal levels of autophagy maintain cellular homeostasis by recycling unnecessary cellular components (reviewed in (Mizushima and Komatsu 2011)). Moreover, during nutrient deprivation and stress conditions, autophagy performs two nonexclusive functions for cellular survival: sequestering cytoplasmic materials and degrading them (Mizushima 2005). To carry out these tasks, the elaborate machinery of autophagy is highly regulated at different steps. Regulation of the autophagy was proposed to be divided at three levels (Esclatine, Chaumorcel et al. 2009). The first level of regulation (Level 1) targets signaling pathways upstream of the autophagy machinery. Many pathways converge on the evolutionary conserved target of rapamycin (TOR) signaling pathway, while others, such as the inositol phosphate pathway, act independently from TOR signaling (reviewed in (Meijer and Codogno 2009)). These pathways are important for autophagy upregulation during stress conditions; however, regulation of basal autophagy is still poorly understood. The second level of regulation (Level 2) modulates the autophagic machinery via posttranslational modifications or protein-protein interactions, such as Beclin1/Bcl-2 interactions (Ohsumi 2001, Pattingre and Levine 2006, Maiuri, Criollo et al. 2007). The third level of regulation (Level 3) involves autophagosome maturation and fusion with the lysosome (Eskelinen 2005). Problems at this level of regulation were implicated in various neurodegenerative diseases, such as Alzheimer’s (Yu, Cuervo et al. 2005). Although there are three proposed levels of autophagy regulation, they are
not mutually exclusive, and any given autophagy regulator can target several levels. For example, starvation induces autophagy at Level 1 and 2 (Pattingre, Espert et al. 2008) and some viruses (such as poliovirus) induce autophagy at Level 2 and 3, for their own benefit (Taylor and Kirkegaard 2008).

1.1.3.1. Signaling pathways that regulate autophagy

Autophagy can be induced by a variety of factors, such as lack of nutrients and growth factors, hypoxia, DNA damage, and viral infections. In mammals, insulin and insulin-like growth factors regulate mTOR. TOR signaling controls phosphorylation of Atg13, thus in turn, alters Atg1-Atg13 interactions. Under starvation conditions, dephosphorylated or hypophosphorylated Atg13 induces autophagy by forming the Atg1-Atg13-Atg-17 complex (Klionsky 2005, Suzuki and Ohsumi 2007).

Induction of autophagy, by mTOR downregulation, results in ATP production by nutrient recycling, and thus, is a key to cell survival. The TOR kinase is a major evolutionarily conserved nutrient availability sensor. Mammalian TOR (mTOR) functions in two different complexes: mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2) (Schmelzle and Hall 2000). These complexes share some common components, but display distinct cellular functions and lead to the activation of different downstream substrates (Loewith, Jacinto et al. 2002, Jacinto, Loewith et al. 2004). In growing cells, activation of mTORC1 requires adequate energy resources, nutrient availability, oxygen abundance, and proper growth factors to begin mRNA translation (Wullschleger, Loewith et al. 2006). mTORC1 is composed of mTOR, regulatory-associated protein of mTOR (Raptor), the
mammalian lethal with SEC13 protein 8 (mLST8), proline-rich AKT1 substrate 1 (PRAS40) and DEP domain-containing mTOR-interacting protein (DEPTOR) (Wullschleger, Loewith et al. 2006). This mTORC1 complex is controlled by insulin, growth factors, oxidative stress, and amino acids (particularly leucine), to control protein synthesis (Hay and Sonenberg 2004, Esclatine, Chaumorcel et al. 2009). Insulin-like growth factors activate the class I PI3K/Akt/mTOR pathway and consequently inhibit autophagy. The class I phosphoinositide 3 kinase (PI3K) enzyme phosphorylates phosphatidylinositols (PtdIns(4)P, PtdIns(4,5)P2 and PtdIns(3,4,5)P3), which activate serine/threonine-specific protein kinase (Akt) (also known as protein kinase B (PKB)), and mTORC1 that in turn, inhibits autophagy. The phosphatase and tensin homolog (PTEN) stimulates autophagy by hydrolyzing PtdIns(3,4,5)P3 and abolishing PI3K/Akt inhibition (Meijer and Codogno 2004).

Additionally, during metabolic stress and low levels of cellular energy, activation of autophagy is essential for cell survival. In mammalian cells, low levels of ATP lead to inactivation of the TORC1 complex, and autophagy induction (Inoki, Zhu et al. 2003). The mTORC1 complex can be inhibited by AMP-activated kinase (AMPK), which reflects the energy status of the cell. Low ATP levels increase the AMP to ATP ratio and activate AMPK (5′ adenosine monophosphate-activated protein kinase). AMPK will inhibit energy consuming pathways, such as protein synthesis, and promote recycling pathways, such as autophagy (Hardie 2007). Moreover, a number of other extra- and intracellular stresses are involved in the activation of autophagy such as hypoxia, oxidative stress, ER- stress, and Ca^{2+} efflux from the ER (reviewed in (He and Klionsky 2009)). Furthermore, amino acid deprivation stimulates autophagy (Poso, Wert et al.
In mammals amino acids mediate mTORC1 signaling by Ca^{2+}/calmodulin dependent activating class III PI3K/Vps34 (Nobukuni, Joaquin et al. 2005, Gulati, Gaspers et al. 2008). However in Drosophila melanogaster, class III PI3K does not act upstream of TOR to regulate autophagy, illustrating the difference in signaling pathways between species (Juhasz, Hill et al. 2008).

### 1.1.3.2. Autophagosome formation regulation

Several studies propose autophagy regulation at the transcriptional level. Little is known about upstream regulation of the rapamycin insensitive complex, mTORC2. Rapamycin-insensitive companion of mammalian target of rapamycin protein, RICTOR, integrates nutrient- and growth factor-derived signals to activate Akt, which then blocks activation of FoxO3 transcription factor and thus, induces autophagy (Guertin, Stevens et al. 2006, Mammucari, Milan et al. 2007). Interestingly, during muscle atrophy, autophagy is induced independent of mTOR, due to decreased IGF-1/PI3K/Akt signaling, by a much slower FoxO3 transcription-dependent mechanism (Zhao, Brault et al. 2007). Thus, it is interesting to note that decreased PI3K/Akt signaling activates autophagy, not only through mTORC1, but also through a slow transcription-dependent mechanism involving FoxO3, affecting autophagy proteins, such as LC3/Atg8, Atg12 and Beclin 1/Atg6 (Zhao, Brault et al. 2007). Furthermore, another transcription factor, E2F1, which mediates both cell proliferation and apoptosis, was shown to induce autophagy. In mammals, E2F1 activation upregulates the expression of LC3, Atg1, Atg5 and damage regulated autophagy modulator DRAM (Polager, Ofir et al. 2008).
Moreover, a high-affinity E2F binding site was found on the Beclin 1 promoter, however, the effect of E2F1 on Beclin 1 is still not clear (Weinmann, Bartley et al. 2001).

During accumulation of misfolded proteins in the endoplasmic reticulum (ER) in yeast, protein kinase, R-like endoplasmic reticulum kinase (PERK), is activated to phosphorylate eukaryotic translation initiation factor 2A (eIF2a) and stimulates autophagy by upregulation of Atg12, and activation of the Atg5–Atg12–Atg16 complex (Kouroku, Fujita et al. 2007). Interestingly, rapamycin- and starvation-induced autophagy are not mediated by the PERK/eIF2a pathway, showing selective modulation of autophagy by eIF2a (Yorimitsu and Klionsky 2007). Moreover, the transcription factor EB (TFEB), a master gene for lysosomal biogenesis, activates a transcriptional activation of autophagy and lysosomal genes involved in autophagosome formation and autophagosome-lysosome fusion (Settembre, Di Malta et al. 2011). In cardiomyocytes, overexpression of Atg7 induced basal autophagy levels and reduced accumulation of misfolded proteins and aggregates (Pattison, Osinska et al. 2011). These examples highlight the complexity and variety of pathways that regulate autophagy.

1.1.3.3. Maturation step regulation

Maturation of autophagosomes is crucial for the degradation of the autophagosomal cargo, since degradation prevents depletion of nutrients and ATP. In yeast, autophagosomes fuse with the vacuole where the Atg15 lipase directs available autophagic cargo to hydrolysis for the final degradation (Epple, Suriapranata et al. 2001). In mammals, two different scenarios are possible. First, the autophagosome can
fuse with the early or late endosomes to form acidic and degradative amphisomes, followed by final fusion with the lysosome to complete the degradation (Eskelinen 2005). Second, autophagosomes can directly fuse with the lysosomes to degrade and recycle materials (reviewed in (Luzio, Pryor et al. 2007)). There are several crucial events that have to be maintained and regulated, such as vesicular fusion and proper acidification of the autophagic compartments.

Maturation of autophagosomes depends on the fusion of several vesicular compartments, such as autophagosomes, endosomes, and lysosomes. Multiple groups of proteins are responsible for mediating those events. Group of soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) are required for intracellular membrane fusion during autophagosome maturation (Rothman and Wieland 1996, Gurkan, Koulov et al. 2007). A family of Rab GTPases regulates several steps of membrane trafficking, such as membrane fusion and vesicle movement along actin and tubulin networks. Furthermore, microtubule (MT) stability is crucial for the proper fusion of the autophagic vacuoles with the lysosomes, where change of the MT stability alters autophagic flux (reviewed in (Monastyrska, Rieter et al. 2009)). The alteration of autophagosomal flux can also be due to variations in lysosomal pH. A group of vacuolar ATPases (v-ATPases), proteins located in acidic compartments, pump H+ ions into the lysosome, to maintain low pH for lysosomal enzyme function (Forgac 2007). The inhibition of the activity of ATPases, by incubation with bafilomycin, blocks lysosomal degradation (Fass, Shvets et al. 2006).
1.1.4. Beclin1

1.1.4.1. Beclin1 structure and complexes

Mammalian Beclin 1 belongs to an evolutionarily conserved protein family and shares 30% sequence identity with the yeast Atg6/Vps30 protein (Kametaka, Okano et al. 1998, Melendez and Levine 2009). The C. elegans ortholog of Beclin 1 is BEC-1 and has 28% sequence identity with yeast Atg6/Vps30 and 31% with mammalian Beclin 1. Beclin 1 is a peripheral membrane protein that consists of several domains: an N-terminal intrinsically disordered region (Mei, Su et al. 2014), a BH3-only domain (Oberstein, Jeffrey et al. 2007), a central coiled-coil domain (CCD) (Li, He et al. 2012), and a C-terminal BARA (β-α repeated, autophagy specific) domain (Huang, Choi et al. 2012). These domains provide a platform for binding other proteins and to form distinct complexes that are involved in autophagy and endocytosis (Furuya, Yu et al. 2005).

increases production of phosphatidylinositol 3-phosphate (PIP3) leading to phagophore elongation and recruitment of other Atg proteins to the phagophore (Fan, Nassiri et al. 2011). Mammals have at least two other stable Vps34/Beclin1 complexes, which include UVRAG (human ultraviolet irradiation resistance-associated gene), and the negative regulator Rubicon (Run domain protein as Beclin 1 interacting and cysteine-rich containing). A complex with UVRAG alone functions in the vacuolar protein sorting /endocytic trafficking pathway (Sun, Fan et al. 2009), and autophagosome and lysosome fusion. The mammalian complexes also contain other Beclin 1 binding proteins, such as the B-cell lymphoma 2 (Bcl-2), Ambra, Bif-1, and the vacuole membrane protein 1 (VMP1) (Funderburk, Wang et al. 2010). The presence of Ambra1 (activating molecule in Beclin 1 regulated autophagy protein1) favors the Beclin 1-VPS34 interaction and upregulates autophagy (Fimia, Stoykova et al. 2007). UVRAG interacts with the membrane either through its phospholipid-binding C2 domain, or indirectly, through Bif-1/endophilin B1 (Bax interacting factor), that binds to the UVRAG and regulates membrane curvature, to promote autophagosome formation (Farsad, Ringstad et al. 2001, Takahashi, Coppola et al. 2007). Interestingly, UVRAG-Beclin1–PI3K complex seems to be functional in both endocytic trafficking and autophagosomal maturation; however it is still unclear if UVRAG mediates autophagosomal maturation directly, or indirectly through its endocytic function (Liang, Yu et al. 2001, Liang, Feng et al. 2006, Itakura, Kishi et al. 2008). Moreover, Beclin 1 was shown to bind to membranes through aromatic amino acids at the tip of a surface loop in its BARA domain, and possibly facilitates membrane interaction with the PI3K complex (Huang, Choi et al. 2012). In addition to autophagy and endocytosis, Beclin1–PI3K participates
in the LC3 associated phagocytosis that aids in maturation of the phagosomes containing apoptotic cells and pathogens (Sanjuan, Milasta et al. 2009).

1.1.4.2 Regulation of autophagy by Beclin 1

Several studies have confirmed that Beclin 1 regulates the autophagic machinery through protein-protein interactions via the phosphorylation status of Beclin1 or the presence of unique proteins bound to Beclin 1 (Zalckvar, Berissi et al. 2009, Zalckvar, Berissi et al. 2009, Furuya, Kim et al. 2010). Autophagy can be modulated by diverse cellular stimuli that ultimately regulate the lipid kinase activity of the Vps34 complex. Beclin 1 activity can be altered by its sequestration to subcellular locations, post-translational modifications, or protein-protein interactions (reviewed in (Kang, Zeh et al. 2011, Reidick, El Magraoui et al. 2014, Levine, Liu et al. 2015).

Beclin 1 was initially isolated as a B-cell lymphoma 2 (Bcl-2) -interacting protein (Liang, Kleeman et al. 1998). Under normal conditions, inactive Beclin 1 is bound to Bcl-2 to form a Beclin1-Bcl-2 complex that results in the homodimerization of Beclin 1. During homodimer formation, the Beclin 1 N-terminal phosphorylation sites are blocked by its binding partner and Bcl-2, to prevent the phosphorylation of Beclin1, and inhibit autophagy (Wei, An et al. 2015). During starvation, Bcl-2 is phosphorylated and dissociates from Beclin 1, leading to the formation of the PI3K complex with Vps34, Vps15 and Atg14L (Russell, Tian et al. 2013).

The BH3 domain of Beclin 1 forms an amphipathic helix and is important for regulating Beclin1 activity (Oberstein, Jeffrey et al. 2007). BH-only proteins, including
the Bcl-2-associated death promoter (Bad) and the Bcl-2-interacting mediator of cell death (Bim), disrupt the Beclin1-Bcl-2 complex and stimulate autophagy (Maiuri, Criollo et al. 2007). Moreover, it was recently shown that the vacuole membrane protein 1 (VMP1) binds to the BH3 domain of Beclin1, promoting the dissociation of Bcl-2 from the Beclin1-Bcl-2 complex. VMP1 was proposed to be essential for bringing the mammalian Beclin 1 –PI3K complex to the site of autophagosome formation (Molejon, Ropolo et al. 2013).

The N-terminus of Beclin 1 contains multiple regulatory amino acids that are targets for different kinases, such as the serine/threonine-protein kinase ULK1, MAP kinase activated protein kinase 2/3 (MAPKAPK2/ MAPKAPK3), AMPK, DAPK (Zalckvar, Berissi et al. 2009, Kim, Kim et al. 2013, Russell, Tian et al. 2013, Wei, An et al. 2015). Phosphorylation by these kinases stimulates the lipid kinase activity of the Vps34 complex and leads to the induction of autophagy. On the contrary, the multisite phosphorylation of Beclin 1 by epidermal growth factor receptor (EGFR) or AKT decreases the Beclin 1-associated VPS34 kinase activity and downregulates autophagy (Wang, Wei et al. 2012, Wei, Zou et al. 2013). Additionally, the tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6)-mediated ubiquitination of Beclin-1 is critical for autophagy induction (Shi and Kehrl 2010). Thus, posttranslational modifications are important in regulating Beclin 1 activity, and thus autophagy.

Recent research in mammals found that Beclin 1 is regulated by cytoskeletal sequestration. It was shown that Bim inhibits autophagy by interacting with Beclin 1 through the cytoplasmic enzyme dynein light chain 1 (DYNLL1/LC8) and localizing Beclin 1 to the dynein motor complex. Starvation induces dissociation of Bim and Beclin
1, leading to the upregulation of autophagy (Luo, Garcia-Arencibia et al. 2012). Furthermore, Beclin 1-Vps34 is tethered to microtubules through dynein and AMBRA1. During autophagy induction, Ulk1 phosphorylates AMBRA1, releasing the Beclin 1-Vps34 complex from dynein, resulting in the subsequent relocalization of Beclin1 to the endoplasmic reticulum for autophagosome nucleation (Di Bartolomeo, Corazzari et al. 2010). In addition, AKT-mediated phosphorylation of Beclin 1 promotes the formation of the autophagy-inhibitory Beclin 1/14-3-3/vimentin intermediate filament complex (Wang, Wei et al. 2012). Thus, a regulatory relationship between cytoskeleton dynamics and autophagosome formation has been shown.

In addition to all known roles of autophagy, it was recently established that PI3K Class III complex (main initiation complex of autophagy) plays an important role in cytokinesis (Nezis, Sagona et al. 2010, Sagona, Nezis et al. 2010, Thoresen, Pedersen et al. 2010). Depletion of either Vps34 or Beclin 1 causes an increase in cellular arrest at cytokinesis, as well as an increase in binuclear and multinuclear cells (Sagona, Nezis et al. 2010). In addition, they showed that autophagy may be inhibited during mitosis. Interestingly, the formation of autophagic vacuoles was inhibited during the metaphase and anaphase stages of cultured animal cells, and was restored after formation of the nuclear envelope in telophase/G1 (Eskelinen, Prescott et al. 2002). A decrease of autophagy during mitosis was associated with the reduction of PI3P levels (Furuya, Kim et al. 2010).

The growing spectrum of Beclin1 regulators, and Beclin1 interactors, underlines the importance of Beclin1 as an autophagy regulator. However, given the multitude of protein-protein interactions and different functions of Beclin1, it is still unclear how
Beclin 1 is spatiotemporally regulated, and how its various modes of regulation are altered/controlled in various tissues, in respect to different physiological contexts.

1.1.5. Cell proliferation and cancer

In somatic cells, autophagy controls the lifespan of long-lived proteins and organelles. Autophagy targets and degrades misfolded proteins, or functionally impaired organelles, preventing the toxic effects caused by their accumulation. Autophagy will also supply the cell with building blocks for cellular remodeling. A deficiency in autophagy is associated with several pathological conditions that affect the aging population, such as the neurodegenerative Parkinson's, Alzheimer's, or Huntington's disease, cardiac disease, and cancer (Mizushima, Levine et al. 2008). While our knowledge of autophagy in somatic cell physiology is extensive, the role of autophagy in actively proliferating cells is much less understood.

1.1.5.1. Role of Beclin 1 and autophagy in cell proliferation and cancer

Recent studies established a requirement for Beclin 1 and autophagy in progenitor cell proliferation/maintenance in mammalian tissue culture cells. Beclin 1 and autophagy are required for cell proliferation of breast cancer stem-like/progenitor cells (Hoyer-Hansen, Bastholm et al. 2005, Gong, Bauvy et al. 2013). Vps34 was shown to be required for the peripheral maintenance and function of T cells (Parekh, Wu et al. 2013). Furthermore, a Beclin 1 protein, but not Atg5 or Atg7, deficiency leads to defects in thymocyte progenitor maintenance and results in severe depletion of T and B cell precursors (Arsov, Adebayo et al. 2011). It was also shown that Atg7 is essential for the
maintenance of hematopoietic stem cells, and Atg5 is essential for lymphocyte survival and proliferation (Pua, Dzhagalov et al. 2007, Hubbard, Valdor et al. 2010, Mortensen, Soilleux et al. 2011). These examples outline similarities and possible differences between Beclin 1 and other autophagy genes, and their affect on cell proliferation.

The connection between autophagy and cancer was first established when Beclin 1 was shown to act as a tumor suppression in mammalian cells (Liang, Jackson et al. 1999). Beclin 1 was shown to be a haploinsufficient tumor suppressor protein, where mice heterozygous for beclin 1 display a high incidence of tumors (Qu, Yu et al. 2003, Yue, Jin et al. 2003). Moreover, it was shown that Beclin 1 is monoallelically deleted and/or its expression is decreased in ovarian, breast and prostate cancers (Karantza-Wadsworth and White 2007, Gawriluk, Ko et al. 2014). It was also demonstrated that increased expression of beclin 1 inhibits tumorigenesis (Liang, Jackson et al. 1999). Beclin 1 can inhibit cell proliferation by increasing apoptotic and/or autophagic cell death (reviewed in (Maiuri, Zalckvar et al. 2007, Fu, Cheng et al. 2013)). Furthermore, mutations in Atg2, Atg5, and Atg9 (alone or in combination) were found in gastric and colorectal cancers (Kang, Kim et al. 2009). Multiple studies have implicated autophagy in the homeostatic control, and maintenance, of the self-renewal capacity of stem cell populations and in cancer stem cells (reviewed in (Guan, Simon et al. 2013, Hamaï, Codogno et al. 2013)).

There is evidence that autophagy plays a role in both tumor suppression and tumor promotion. This paradox is thought to depend on tumor type, context, and stage. Genetic evidence suggests that during the initial stages of tumor development, autophagy has a tumor-suppressing role. However, during the later stages of tumor
development and metastasis, cancer cells may upregulate basal levels of autophagy to survive in the hostile tumor microenvironment (Ding, Shi et al. 2008, Fujii, Mitsunaga et al. 2008, Chen and White 2011). Moreover, relapsed tumors that utilize autophagy to survive metabolic deprivation present an even bigger problem: they proliferate more aggressively and are often the main cause of lethality in patients (reviewed in (Mathew, Karantza-Wadsworth et al. 2007)). Therefore, during cancer formation and progression, autophagy acts as a “double-edged sword” and its modulation is essential for tumor survival (White and DiPaola 2009). All these examples, and many other studies, outline Beclin1 and other autophagy proteins, as necessary mediators of cell proliferation, however, the exact role of autophagy in progenitor and cancer cell proliferation still remains elusive. Thus, to understand the role of basal autophagy in cell proliferation is crucial, as inhibition of autophagy may develop into a new therapeutic strategy to accompany existing chemotherapy treatments for cancer, as well as an independent treatment for relapsed cancers.

1.2. C. elegans as a model organism

To study autophagy in vivo we use a free-living, transparent nematode (roundworm), Caenorhabditis elegans. The animals are about 1 mm in length and can be easily maintained in laboratory settings where they are grown in liquid culture or on agar plates using E. coli as the food source. Under optimal conditions, C. elegans reproduces, with a life cycle of about 3 days at 20°C, during which animals undergo four larval stages (L1 - L4). During unfavorable conditions, such as lack of food, population overcrowded, or high temperature, animals arrest as “dauer larvae,” a metabolic and
physiological state that allows them to survive until conditions improve (Golden and Riddle 1984). *C. elegans* makes a beautiful genetic model due to its large number of progeny per adult, relatively fast life cycle, and short lifespan.

Moreover, due to their transparency, *C. elegans* development can be examined with single-cell resolution, in live preparations, by differential interference contrast (DIC) microscopy. The adult hermaphrodites contain approximately 959 somatic cells, and we know the history of every cell in their mostly invariant cell lineage (Edgar and Wood 1977, Sulston, Schierenberg et al. 1983, Denich, Schierenberg et al. 1984, Altun and Hall 2008). Moreover, *C. elegans* was the first multicellular organism to have its whole genome sequenced, and as of 2012, the only organism to have its connectome (neuronal "wiring diagram") completed (White, Southgate et al. 1986). The short life cycle, stereotypical development, ease of propagation, small size and compact genome established *C. elegans* as a powerful model organism for in vivo biological research in various fields such as genomics, cell biology, neuroscience and aging (Girard, Fiedler et al. 2007).

### 1.2.1. Genetic model of the germline

In my research, I use the *C. elegans* germ line as an in vivo genetic model to study cell proliferation. The *C. elegans* germ line is a powerful system to investigate the crosstalk between genes and the animal’s environment, during cell proliferation and/or differentiation. Moreover, *C. elegans* provides a simple in vivo model to study self-renewable cells and their proliferation in the stem cell niche.
C. elegans animals have two sexes: hermaphrodite (XX) and a male (XO). Males can arise by spontaneous non-disjunction of the X chromosomes, at a very low frequency (0.1%); or through mating, at higher frequency (up to 50%) (Altun and Hall 2008). Hermaphroditic self-fertilization allows for the maintenance of genetically identical progeny. On the contrary, male mating allows for genetic variability and facilitates the isolation and maintenance of mutant strains. In both sexes, the germ line is the only lineage that continues to proliferate during adulthood (Hirsh, Oppenheim et al. 1976). Germ cells are spatiotemporally oriented in the germline syncytium and differentiate as they move proximally to develop into sperm and/or oocytes (FIG. 1.2) (Hubbard and Greenstein 2005, Kimble and Crittenden 2005).

**Figure 1.2. Basic anatomy of the C. elegans hermaphrodite gonad.**

(A) Schematic representation of the zones of the gonad in C. elegans. At the distal end (capped by the distal tip cell - DTC) is a mitotic cell population (green), followed by the transition zone and a meiotic cell population (red), where cells differentiate into oocytes (yellow) or sperm (blue)

(B) Structure of the distal area of the gonad. Red - area under strong influence of GLP-1, yellow - mitotic region of the gonad, green - transition zone
1.2.2. Development of the gonad

The gonad of *C. elegans*, at the L1 stage, comprises of four cells (Z1, Z2, Z3, Z4) that can be observed. The primordial germ cells (Z2 and Z3) are flanked by the somatic gonad precursors (Z1 and Z4) and surrounded by the basement membrane ((Sulston, Schierenberg et al. 1983), reviewed in (Hubbard and Greenstein 2005, Kimble and Crittenden 2005)). During the L1 larval stage, the somatic gonad and germline continue to develop. The somatic gonad precursors (Z1 and Z4) divide to give rise to 12 cells, two Distal Tip Cells (DTC) and 10 cells that will give rise to somatic sheath cells, spermatheca, and uterus (Kimble and Hirsh 1979). The hermaphrodite DTC has two functions: migratory, where it acts as a leader to establish the U-shaped structure of each gonad arm (only in hermaphrodites), and signaling, to promote germ cell proliferation (Kimble and White 1981, Hedgecock, Culotti et al. 1987). The sheath cells have several roles, for meiotic prophase progression, robust germ cell proliferation, oocyte maturation, and ovulation (McCarter, Bartlett et al. 1999, Killian and Hubbard 2005, Govindan, Nadarajan et al. 2009).

The primordial germ cells Z2 and Z3 divide to give rise to mitotic germ cells in the gonad. During the third larval stage (L3), polarity of the germ line is established by the most proximal germline nuclei entering meiosis and removing itself from the pool of actively dividing cells. The primordial cells are distinct, but, by the second larval stage, the proliferating germ line becomes a syncytium where cellular nuclei are joined by a common cytoplasm. Interestingly, germ cells in the syncytium do not divide synchronously (Maciejowski, Ugel et al. 2006), which may be due to partial enclosure of each nucleus by a plasma membrane (Hirsh, Oppenheim et al. 1976). The course of
proliferation is similar in two sexes, where hermaphrodites produce ~2000 germ cells in two gonadal arms, and males produce ~1000 germline cells in a single arm (Kimble and White 1981).

1.2.3. Anatomy of the gonad

The adult hermaphrodite germ line consists of a "mitotic region" at its distal end, a "transition zone", and a proximal "meiotic zone" of differentiated cells (FIG. 1.2). Germ cells in the mitotic region serve as a stem cell population that is able to self-renew or to produce gametes. In young adults (24 hours past L4), the mitotic region hosts approximately 225–250 germ cells that extend to ~20 germ cell diameters along the distal-proximal axis (Crittenden, Troemel et al. 1994, Eckmann, Crittenden et al. 2004, Hansen, Hubbard et al. 2004). Once the germline nuclei start to transition into the meiotic prophase stage, chromosomes begin to pair and nuclear chromatin assumes a crescent-shaped morphology (Francis, Maine et al. 1995, Dernburg, McDonald et al. 1998). The most proximal region of the gonad contains cells that have differentiated into sperm (stored in the spermatheca) or oocytes. Upon fertilization, eggs are stored in the common uterus and are laid through the vulva.

1.2.3.1. Stem Cell Proliferation/Proliferative fate

The C. elegans germ line contains a comparatively large population of stem cells that produce equivalent daughters that eventually differentiate. The GLP-1/Notch receptor pathway is required to keep germ cells in the mitotic undifferentiated state and
inhibits differentiation and meiotic entry (Austin and Kimble 1987, Berry, Westlund et al. 1997). GLP-1 is an N-glycosylated transmembrane protein that is a member of the LIN-12/Notch family of receptors (Austin and Kimble 1987, Yochem and Greenwald 1989, Berry, Westlund et al. 1997, Pepper, Lo et al. 2003). During the late L2 stage, the somatic gonad cells reorganize, forming a central primordium, flanked by two gonad “arms,” each of which is each capped by a single distal tip cell (DTC) (Kimble and Hirsh 1979). The somatic DTC expresses the transmembrane DSL (Delta-Serrate-LAG-2) ligand LAG-2, which activates GLP-1/Notch receptors in adjacent germ cells (Henderson, Gao et al. 1994, Nadarajan, Govindan et al. 2009). Both, the ligand and receptor proteins, are single pass transmembrane l proteins. Upon ligand-binding, the GLP-1/Notch receptor undergoes two proteolytic cleavages that result in the intracellular domain of the receptor translocating into the nucleus, where, in concert with other nuclear factors, it produces changes in gene expression (Greenwald 2005, Greenwald and Kovall 2013).

In the absence of GLP-1/Notch activity, only a few germ cells are produced, which enter meiosis prematurely and differentiate as sperm (Austin and Kimble 1987). Reduction of GLP-1/Notch signaling to the germ cells changes the relative balance between proliferation and differentiation, without altering the cell cycle rate of the proliferative cells (Michaelson, Korta et al. 2010). In contrast, a constitutively active (mostly signal-independent) GLP-1/Notch receptor results in the proliferative fate causing a tumorous (Tum) phenotype (Berry, Westlund et al. 1997, Pepper, Killian et al. 2003, Maciejowski, Ugel et al. 2006).
1.2.3.2. Cell-cycle kinetics

The distal proliferative zone of wild-type young adult animals consists of ~230 cells. Kinetic analysis of the proliferative zone suggests that ~130–160 of these cells are actively cycling, with the remaining ~70–90 cells undergoing meiotic S-phase (Fox, Vought et al. 2011). Generally, from the total population of ~230 mitotic cells, only ~20 cells enter meiosis per hour (Fox, Vought et al. 2011). Most mitotic cells function as nurse cells for the forming oocytes, prior to their undergoing physiological germ cell apoptosis during the late pachytene stage. Analysis of the gametogenesis output during oogenesis, under optimal growth conditions, leads to estimates that 90% or more of the pachytene germ cells undergo physiological apoptosis (McCarter, Bartlett et al. 1999, Jaramillo-Lambert, Ellefson et al. 2007, Fox, Vought et al. 2011).

Interestingly, two different groups used similar methods to analyze the total time for the proliferating cell to complete the cell cycle in C. elegans, and obtained different results, as 6.5–8 h vs. 16–24 h total cell cycle length (Crittenden, Leonhard et al. 2006, Fox, Vought et al. 2011). The reason for this difference in the estimates is unknown. Furthermore, cell-cycle kinetics analysis of C. elegans germ cells indicates that, in cycling cells, the G1-phase is either very short, or absent, and that G2 may be a major phase for cell cycle regulation (Fox, Vought et al. 2011). Consistent with this idea, it was shown that during starvation of L1 diapause or dauer worms, germ cells arrest during G2 (Fukuyama, Rougvie et al. 2006, Narbonne and Roy 2006).
1.2.3.3. Stem Cell Differentiation/Meiotic Fate

The GLP-1/Notch signaling pathway maintains the proliferative identity by inhibiting the two main regulatory pathways, GLD-1 and GLD-2, which promote meiotic entry and/or inhibit the proliferative fate (Francis, Barton et al. 1995, Kadyk and Kimble 1998). Thus, GLD-1 and GLD-2 activities are noted to be low or absent in the distal end of the gonad, where GLP-1/Notch signaling levels are high. As cells move proximally along the gonad syncytium, the influence of the GLP-1/Notch signaling from DTC decreases, resulting in increased activity of two redundant pathways, GLD-1 and GLD-2, which take precedence over GLP-1/Notch pathway and act in the transition into meiosis. Due to the redundancy of the GLD-1 and GLD-2 pathways, if activity of either one pathway is eliminated or reduced, its function is overcompensated by the activity of the other pathway. Thus, either pathway alone is sufficient to result in germ cells that enter meiosis. However, the simultaneous reduction or elimination of the GLD-1 and GLD-2 pathways activities, leads to an overproliferation that results in a Tumorous (Tum) germline phenotype (Kadyk and Kimble 1998, Eckmann, Crittenden et al. 2004, Hansen, Hubbard et al. 2004).

Within the GLD-1 pathway, there are two RNA-binding translational inhibitors: GLD-1 (homolog of mouse Quaking), which inhibits translation by binding to the 3’UTRs of mRNAs (Jones and Schedl 1995, Lee and Schedl 2001, Lee and Schedl 2010, Jungkamp, Stoeckius et al. 2011) and NOS-3 (a homolog of the Drosophila translational regulator Nanos), required in the hermaphrodite germline for the switch from sperm to oocyte production (Kraemer, Crittenden et al. 1999, Hansen, Wilson-Berry et al. 2004). The GLD-1 protein acts in the regulation of the transition into meiosis,
the GLD-1 protein levels are absent or low in the distal gonad and rise gradually toward transition zone, where they reach maximum levels and allow cells to enter meiotic prophase (Jones, Francis et al. 1996). Increasing GLD-1 levels in the distal gonad drives progenitor cells to premature differentiation; moreover, elimination of GLP-1/Notch signaling increases GLD-1 levels (Hansen, Wilson-Berry et al. 2004). This suggests that GLP-1/Notch suppresses GLD-1 accumulation, and keeps GLD-1 at low level in the distal end, which allows cells to remain proliferative and increase in GLD-1 levels in wild-type animals promotes cells from proliferation to differentiation.

Within the GLD-2 pathway, the GLD-2 and GLD-3 proteins promote meiotic fate (Eckmann, Crittenden et al. 2004). *gld-2* encodes the catalytic portion of a poly(A) polymerase and *gld-3* encodes a BicC homolog (Kadyk and Kimble 1998, Eckmann, Kraemer et al. 2002). The GLD-3 protein enhances GLD-2 activity by binding to it (Eckmann, Crittenden et al. 2004). GLD-2, the catalytic subunit of a poly(A) polymerase, targets certain mRNAs via GLD-3; thus, providing the translational regulation and/or mRNA stability control of the downstream genes in the pathway, identification of which is still in its early stages. Nevertheless, GLD-2/GLD-3 promotes the activity of *gld-1*, but probably has additional targets that have not been established. (Hansen, Hubbard et al. 2004, Suh, Jedamzik et al. 2006). Taken together, GLD-1 and GLD-2/GLD-3 likely target RNAs with opposite functions. GLD-1, being an RNA-binding translational inhibitor, likely represses the proliferative fate, while GLD-2/GLD-3 functions as a poly(A) polymerase that likely stabilizes mRNAs that promote the meiotic fate.
1.2.4. Physiological control of germline proliferation

Constant coordination between cell proliferation and differentiation is required during organismal development and throughout life. Although major regulators of cellular proliferation are understood, less is known about the control of cell proliferation by growth factors and environmental conditions that contribute to organismal development (Edgar and Lehner 1996, Fichelson, Audibert et al. 2005, Orford and Scadden 2008).

1.2.4.1. The Insulin receptor signaling in the germ cell proliferation

The insulin-like/IGF receptor (IIR) family is conserved across metazoans and controls multiple aspects of cell growth and metabolism, in addition to organismal homeostasis, growth, and survival (Taguchi and White 2008). The study of IIR signaling in invertebrate model systems, in the cellular and whole-organismal context, and particularly from the perspective of metabolism, developmental decisions and aging, has significantly improved our understanding of the role of this pathway within the context of the organism.

The IIR signaling cascade is highly conserved from C. elegans to mammals. The genome of C. elegans includes one insulin receptor (IIR) gene, daf-2 (Kimura, Tissenbaum et al. 1997) and 40 recognized insulin-like peptide genes (Pierce, Costa et al. 2001). DAF-2/IIR activates signals through a class I PI3-kinase (C. elegans AGE-1) (Morris, Tissenbaum et al. 1996) cascade that causes phosphorylation of a FOXO transcription factor (C. elegans DAF-16) and prevents its translocation to the nucleus,
which in turn, inhibits translation of DAF-16/FOXO transcriptional targets that modulate various processes, such as metabolism, stress response, immunity, lifespan and reproduction (reviewed in (Kenyon 2010, Landis and Murphy 2010, Hubbard 2011)). Mutants with reduced daf-2 activity enter dauer even under replete conditions, and have an extended lifespan, as well as extended reproductive timing (Vowels and Thomas 1992, Kenyon, Chang et al. 1993, Gottlieb and Ruvkun 1994, Larsen, Albert et al. 1995).

In the C. elegans germ line, signals from the DAF-2/Insulin (IIR) pathway promote robust proliferation to expand the larval germline progenitor pool during development (Michaelson, Korta et al. 2010). Hubbard’s lab found that, independently from GLP-1/Notch pathway, DAF-2/IIR signaling stimulates germline proliferation, through the activity of DAF-18/PTEN and DAF-16/FOXO. Furthermore, they established that the decrease in progenitor stem cell population in daf-2/IIR mutants is attained through the alteration of the larval germline cell cycle. A decrease in daf-2 activity results in a reduction in the proportion of germ cells in M- and in S-phase of the cell cycle. Moreover, based on measurements of DNA content in germ nuclei, they suggested that a reduction of DAF-2 activity delays the G2 phase of the cell cycle. Finally, they determined that the DAF-2/IIR pathway and its downstream components are required to promote robust larval proliferation in the germline. Interestingly, stimulation of this DAF-2/IIR signaling cascade occurs in a germline-autonomous manner, and requires the activity of somatic insulin-like ligands, INS-3 and INS-33. IIR signaling in germline proliferation acts through the canonical PI3K pathway, inhibiting DAF-16/FOXO. However, neurons and intestine were previously implicated in other
insulin signaling roles (Hung, Wang et al. 2014) and, interestingly, signaling from INS-3 and INS-33 ligands does not inhibit DAF-16 function in either neurons or intestine (Michaelson, Korta et al. 2010, Hubbard 2011).

During the life of the organism, the proliferation/differentiation balance of stem and progenitor cell populations must be receptive to developmental signals, but also be responsive to the physiological needs of the organism, in response to environmental cues. The *C. elegans* germ line serves as a tractable system to study the influence of the environment on progenitor cells. In the germ line, GLP-1/Notch signaling from the DTC niche maintains the progenitor cell pool (Hansen, Hubbard et al. 2004, Hubbard 2007, Kimble and Crittenden 2007) and the DAF-2/IIR signaling boosts larval germline cell cycle (Michaelson, Korta et al. 2010). It was also shown that DAF-7/TGFβ signaling tightly regulates germ cell proliferation during the lifespan of the animal, also as part of the response to environmental conditions.

1.2.4.2. DAF-7/TGFβ signaling in the germ cell proliferation

The DAF-7/TGFβ pathway was first revealed by its regulation of the dauer development. There are several core components of this pathway: the ligand DAF-7, which binds to the type I receptor DAF-1 and type II receptor DAF-4 heteromeric complex, and leads to the phosphorylation of the R-Smads DAF-8 and DAF-14 (Georgi, Albert et al. 1990, Ren, Lim et al. 1996, Inoue and Thomas 2000, Estevez, Estevez et al. 2004, Park, Estevez et al. 2010). Downstream of the pathway, the Sno/Ski homolog DAF-5 binds to the DAF-3/Co-Smad to induce reproductive development. In
general, the *C. elegans* DAF-7 pathway components are highly diverged from the TGF-β signaling components in other species, but the DAF-5/Sno/Ski and DAF-3/Co-Smad interaction is conserved in other systems (Patterson, Koweek et al. 1997, da Graca, Zimmerman et al. 2004, Inoue and Imamura 2008). DAF-7, DAF-1, DAF-4, DAF-8 and DAF-14, promote expansion of the proliferative zone, while DAF-3 and DAF-5 limit the DAF-7-mediated proliferation (Dalfo, Michaelson et al. 2012). In response to favorable environmental conditions, DAF-7 expressed from the environment-sensing ASI neurons, binds to the DAF-1/DAF-4 receptor in the DTC and interacts with the downstream components of the pathway to promote germline progenitor cell accumulation (Patterson, Koweek et al. 1997, Inoue and Thomas 2000, da Graca, Zimmerman et al. 2004, Park, Estevez et al. 2010, Dalfo, Michaelson et al. 2012). The DAF-7/TGFβ pathway influences the proliferation versus differentiation decision of larval germ cells in non-cell autonomous fashion, without influencing the cell cycle in a GLP-1/Notch-independent manner. DAF-7 regulates the proliferative zone expansion independently of the DAF-12/nuclear hormone receptor, DAF-18/PTEN, or DAF-16/FOXO. In contrast, DAF-12 is required for the DAF-7 mediated dauer development and DAF-18 with DAF-16 are necessary for the life-span regulation (Dalfo, Michaelson et al. 2012). Thus, DAF-7/TGFβ signaling operates as quality control of the environment to alter germline stem cell proliferation.
1.2.4.3. TOR signaling pathway in the germ cell proliferation

In mammals, cell growth and proliferation respond to nutrient and energy availability through the evolutionary conserved TOR pathway. The TORC1 complex responds to PI3K-mediated growth factor signaling via the conserved substrate S6K (p70 ribosomal S6 kinase) (reviewed in (Wullschleger, Loewith et al. 2006). Recent work in *C. elegans* indicates that TOR and S6K are important for larval germline progenitor cell accumulation, in response to food availability (Korta et al. 2012). In *C. elegans*, mutations in the components of the TORC1 complex *let-363/Tor* or *daf-15/Raptor* cause an L3-stage larval arrest phenotype that is similar to, but distinct from dauer (Long, Spycher et al. 2002, Jia, Chen et al. 2004). It was shown that the only S6K-encoding gene in *C. elegans*, *rsks-1/S6K*, influences germline progenitor cell proliferation cell-autonomously by both, promoting overall cell cycle progression, and inhibiting differentiation. Similar to DAF-2/IIR, reducing RSKS-1/S6K signaling promotes overall cell cycle progression, whereas DAF-2/IIR is required for appropriate G2 progression (Pinkston, Garigan et al. 2006, Korta, Tuck et al. 2012). Interestingly, RSKS-1/S6K is also required for the effects of a restrictive diet on the larval germline progenitor pool accumulation and thus was suggested that S6K (and likely TOR) act as a mediator of nutrition on the establishment of the germline progenitor pool (Korta, Tuck et al. 2012).
1.3. Introduction to this thesis and rationale

Autophagy is a catabolic recycling process, by which, cellular proteins and organelles are sequestered into autophagosomes, degraded in lysosomes, and recycled to sustain cellular homeostasis. Autophagy is viewed as an adaptive response and is upregulated to assure cellular survival during stressful conditions, whereas in other cases, it appears to promote cell death and lethality. Recent studies have implicated autophagy in the homeostatic control and maintenance of the self-renewal capacity of stem cell population and in cancer stem cells (reviewed in (Pan, Cai et al. 2013)). Multiple studies have implicated the dual role of autophagy in cancer. Autophagy can either suppress tumor proliferation, by preventing accumulation of damaged proteins and organelles along with inducing autophagic cell death, or promote survival of the tumors (reviewed in (White 2015)). During increased metabolic demands, rapidly proliferating tumor cells have been shown to activate autophagy in response to cellular stress. Acquired autophagy-related stress tolerance, enables cancer cell survival by restoring homeostatic energy production that can lead to tumor growth and therapeutic resistance (Jin and White 2007). Several preclinical models have shown that inhibition of autophagy restores sensitivity to drugs used in chemotherapy and enhances tumor cell death (reviewed in (Yang, Chee et al. 2011)). Thus, autophagy has been established as a potential novel therapeutic target, and multiple early phase clinical trials are in place to evaluate the effects of autophagy inhibition with combination of chemotherapy or other targeted agents (McCrea and De Camilli 2009, Yang, Chee et al. 2011, Kubisch, Turei et al. 2013, Feitelson, Arzumanyan et al. 2015). As the role of autophagy, and its regulation in proliferating cells, emerges and studies aim to define
optimal strategies to modulate autophagy for therapies, targeting autophagy in cancer will provide potential new opportunities for drug development. More potent and specific inhibitors of autophagy are needed. Our knowledge of the regulation of autophagy in mammalian cells has been derived mainly from analyses of transformed cell lines. These studies have been very useful in expanding our understanding of the mechanism of autophagy regulation. However, this type of analysis fails to recognize that the processes involved, for example in cellular metabolism after nutrient deprivation, may be distinct during development or in the context of different tissues, in a whole organism. In multicellular organisms, cell context, cell-to-cell contact, cellular communication, the physiology of the animal, and the developmental stage, may affect the regulation and requirements for autophagy. Multiple studies have shown that the role of autophagy in cell proliferation is dependent on the surrounding cellular environment, and moreover, it is cell-type, mutation and tumor-stage dependent. Thus, these observations highlight the importance of further in vivo studies where different signaling pathways are considered and how they regulate autophagy can be explored in the context of the whole organism.

In this work, we investigate the role of BEC-1 and other autophagy genes in the control of proliferation of germ cell progenitors in C. elegans. The C. elegans germline stem cells are located in the stem cell niche and their proliferation is modulated in response to various signals. BEC-1 is the C. elegans ortholog of mammalian beclin1 and yeast Atg6/Vps30 (Melendez and Neufeld 2008). C. elegans BEC-1 is crucial for viability, development, normal movement, dauer morphogenesis, dauer larval survival and longevity. Previously, our lab showed that bec-1 is required for the retrograde
transport of the MIG-14/Wntless protein cargo, from endosomes to Golgi (Ruck, Attonito et al. 2011). MIG-14/Wntless is a transmembrane protein that is required in Wnt-producing cells. Retromer-dependent recycling of MIG-14/Wntless between the Golgi and plasma membrane mediates Wnt secretion and regulates Wnt signaling (Yang, Lorenowicz et al. 2008). In *C. elegans*, the lack of BEC-1 activity leads to the mislocalization and degradation of the MIG-14 in lysosomes (Ruck, Attonito et al. 2011). This phenotype is accompanied by an alteration in the localization of retromer complex proteins such as a decrease in the intensity and number of RME-8::GFP labeled vesicles, and the concurrent increase in number and size of GFP::SNX-1 positive puncta, suggesting that BEC-1 functions in retrograde transport, possibly in concert with RME-8. In addition, RAB-7 positive maturing endosomes were increased in *bec-1* mutants, as if there is a defect in the maturation of endosomes. This has also been demonstrated in mammalian cells (McKnight, Zhong et al. 2014). These results support the idea that BEC-1 plays evolutionarily conserved roles in retrograde transport, and autophagy in *C. elegans*. Here, we hypothesize that basal levels of autophagy are necessary for germ cell proliferation and that autophagy is required for the proper dynamics of cell cycle progression in the germline. Chapter 2 discusses the results and conclusions on our investigation of the role of BEC-1-mediated autophagy in the control of stem/progenitor cell proliferation. Chapter 3 discusses more in depth the phenotypes associated with the loss of other autophagy genes in the *C. elegans* germ line. Loss of autophagy gene activity results in a cell cycle delay, and had an effect on the G2/M phase transition. Moreover, the modulation of cell cycle progression by BEC-1 and several other autophagy genes, appears to be required in somatic cells, and not in the
germ line, thus the focus of BEC-1-mediated autophagy is cell non-autonomous. Interestingly, we noticed that autophagy genes modulate cell proliferation through the DAF-2/IIIR signaling pathway in a DAF-18/PTEN and DAF-16/FOXO dependent manner (that is atg-16.2 and atg-18) or independent of DAF-16/FOXO (that is bec-1). Note that this last observation of bec-1 loss of function being independent of daf-16/FOXO will be further tested in the future. Furthermore, our findings show that ATG-7 possibly interacts with the TGF-β signaling pathway to promote germ cell proliferation. Although it is not novel that the autophagy process can be regulated at different levels, it is interesting to note that with the same conditions, cellular proliferation can be altered by distinct autophagy genes to different levels, and via diverse signaling pathways. Given the evolutionary conservation of the autophagy pathway, understanding how it modulates stem cell proliferation in the germ line may provide new and important insights for anti-tumorigenic treatments and may lead to novel therapeutic approaches.
Chapter 2

2. Role of the BEC-1/Beclin 1 in germcell proliferation

The decision of a stem cell to proliferate and/or differentiate is finely controlled. The *C. elegans* germline provides a tractable system to study the mechanisms that control this decision (Hansen, Wilson-Berry et al. 2004, Hubbard 2007, Korta and Hubbard 2010, Hubbard 2011, Hubbard, Korta et al. 2013). Autophagy is an evolutionarily conserved cellular recycling process crucial for cellular homeostasis (reviewed in (Mizushima 2007)), that acts with the Notch/GLP-1, DAF-2/Insulin-like growth factor (IIR), and TGF-β pathways, in several contexts (Melendez, Talloczy et al. 2003, Suzuki, Kiyono et al. 2010, Lapierre, Gelino et al. 2011, Wang, Wei et al. 2012, Barth and Kohler 2014, Ghavami, Cunnington et al. 2015). Autophagy may suppress or promote cellular growth in tumors, depending on the cell type and metabolic state of the cell (reviewed in (Mizushima and Levine 2010, Kenific and Debnath 2015, White 2015)), where autophagy is generally believed to mediate these functions cell-autonomously. Here we evaluate the role of BEC-1 in cell proliferation, using the germ line as an *in vivo* model. BEC-1 is the *C. elegans* ortholog of human BECN1/Beclin 1, an essential autophagy regulator and tumor suppressor protein (Qu, Yu et al. 2003, Yue, Jin et al. 2003). We show that BEC-1/BECN1 acts independently of the GLP-1/Notch or DAF-7/TGF-β pathways, but interacts with components of DAF-2/IIR signaling pathway to potentiate germline proliferation during development. Moreover, BEC-1/BECN1 requires DAF-18/PTEN but not DAF-16/FOXO for this function and can both promote and inhibit germ cell proliferation depending on the genetic context. Our findings indicate that BEC-
1/BECN1 functions non-cell autonomously to control germ line proliferation by facilitating cell cycle progression from G2 to M phase. Given the evolutionary conservation of autophagy genes from C. elegans to humans, understanding the molecular mechanisms by which autophagy genes modulate the proliferation and/or maintenance of the stem progenitor cell population in vivo may lead to novel autophagy based chemotherapeutic approaches in the future.

2.1. BEC-1 is required for the normal accumulation of germline progenitor cells during larval development

Stem cell proliferation is tightly controlled in response to changes in physiological demands (reviewed by (Drummond-Barbosa 2008)). The distal gonad in C. elegans contains a stem cell population that divides to give rise to oocytes or sperm cells (FIG. 2.1 A, B) (reviewed by (Hansen and Schedl 2006, Hubbard 2007, Kimble and Crittenden 2007)). BEC-1 is the ortholog of mammalian BECN1/Beclin 1 and yeast Atg6/Vps30 and is involved in mediating the nucleation step of the autophagosome, together with the Vps34 Class III PI3 Kinase (reviewed in (Melendez and Neufeld 2008)). BEC-1/BECN1 is also involved in endocytosis, in the maturation step of endosomes and in the retromer transport from the endosome to the Golgi network (Ruck, Attonito et al. 2011, McKnight, Zhong et al. 2014). In C. elegans, loss-of-function mutations in bec-1/BECN1 result in a sterile phenotype (Ruck, Attonito et al. 2011), suggesting a role for BEC-1/BECN1 in gonadogenesis or in germline development. To distinguish between these possibilities, we analyzed germ lines of animals with depleted
Figure 2.1. BEC-1 controls germ cell population in the distal gonad.

(A) Schematic representation of the distal part of the gonad. (B) Representative image of extracted DAPI stained gonad of wild type animal depicting the organization of nuclei in the distal gonad. (C) Representative images of extracted and DAPI stained gonads of wild-type animals and animals lacking BEC-1 function by mutation (bec-1(ok691), bec-1(ok700) single mutants) or RNAi depletion. (D) Quantification of nuclei in the mitotic region of wild-type animals, bec-1 mutants and bec-1 RNAi depleted animals. bec-1(ok691) or bec-1(ok691) homozygous animals segregated from bec-1/nT1 heterozygous parents. Empty vector (L4440) was used as a control.

(E) Time course analysis of germline proliferation during development. The number of mitotic nuclei in wild type, bec-1(ok691), or daf-2(e1370) single mutants are shown at the indicated stages, L3-L4, L4, and young adult stage. For (B)-(E), animals were DAPI stained for analysis as young adults. For (C) and (D), animals grown at 15°C, shifted to 20°C as L4 larvae, and analyzed as young adults. For (E), animals grown at 15°C and switched to 20°C at L3 larval stage and analyzed at the specified stage. Results reflect the average of three biological replicates shown as the mean ± SEM (shown as error bars). Significance determined by two-tailed Student’s T-test ** P≤0.01 *** P≤0.001; number of analyzed gonads N≥20.
bec-1/BECN1 function by RNAi or by chromosomal deletion, in deletion mutants such as bec-1(ok691) or bec-1(ok700). The ok691 allele is a predicted molecular null, as it deletes the ATG start codon together with the first six out of seven exons (Takacs-Vellai, Vellai et al. 2005). The ok700 allele is a strong hypomorph (possibly null, with a deletion of the "evolutionarily conserved domain" (ECD), which causes frameshift in the open reading frame of the remaining portion of the transcript (Ruck, Attonito et al. 2011). Both ok691 and ok700 alleles are embryonic lethal, thus we maintain them as heterozygote with nT1 balancer and segregate homozygote progeny for each experiment. We found that compromising BEC-1/Beclin 1 function resulted in a significant decrease (up to 50%) in the number of stem cells in the distal proliferative zone, when compared to wild-type animals of the same stage (FIG. 2.1 C, D and FIG. 2.2). Moreover, the proliferating zone was shortened, from an average length of 20 cell diameters in wild-type animals, to an average of 15 cell diameters in bec-1/BECN1 mutant animals (FIG. 2.1C, D and FIG. 2.2). bec-1 homozygous animals segregate from a heterozygous parent, and are maternally rescued for the lethal phenotype (Ruck, Attonito et al. 2011), however, since the germline phenotype of homozygous bec-1 and bec-1 RNAi depleted animals are very similar, and RNAi targets both the maternal and zygotic mRNA, we conclude that there is no maternal rescue of the bec-1 germline phenotype.

Under normal growth conditions, wild-type germ line stem cell progenitors rapidly accumulate during the third and fourth larval stage to establish an adult pool of germ cells (Killian and Hubbard 2005). A developmental time course analysis of bec-1 mutant animals determined that bec-1 is essential for the late larval accumulation of germline
Figure 2.2. BEC-1 mutants exhibit temperature sensitive phenotype during germline development.

(A) Representative DIC images of wild-type and bec-1(ok691) homozygous animals raised at 15ºC, 20ºC, 25ºC temperatures. Magnification 400X. (B) Quantitative analysis of egg development and egg lay for bec-1 homozygote animals, ok691 and ok700, raised at different temperatures. Number of analyzed animals per each strain N≥95. (C) Representative gonad confocal images of animals treated with RNAi against control and bec-1 gene. Animals express GFP::PHPLC1δ1 to visualize the plasma membrane. At top focal plane, the membrane in the germline displays hexagonal shapes of equal size. At midfocal plane, wild type germline membranes are observed to have a T-shape. In bec-1 RNAi mutants, the germline defects on the membrane are observed with severe aneuploidy, and the germline membranes T-shape at the midfocal plane is not maintained. Magnification 630X. (A,C) bec-1 homozygous animals segregated from bec-1/nT1 parents. Developed eggs do not hatch to give a viable progeny at any temperature.
stem cell progenitors (FIG. 2.1 E). These results suggest that BEC-1 is necessary for proliferation of stem/progenitor cells during larval development, specifically during the germ cell expansion that occurs during the fourth larval stage and thereafter.

Besides its role in the nucleation of autophagosomes (Melendez, Talloczy et al. 2003), BEC-1 has been shown to function in a complex with the Class III phosphatidylinositol-3-kinase VPS-34 in retrograde transport (Ruck, Attonito et al. 2011). To determine whether the autophagy activity of BEC-1 was required for germline proliferation in bec-1 mutants, we inhibited other genes required at different steps of the autophagy process, as well as genes involved in retromer function. RNAi depletion of vps-34, epg-8, atg-7, atg-12, atg-13, atg-18, and atg-9, or a genomic mutation in atg-16.2 or atg-18, resulted in a reduction in germline proliferation similar to that of bec-1 mutants (FIG. 2.3 A, B). In contrast, RNAi against retromer genes snx-1 or rme-8 had no effect on germline stem cell proliferation (FIG. 2.3 A). Interestingly, cup-5, required for lysosomal degradation (Fares and Greenwald 2001, Sun, Wang et al. 2011), is also required for germline proliferation (FIG. 2.3 A). We conclude that BEC-1/BECN1-mediated autophagy and lysosomal degradation are required for the proliferation of stem cells during germline development, while the retromer may not serve a major function.

To obtain further insight into the germline defects in bec-1/BECN1 loss of function animals, we examined the plasma membrane of germ line by using a GFP::PLC1 (phospholipase C delta) reporter (FIG. 2.2 C). PLC1 (derived from rat PLC1) comprises a PH domain that specifically binds to the plasma membrane associated phosphatidylinositol 4,5-biphosphate (Audhya, Hyndman et al. 2005). In
**Figure 2.3.** BEC-1-mediated autophagy controls stem cell proliferation.

(A) Quantification of nuclei in the mitotic region of wild-type animals after RNAi depletion against the autophagy genes: *epg-6, atg-7, atg-12, atg-13, atg-18,* and *atg-9.* The retromer genes, *snx-1* and *rne-8,* were also depleted to be compared to *bec-1* and *vps-34* RNAi depleted animals. *gfp(RNAi)* was used as a control. (B) Quantification of nuclei in the mitotic region of autophagy mutants *atg-16.2(ok3224), atg-18(gk378),* and *bec-1(ok691)* single mutants. For (A) and (B), animals were DAPI stained for analysis as young adults. Results reflect the average of at least three biological replicates shown as the mean ± SEM (shown as error bars). Significance determined by two-tailed Student’s T-test **P≤0.01*** **P≤0.001; number of analyzed gonads N≥20 and magnification 630X.
wild-type hermaphrodites, the membranes of the germline syncytium can be viewed as uniformly arranged hexagons at the top focal plane (FIG. 2.2 C). At the midfocal plane, the membranes, in the distal region of the gonad, line up in at the periphery and surround the shared cytoplasm (rachis) in the shape of a T-letter. However, in bec-1 mutants, the membranes are randomly shaped, and discontinuous, with multiple nuclei surrounded by hexagon shapes. At the midfocal plane, the membranes of bec-1/BEC-1 mutants are disorganized, and the T-shapes are no longer visible (FIG. 2.2 C). Our results suggest that BEC-1 is necessary for the membrane formation and/or stability during germline development. A function for autophagy in membrane trafficking has been shown which could underlie the observed phenotypes (reviewed in (Stolz, Ernst et al. 2014)). Alternatively, but not mutually exclusive BEC-1/Bclin1 may have a role on the polarity or orientation of cell division. However, whether BEC-1 is required in a cell autonomous or cell non-autonomous fashion to maintain the germline membrane structure has to be further investigated.

2.2. BEC-1 promotes proliferation in adult germcell tumors independently of GLP-1/Notch

Several pathways have been shown to be important for germ cell proliferation. GLP-1/Notch signaling from the DTC maintains the proliferative fate of the germ line (Austin and Kimble 1987, Crittenden, Troemel et al. 1994). A weak gain of function mutation in glp-1(ar202), results in germ cells that fail to differentiate (Pepper, Killian et al. 2003, McGovern, Voutev et al. 2009), and display an overproliferation of germline
stem/progenitor cells, referred to as a Tumorous (Tum) phenotype (Pepper, Killian et al. 2003). Loss of BEC-1 function by RNAi in \textit{glp-1(ar202gf)} animals, or in the \textit{glp-1(ar202gf); bec-1(ok691)} double mutants, leads to a significant decrease in the number of stem/progenitor cells (\textbf{FIG. 2.4 A, B}). Thus, \textit{bec-1} gene function is required for the excessive proliferation of progenitor/stem cells in \textit{glp-1(ar202gf)} mutants. To further investigate the role of BEC-1 in GLP-1 signaling, we tested the effect of the loss of BEC-1 on the \textit{glp-1} loss of function allele, \textit{e2141}. \textit{glp-1(e2141lf)} mutants have a reduction in germline progenitors, and a severe Glp (Germ line abnormal proliferation) phenotype, where all germ cells differentiate prematurely, at the restrictive temperature (Priess, Schnabel et al. 1987). Loss of BEC-1 function in the \textit{glp-1(e2141); bec-1(ok691)} double mutants, further decreased germ cell proliferation (\textbf{FIG. 2.4 C}). Therefore, reducing \textit{bec-1} activity enhanced the phenotype associated with a reduction in \textit{glp-1} function, and suppressed the phenotype associated with elevated \textit{glp-1} activity. A hypothesis consistent with these findings is that BEC-1 acts to modulate the production or localization of a DTC-expressed Notch ligand or ligands. To examine this possibility, we tested the proliferation phenotype of loss of BEC-1 in a strain lacking GLP-1 activity. To circumvent the requirement for GLP-1 in differentiation, we depleted BEC-1 in \textit{gld-2(q497) gld-1(q1485); glp-1(q175)} mutants, where proliferative germ cells are observed, despite the complete absence of GLP-1 activity (Hansen, Hubbard et al. 2004). We found that RNAi against \textit{bec-1} still reduced the number of proliferative germ cells in the \textit{gld-2(q497) gld-1(q1485); glp-1(q175)} triple mutants to a similar extent as in wild-type animals (\textbf{Fig. 2.4 D}). These results suggest that BEC-1 functions independently of GLP-1/Notch activity to promote germ cell proliferation.
Figure 2.4. BEC-1 acts in parallel to GLP-1/Notch signaling.

(A) Representative confocal images of DAPI stained gonads of wild type, glp-1(ar202), glp-1(ar202), bec-1(ok691) and bec-1(ok691) mutant animals. * labels the distal tip of the gonad. White line shows the end of the mitotic zone. Magnification 630X. 

(B) Number of nuclei in the mitotic zone in wild type, glp-1(ar202), glp-1(ar202), bec-1(ok691) and bec-1(ok691) mutant animals, and in the glp-1(ar202) animals fed with control (L4440) and bec-1 RNAi.

(C) Number of nuclei in the mitotic zone of the glp-1(e2141lf), glp-1(e2141lf);bec-1(ok691) and bec-1(ok691) mutant animals. Animals were raised at 15°C and switched to 25°C as L4 larvae. Young adults were DAPI stained and analyzed. Results reflect the average of at least three biological replicates shown as the mean ± SEM (shown as error bars). Number of analyzed gonads N=30. Significance determined by two-tailed Student’s T-test * P ≤ 0.05 , *** P ≤ 0.001.

(D) Number of nuclei in the distal mitotic zone in gld-2(q497) gld-1(q485)/hT2 [dpy-18(h662)] I; unc-32(e189) glp-1(q175)/hT2 [bli-4(e937)] III after RNAi against control empty vector (L4440) or bec-1.
2.3. BEC-1 affects germline proliferation independently of DAF-7/TGFβ signaling

Another pathway shown to regulate germline proliferation is the DAF-7/TGFβ signaling pathway, which acts through the daf-3/CoSMAD and daf-5 genes (Dalfo, Michaelson et al. 2012). TGFβ signaling regulates the balance of proliferation versus differentiation in response to sensory cues that report on food availability and population density independently of its role in dauer development (Narbonne and Roy 2006, Dalfo, Michaelson et al. 2012). Similar to GLP-1, TGFβ promotes proliferation without affecting the cell cycle, but affecting the later decision to proliferate or differentiate (Dalfo, Michaelson et al. 2012). To investigate whether BEC-1 could affect TGFβ signaling, we investigated whether bec-1/BECN1 required daf-3/CoSMAD or daf-5 in the regulation of germ cell proliferation. We found neither enhancement, nor suppression, of the germline proliferation defect of bec-1 mutants after depletion of daf-3 or daf-5 in (FIG. 2.5). In addition, the reduction in adult germline proliferation observed as a consequence of dietary restriction, in eat-2 mutants, was exacerbated by bec-1 RNAi (FIG. 2.6). We conclude that, BEC-1 acts in an independent pathway from DAF-7/TGFβ to promote germline proliferation.
Figure 2.5. **BEC-1 acts independently of the TGFβ/DAF-7 signaling pathway.**
Quantification of the number of mitotic cells in wild type, daf-7(e1372), daf-3(ck3610)) and daf-5(e1386) animals, treated with RNAi against control empty vector (L4440) and bec-1. Animals were grown at 15°C, shifted to 20°C, as L3 larvae, and analyzed as young adults. Results reflect the average shown as mean ± SEM (shown as error bars). Number of analyzed gonads N≥12. Significance determined by two-tailed Student’s T-test * P≤0.05, ** P≤0.01, *** P≤0.001.
Figure 2.6. The effect of dietary restriction germline proliferation is independent of BEC-1.
Quantification of the mitotic nuclei in the *eat-2(ad1116)* dietary restricted animals treated with, control (L4440), *bec-1* and *lag-2* (as a positive control) RNAi. Results reflect the average and are shown as the mean ± SEM (shown as error bars). Significance determined by two-tailed Student's T-test **P≤0.01, ***P≤0.001. Number of analyzed gonads N≥20.
2.4. BEC-1 acts in parallel or downstream of DAF-2/IIR signaling to control germ cell proliferation

A third pathway known to regulate germ cell proliferation is the DAF-2/IIR pathway (Michaelson, Korta et al. 2010) (FIG. 2.7 A). Thus, we investigated possible interactions between the DAF-2/IIR pathway and BEC-1, and whether BEC-1 affected larval germline proliferation independently of DAF-2. We found the number of germline progenitor cells in \textit{bec-1(ok691);daf-2(e1370)} double mutants indistinguishable from the \textit{bec-1} single mutant (P>0.05) but different from the \textit{daf-2} single mutant (P≤0.05) (FIG. 2.7 B). Thus, we conclude that \textit{bec-1} is epistatic to \textit{daf-2/IIR}. DAF-2/IIR signaling promotes germ cell proliferation through the canonical PI3K pathway, which requires DAF-18/PTEN to inhibit DAF-16/FOXO function (Michaelson, Korta et al. 2010). Interestingly, RNAi depletion of \textit{daf-18/PTEN} but not \textit{daf-16/FOXO} in \textit{bec-1(ok691 or ok700)} mutant animals, suppressed the germline proliferation defect (FIG. 2.7 C, D). Thus, BEC-1/Beclin 1 functions, at least partially, in a non-canonical pathway, which involves DAF-18/PTEN, but not DAF-16/FOXO, to control stem cell proliferation. Similar observations have been made for the quiescence of germline development during L1 diapause, which is also regulated by DAF-2/IIR in a DAF-18/PTEN-dependent but DAF-16/FOXO independent manner (Fukuyama, Rougvie et al. 2006). The L1 diapause arrest is a response to nutrient deprivation, dependent on the activity of AGE-1, the class I PI3K, and AKT-1/PKB kinases, where germline precursors undergo G2 arrest and chromosome condensation.

Signaling downstream of the DAF-2/IIR receptor is mediated by a cascade of kinases, including PDK-1, AKT-1, and AKT-2. We thus tested for possible interactions
Figure 2.7. BEC-1 role in the germ cell proliferation is DAF-18 dependent and DAF-16 independent.

(A) Schematic representation of the insulin pathway

(B) Quantification of the number of nuclei in the mitotic zone of wild-type, bec-1(ok691), daf-2(e1370) single mutant and daf-2(e1370);bec-1(ok691) double mutant animals.

(C) Quantification of the number of nuclei in the mitotic zone of wild-type, bec-1(ok691) or daf-2(e1370) single mutant animals treated with RNAi against control (L4440), or daf-18.

(D) Quantification of the number of nuclei in the mitotic zone of wild-type, bec-1(ok691) or daf-2(e1370) single mutant animals treated with RNAi against control (L4440), or daf-16.

(E) Quantification of the number of nuclei in the mitotic zone of wild-type, pdk-1(mg142gf), pdk-1(sa709lf), akt-1(ok525lf), or akt-2(ok393lf) mutant animals treated with RNAi against bec-1 or daf-2. For (B)-(E), animals were raised at 15°C, shifted to 20°C as L3 larvae, and analyzed as young adults. Results reflect the average of at least 2 trials and shown as mean ± SEM (shown as error bars). Number of analyzed gonads N≥21. Significance determined by two-tailed Student’s T-test * P≤0.05, ** P≤0.01, *** P≤0.001.
between these kinases and BEC-1, and inactivated bec-1 by RNAi in the background of the loss of function mutations pdk-1(sa709lf), akt-1(ok525lf), akt-2(ok393lf) or a gain of function mutation in pdk-1(mg142). Interestingly, only pdk-1(sa709lf) loss of function mutants displayed a reduction in germline proliferation, similar to that caused by the loss of bec-1 activity (RNAi depleted or chromosomal mutants), or that of daf-2/IIIR (RNAi or chromosomal mutations). Loss of function mutations in akt-1 or akt-2 had no effect in germline proliferation, probably due to redundancy between the akt-1 and akt-2. We found that the loss of function in akt-2, but not akt-1 suppressed the germline proliferation defect of bec-1 RNAi depleted animals (FIG. 2.7 E). Surprisingly, we found that the decrease in proliferation observed in pdk-1 loss of function mutants requires bec-1, as the number of germ cells was restored in pdk-1 mutants that were depleted of bec-1 (FIG. 2.7 D). As a control experiment, we also tested daf-2/IIIR RNAi depletion together with loss of function mutations in akt-1(ok525lf), akt-2(ok393lf) or pdk-1(sa709lf), . We also tested the pdk-1 gain of function mutation, mg142. We note that the daf-2 RNAi proliferation phenotype was not restored when combined with pdk-1(sa709lf), akt-1(ok525lf), akt-2(ok393lf) or the gain of function mutation pdk-1(mg142) (FIG. 2.7 E). In contrast to the suppression of pdk-1(sa709lf) mutants when depleted of BEC-1, the phenotype of pdk-1(sa709lf) animals depleted of daf-2 was enhanced, when compared to either pdk-1(sa709lf) single mutants or the daf-2 depleted animals. Thus, our results indicate that BEC-1/BECN1 acts together with DAF-2/IIIR and shares some of the components of the DAF-2/IIIR signaling to control germline proliferation. However, there are some differences between the BEC-1 and DAF-2 pathways, such as the lack of suppression by daf-16 RNAi or the interaction with pdk-1 loss of function. Our data
also suggests that the loss of BEC-1/BECN1 may have different effects on proliferation depending on the mutation in the background. For example, in wild-type animals, wild-type BEC-1 activity is required for proliferation; in contrast, in \textit{pdk-1(lf)} loss of function mutants, BEC-1 is required to inhibit proliferation. This has been previously observed in glandular structures where autophagy simultaneously mediated tumor suppressive and tumor promoting functions (Chen, Pang et al. 2013).

We found that the phenotype for the loss of bec-1 was not suppressed by daf-16 RNAi. To explore the possibility that other transcription factors may act together with BEC-1 to promote cell proliferation, we assayed for suppression of the \textit{bec-1} loss of function phenotype. Another transcription factor that acts downstream of DAF-2/IIIR is SKN-1/Nrf, which is directly phosphorylated by Akt/PKB and related kinases to promote cytoplasmic sequestration (Tullet, Hertweck et al. 2008). To test if BEC-1 interacts with SKN-1 to promote cell proliferation, we depleted \textit{skn-1} function by RNAi in wild-type and \textit{bec-1} mutant animals. Interestingly, the lack of \textit{skn-1} gene function significantly suppressed the lack of proliferation phenotype in \textit{bec-1} mutants, suggesting that BEC-1 promotes cell proliferation in the germline via SKN-1 transcription factor. In contrast, depletion of another transcription factor, \textit{efl-1/ E2F4}, which regulates G1/S cell cycle transition (DeGregori, Kowalik et al. 1995, Ceol and Horvitz 2001), did not suppress the proliferation defect of \textit{bec-1} mutants, suggesting that BEC-1 promotes proliferation independently from EFL-1/E2F4 (\textbf{FIG. 2.8}). A fascinating new mechanism of germline cells-to-soma communication has been proposed, which involves SKN-1/Nrf protein activation by fatty acid signals in germline-less animals (Steinbaugh, Narasimhan et al. 2015), which opens an interesting possibility for a role of autophagy in the direct or indirect regulation of the SKN-1 transcription factor activity.
Figure 2.8. BEC-1 possibly controls germline proliferation through SKN-1. Quantification of the number of mitotic cells in wild type and *bec-1(ok691)* with depleted *skn-1, daf-16, efl-1* gene function by RNAi, *gfp(RNAi)* was used as control. Animals were grown at 15°C and shifted to 20°C, as L3 larvae, and analyzed as young adults. Results are shown as mean ± SEM (shown as error bars). Significance determined by two-tailed Students T-test: *P*≤0.05, ***P*≤0.001. Number of analyzed gonads N≥15.
2.5. BEC-1 affects germline proliferation cell non-autonomously through the somatic tissues

Germline proliferation can be regulated cell non-autonomously, where signals originate from the surrounding somatic tissue, or cell autonomously where signals are required within the germ line. To interrogate the focus of action of BEC-1/Beclin 1, we tested the efficacy of \textit{bec-1/BECN1} RNAi in the \textit{rrf-1} mutant background that is mostly resistant to RNAi treatment in somatic tissues (Sijen, Fleenor et al. 2001), and in the \textit{ppw-1} mutant background that reduces RNAi effectiveness in the germ line (Tijsterman, Okihara et al. 2002). We found that RNAi depletion of \textit{bec-1/BECN1} in \textit{rrf-1} mutants had no effect on germline proliferation, whereas depletion of \textit{bec-1/BECN1} in \textit{ppw-1} mutants resulted in the reduced germ cell progenitor pool (FIG. 2.9 A-C). These results suggest that BEC-1 functions cell non-autonomously to control germcell proliferation. To further investigate this notion, we conducted cell specific rescue experiments. We found that BEC-1 expression from a hypodermal (\textit{dpy-7}), muscle (\textit{myo-3}), or its own promoter rescued the germline proliferation defects in \textit{bec-1(ok691)} mutant animals. Similarly, expression from a panneuronal promoter also provided some BEC-1 function, although not to the same extent (FIG. 2.9 D). In contrast, expression of \textit{bec-1/BECN1} from the \textit{glo-1} intestinal promoter failed to rescue the defects (FIG. 2.9 D). As multi-copy extrachromosomal arrays are often silenced in the germ line (Kelly, Xu et al. 1997, Dernburg, Zalevsky et al. 2000), rescue by the array that contained \textit{bec-1} under control
Figure 2.9. BEC-1 controls germline proliferation cell non-autonomously.

(A) Quantification of the number of mitotic nuclei in wild-type and *rrf-1(ok589)* mutant animals RNAi depleted against *bec-1* or *lag-2*. (B) Quantification of the mitotic index in wild-type, *bec-1(ok691)* mutant animals, and wild-type or *rrf-1(ok589)* animals treated with RNAi against control (*L4440*) or *bec-1*. (C) Quantification of the number of mitotic nuclei in wild-type and *ppw-1(pk1425)* mutant animals treated with RNAi against *bec-1* or *lag-2*. (D) Tissue specific expression of a BEC-1 cDNA using a neuronal (*rgef-1*), muscle (*myo-3*), hypodermal (*dpy-7*), and intestinal (*glo-1*) promoters. Only one line for each promoter was tested. (E) Tissue specific expression of a BEC-1 from the sheath cells using a *ced-1* promoter. For (A)-(D) animals were grown at 20°C and analyzed as young adults. Results reflect the average of at least three biological replicates shown as the mean ± SEM (shown as error bars). Significance determined by two-tailed Student’s T-test ** P≤0.01, *** P≤0.001; number of analyzed gonads N≥20.
of its own promoter also supported the notion that BEC-1 is required in somatic tissues, rather than the germline, to regulate germ cell proliferation (FIG. 2.10 A, B). Furthermore, we constructed strains \texttt{bec-1(ok691)/nT1;ls[pie-1p::bec-1::mCherry::tbb-2]}, that express a single copy insertion of the \texttt{bec-1} gene expressed from the \texttt{pie-1} promoter, resulting in germline expression. Analysis of three different lines of \texttt{bec-1(ok691)} mutant animals that specifically express BEC-1 in the germ line did not show rescue the proliferation phenotype of \texttt{bec-1(ok691)} mutants (FIG. 2.10 C), further confirming that BEC-1 acts outside of the germ line to promote germ cell proliferation. Consistent with a focus for \texttt{bec-1} in somatic cells, we found that rescued gonads of mosaic animals often retained the rescuing array in the sheath cells of the somatic gonad (data not shown) and we see expression of the BEC-1::RFP in the distal tip cell (FIG. 2.10 D). Thus, we conclude that BEC-1/BECN1 functions non-autonomously to regulate germline proliferation.

### 2.6. BEC-1 regulates cell cycle progression

Our results showed that BEC-1 is necessary for the rapid stem cell proliferation in the developing germ line that occurs during the L4 larval stage and thereafter. We considered three possible mechanisms for how BEC-1/BECN1-mediated autophagy may be required for stem cell proliferation: (1) by ensuring cell survival, (2) by inhibiting the switch between proliferation and differentiation, and/or (3) by controlling cell cycle progression of stem cell progenitors. First, we inquired whether animals lacking \texttt{bec-1/BECN1} function exhibit an increase in cell death/apoptosis of stem cell progenitors.
Figure 2.10. BEC-1 acts cell non-autonomously.

(A) Quantification of the number of mitotic cells in wild-type, bec-1(ok691) homozygous, bec-1/nT1 heterozygous animals that carry (+) or do not carry (-) the extrachromosomal array expressing BEC-1 from its endogenous promoter. The bec-1(ok691) homozygous animals segregated from the bec-1/nT1 or bec-1/nT1; Ex[bec-1(+),sur-5::gfp] parents. (B) Quantification of the number of mitotic nuclei in the wild-type or bec-1(ok691) homozygous animals that carry an extrachromosomal array that expresses wild-type BEC-1 from its endogenous promoter, fused with RFP. (C) Quantification of the mitotic region in the bec-1 mutants with germline specific bec-1 expression under pie-1 promoter (bec-1(ok691);Is[pie-1p::bec-1::mCherry::tbb-2]) three lines were analyzed. (D) Representative confocal images of BEC-1::RFP expression in the distal gonad in bec-1(ok691); Ex[bec-1::RFP, rol-6(su1006)] and DAPI staining of nuclei. Arrow head points BEC-1::RFP expressed in the DTC. In (A)-(C), animals grown at 15°C, as L3 larvae shifted to 20°C, and analyzed as young adults. Results reflect the average of at least three biological replicates shown as the mean ± SEM (shown as error bars). Significance determined by two-tailed Student’s T-test * P≤0.05, ** P≤0.01, *** P≤0.001. Number of analyzed gonads N≥20.
We found no increase in the number of apoptotic corpses at the L4 larval stage (FIG. 2.11A), when the decrease in stem cell proliferation in *bec-1/BECN1* mutants was already visible in young adults. Moreover, no dead cells or debris were noticed in the distal region of the gonad, indicating no increase in cell death (data not shown). Thus, we conclude that the reduction of progenitor/stem cells number in young *bec-1* mutants is not due to a decrease in cell survival, and/or increased cell death.

Second, precocious entry into meiosis could decrease the stem/progenitor cell pool, if *BEC-1/BECN1*-mediated autopagy was required for the decision to transition between the proliferation and differentiation of germ cells. The GLD-1 and GDL-3 RNA binding proteins are important for the transition of germ cells from proliferation to differentiation (Francis, Maine et al. 1995, Eckmann, Crittenden et al. 2004, Hansen, Hubbard et al. 2004). Lack of GLD-1 in *gld-1(q485)* mutants decreases the length of the mitotic zone due to precocious differentiation, whereas lack of GLD-3 in *gld-3(q741)* mutants extends the mitotic zone, due to a delay in the decision to differentiate (Eckmann, Crittenden et al. 2004, Hansen, Wilson-Berry et al. 2004). To investigate a possible role for *BEC-1* in the transition from proliferation into differentiation, we depleted *bec-1/BECN1* function by RNAi in *gld-1(q485)* or *gld-3(q741)* single mutants, and analyzed the length of the mitotic region. We found that RNAi-mediated depletion of *bec-1/BECN1* in *gld-1(q485)* or *gld-3(q741)* single mutants resulted in no change in the length of the proliferative zone (FIG. 2.11 B). This suggests that *BEC-1/BECN1* is not involved in the decision to transition from mitosis to meiosis (i.e. proliferation to differentiation).
Figure 2.11. BEC-1 is not required for cell survival and/or the transition from mitosis to meiosis

(A) Quantification of apoptotic cell corpses at the gonad bend. Animals that carry ced-1::gfp transgene labeling apoptotic nuclei were treated with RNAi against the control (L4440) or bec-1 gene. Animals raised at 20ºC and analyzed as young adults. Results reflect the average and are shown as the mean ± SEM (shown as error bars). Significance determined by two-tailed Student’s T-test, ***P≤0.001. Number of analyzed gonads N≥20. (B) Quantification of the length of the mitotic region in cell diameters of the gld-1(q485) and gld-3(q741) mutants RNAi depleted against control (L4440), bec-1 or lag-2 (as a positive control). Animals grown at 15ºC, switched to 20ºC at L1 larval stage and analyzed as young adults.

NOTE: Strain used (A) MD701: bcls39[lim-7p::ced-1p::GFP+lin-15(+)]. Expression of functional CED-1::GFP fusion protein in the sheath cells. (B) gld-1(q485) animals were segregated from the strain JK3025: gld1(q485)i/hT2[bli-4(e937) let?(q782)qls48](I;III), and gld-3(q741) animals were segregated from the strain JK 3345: gld-3(q741)/mln1[mls14 dpy-10(e128)] II.
Lastly, to investigate the role of BEC-1 in the progression of the cell cycle, we evaluated the number of germ cells in the Mitotic-phase, actively dividing (M-phase), and in DNA Synthesis-phase (S-phase), in *bec-1* mutant animals at the late L4/young adult stage. Compared to wild-type control animals, *bec-1/BECN1* mutant animals displayed a significant reduction (P<0.001) in the number of actively dividing cells (reduced M-index), as well as a reduced S-phase index (FIG. 2.12 A-C). In addition, we found the germ cell nuclei of *bec-1(ok691)* mutants to appear smaller compared to wild-type animals. There was no visible cell cycle arrest in *bec-1/BECN1* mutant gonads, as judged by the absence of highly enlarged nuclei (Fox, Vought et al. 2011)(data not shown). Based on the decrease in both M-and S-phase indices we conclude that the stem/progenitor cells in *bec-1(ok691)* mutants are cycling less frequently.

To investigate whether expression of cell cycle components are affected in *bec-1* mutant animals, we analyzed the expression of several protein reporters that are important during G2/M phase transition: a CYB-1/cyclin B reporter, a reporter for the PAR-5/14-3-3 protein, and the LMN-1/lamin protein reporter. We found that depletion of *bec-1* by RNAi resulted in a reduced accumulation in of CYB-1::GFP (Cyclin B) in the proliferative region (FIG. 2.12 D). Since cyclin B levels rise in G2 and fall during the metaphase-to-anaphase transition assuring proper G2/M phase transition (reviewed in (Lim and Kaldis 2013)), this observation suggests that less cells execute the G2/M transition. Furthermore, the cyclin B1-Cdk1 complex is important for phosphorylation of nuclear lamins to disassemble the nuclear envelope and allow access of the mitotic spindle to the chromosomes during mitosis (Heald and McKeon 1990, Peter, Nakagawa et al. 1990, Courvalin, Segil et al. 1992). In *bec-1* RNAi depleted animals, germline
Figure 2.12. BEC-1 promotes the cell cycle progression.  
(A) Representative confocal images of the extracted gonads show cells in the S-phase and in M-phase.  
(B) Quantification of M-phase index, and (C) S-phase index in wild-type and bec-1(ok691) mutant animals.  
For (A-C), animals were grown at 15°C, shifted to 20°C as young L4 larvae, and DAPI stained for analysis as late L4/young adults.  
Results reflect the average of at least three biological replicates shown as the mean ± SEM (shown as error bars).  
Significance determined by two-tailed Student’s T-test: *** P ≤ 0.001; number of analyzed gonads N≥20.  
bec-1 homozygous animals segregated from bec-1/hT1 heterozygous parents.  
(D-F) Representative DAPI stained confocal images of the distal mitotic zone in wild-type animals and in bec-1(RNAi) depleted animals expressing (D) CYB-1::GFP (Cyclin B), (E) LMN-1::YFP (lamin) and (F) PAR-5::GFP(14-3-3).  
Empty vector (L4440) was used as a control for these experiments.  
For (D-F), animals were raised at 15°C and shifted to 25°C as L4 larvae and DAPI stained for analysis as young adults.
expression of the lamin reporter LMN-1::YFP was greatly enriched in the perinuclear regions of germline nuclei, forming multiple aggregates in the distal portion of the gonad (FIG. 2.12 E). Lastly, we examined the expression of the PAR-5::GFP (the 14-3-3 protein). There are several mechanisms by which 14-3-3/PAR-5 prevents G2/M phase transition, including via indirect stalling or preventing cyclin B1-Cdk1 complex activation (reviewed in (Gardino and Yaffe 2011)). We observed an accumulation of PAR-5::GFP in the distal region of the gonad upon RNAi-mediated knock down of bec-1 (FIG. 2.12 F), whereas in wild-type animals, PAR-5::GFP expression was uniform through the mitotic and transition regions of the gonad. Taken together, our data suggest that BEC-1 directly or indirectly facilitates G2/M phase transition of germ cells. The anatomical focus of BEC-1 for the proper expression of G2/M markers has to be further examined, however we failed to observe a decrease in M-phase and S-phase indices in animals that were RNAi depleted against bec-1 in the rrf-1 (germline RNAi sensitive) strain, which would suggest that BEC-1 is required non-cell autonomously for cell cycle progression.

Growing evidence supports the idea that autophagy has context dependent roles in the regulation of proliferation. Our studies show that BEC-1 is necessary for germ cell pool expansion during development. Moreover, we demonstrate that BEC-1 protein is required cell non-autonomously for the robust germline proliferation and propose a model of somatic BEC-1 regulation of germ cell proliferation (FIG. 2.13). We find that BEC-1 acts parallel to the glp-1/Notch and DAF-7/TGFβ pathways, but interacts with components of the DAF-2/IIR signaling pathway. BEC-1 acts in a partly non-canonical pathway that involves DAF-18, but not DAF-16, and also interacts with AKT-1 and PDK-
1. We note that the interaction between DAF-16 and BEC-1 has to be further tested by using double mutants. However, a pathway previously shown to require DAF-18/PTEN, but not DAF-16/FOXO, is that of the regulation of quiescence of germline development during the L1 diapause (Fukuyama, Rougvie et al. 2006). During L1 arrest, DAF-18 regulates a checkpoint that blocks mitotic progression and growth of germ cells, by opposing the proliferation and growth promoting activity of AGE-1/PI3K and AKT-1 (Fukuyama, Rougvie et al. 2006). Interestingly, our studies show that BEC-1 can regulate proliferation of the germ line differently, depending on the genetic background of the animal. In wild-type animals, BEC-1 is required for proliferation, whereas in pdk-1 mutant animals, BEC-1 inhibits proliferation. It is interesting to note that autophagy has been reported to exhibit tumor promoting and tumor suppressing functions. Given our findings, genetic heterogeneity of tumors may provide the explanation for this apparent paradox. Given that autophagy genes are conserved from *C. elegans* to humans, and BECN1 is an essential autophagy regulator and tumor suppressor (Qu, Yu et al. 2003, Yue, Jin et al. 2003), understanding the molecular mechanisms by which BEC-1 modulates the proliferation and/or maintenance of the stem progenitor cell population *in vivo* may advance our understanding of cell non-autonomous regulation of cell proliferation and may lead to novel chemotherapeutic approaches in the future.
Figure 2.13. Current model of BEC-1 promoting germline proliferation. BEC-1 interacts with components of the DAF-2/IIAR signaling pathway to directly or indirectly facilitate G2/M phase transition of mitotic cell cycle in germ cells.
Chapter 3

3. Autophagy genes and their role in the proliferation of progenitor stem cells

Autophagy involves the engulfment of targeted components such as organelles, aggregated or long-lived proteins, within double-membrane vesicles referred to as autophagosomes. Autophagosomes fuse with lysosomes where the sequestered material is degraded by lysosomal proteases into amino acids and macromolecules that are transported across the lysosomal membrane to the cytosol for reuse in cellular processes, including protein biosynthesis and energy production. This dynamic process involves the formation of several functional protein complexes, and autophagosome formation can be broken down into three stages: initiation, nucleation, and expansion. The vesicle nucleation step includes the formation of the protein complex between the Class III phosphatidylinositol 3-kinase (PI3K) VPS-34, BEC-1/Beclin 1, EPG-8/Atg14 and several other proteins. Vesicle expansion and completion requires two protein complexes: the ATG-12 conjugation system (protein complex of ATG-12, ATG-5, ATG-16) and the LGG-1/Atg8 lipidation system (protein complex of LGG-1/Atg8, ATG-3, and ATG-7) (Ohsumi 2001, Mizushima 2007). ATG-9, which cycles between the pre-autophagosomal structure (PAS) and non-PAS compartments, requires two peripheral membrane proteins, ATG2 and ATG18 for the retrieval step (Reggiori, Tucker et al. 2004). In addition, several lysosomal proteins (CUP-5, ARL-8, VPS-16) are involved in the final steps of autophagosome maturation and recycling of sequestered materials (Nakae, Fujino et al. 2010, Sun, Wang et al. 2011, Wartosch, Gunesdogan et al. 2015).
We showed that BEC-1 has a role in the proliferation of progenitor germ cells (Chapter 2). As BEC-1 has a function in retromer transport, from endosomes to the Golgi network, we have investigated if retromer proteins (RME-8, SNX-1) are involved in the regulation of mitotic cell proliferation in the germline. However, we found that loss of RME-8 or SNX-1 retromer proteins, by RNAi depletion of the *rme-8* or *snx-1* genes, had no effect on the proliferation of germ cell progenitors (Chapter 2). In contrast, we found that other autophagy proteins were required for the normal proliferation of progenitor cells. This chapter investigates the role and possible genetic interactions of several autophagy genes and the pathways that they may interact to regulate mitotic germ cell population.

### 3.1. Autophagy gene activity is required for mitotic proliferation in the *C. elegans* germline

In the *C. elegans* germline, proliferation occurs in mitotic region of the distal gonad. The proliferative zone of wild-type young adult hermaphrodite animals, on average, extends to 20 germ cell diameters from the distal tip (Crittenden, Troemel et al. 1994, Hansen, Hubbard et al. 2004), and consists of 220 stem/progenitor cells. In our studies of self renewable progenitor cell population in the *C. elegans* germ line, we noticed that *bec-1(ok691)* mutants had a decrease in the germ cell pool. Moreover, analysis of the proliferative zone in autophagy mutant animals, such as *bec-1(ok691)*, *atg-16.2(ok3224)* and *atg-18(gk378)*, revealed a significant decrease in the number of mitotic nuclei and the shortening of mitotic zone length from 20 to 15 cell diameters.
Similarly, loss of autophagy proteins that act at different steps of the autophagy pathway, directed by RNAi against bec-1, vps-34, epg-8, atg-7, atg-12, lgg-1, atg-13, atg-18 or atg-9, resulted in a significant decrease in the number of germline progenitor cells (FIG. 3.1C). Moreover, we tested whether lysosomal genes, and possibly lysosomal degradation, were required to promote the proliferation of germ cells. We found that RNAi depletion of the lysosomal genes vps-16, cup-5 and arl-8 also decreased the proliferation of germline progenitor stem cells (FIG. 3.1C). Interestingly, RNAi depletion of lgg-1 function did not cause a significant decrease in the germ cell proliferation, probably due to LGG-1 function being compensated by LGG-2, as these two proteins act synergistically in C. elegans (Alberti, Michelet et al. 2010). Thus, from these experiments, we concluded that autophagy genes are required for the proliferation of progenitor/stem cells in the C. elegans germline and it seems that the process of autophagy, including lysosomal degradation, is necessary for the proper accumulation and/or maintenance of the germline progenitor cells.

3.2 Autophagy genes are required to enhance germ cell proliferation in hermaphrodites and males of C. elegans

Adult male germline stem cells (GSCs) have similar properties with the hermaphrodite GSCs. Both lack cell-cycle quiescence and reproducibly oriented divisions (Morgan, Crittenden et al. 2010). However, it was also shown that GSCs in males complete their cell cycle faster (Morgan, Crittenden et al. 2010). To determine if
Figure 3.1. Autophagy function is necessary for the proliferation of germ cell progenitors in the distal gonad.

(A) Representative images of DAPI stained gonads of wild type, atg-16.2(ok3224), atg-18(gk378), bec-1(ok691) single mutants grown at 15°C, shifted to 25°C as L4 larvae, and analyzed as young adults. Magnification 630X. (B) Quantification of the number of mitotic cells in autophagy mutants: atg-16.2(ok3224), atg-18(gk378) or bec-1(ok691). (C) Quantification of the number of mitotic nuclei in animals RNAi depleted of the autophagy genes bec-1, vps-34, epg-8, atg-7, atg-12, lgg-1, atg-13, atg-18 and atg-9, and the lysosome related genes, vps-16, cup-5, and arl-8. For all (A-C), animals were DAPI stained for analysis as young adults. Empty vector (L4440) was used as a control. Results shown as the mean ± SEM (shown as error bars). Significance determined by two-tailed Student's T-test: ** P≤0.01, *** P≤0.001; Number of analyzed gonads N≥20.
autophagy genes act similarly in both male and hermaphrodite germ cell proliferation, we analyzed mitotic cell pools in autophagy mutant hermaphrodites and males.

Decrease of autophagy gene function by mutation (in him-5(e1490);atg-2(bp576), atg-3(bp412)IV;him-5(e1490), atg-7(bp411);him-5, atg-9(bp564)him-5(e1490), and epg-8(bp521);him-5(e1490) mutants) decreased germ cell proliferation significantly in hermaphrodites and males (FIG. 3.2A,B). Interestingly, the severity of the reduction differs between males and hermaphrodites, which can be due to existing sexual dimorphism of GSCs in the germline cell population.

**Figure 3.2. Autophagy gene function is required for hermaphrodite and male germline proliferation.**

(A) Quantification of the number of mitotic nuclei in autophagy mutant hermaphrodites or (B) males. For (A, B), mutant animals him-5 (control), atg-2;him-5, atg-3;him-5, atg-7;him-5, atg-9;him-5, and epg-8;him-5 were grown in 15°C, shifted to 20°C as L1 larvae and analyzed as young adults. DAPI staining was performed on whole animals. Results are shown as the mean ± SEM (shown as error bars). Significance determined by two-tailed Student’s T-test * P≤0.05, ** P≤0.01, *** P≤0.001; number of analyzed animals N≥15.
3.3 Decline in the germline proliferation in autophagy mutants also reduces brood size

To investigate whether the decrease in germline proliferation had an effect on reproduction and therefore progeny production, we compared broods of wild type vs. \textit{atg-18(gk378)} and \textit{atg-16.2(ok3224)} mutant animals. To ensure that all hatched animals are included in the brood count and to eliminate the possibility of L1 larval lethality influencing the results, all hatched progeny were counted as L1 larvae. Compared to wild-type animals, autophagy mutant hermaphrodites generated approximately 40\% less L1 progeny (\textbf{FIG. 3.3}). In addition to the reduction in brood size of \textit{atg-18(gk378)} mutant hermaphrodites, we observed L1 lethality, which was not observed in wild type or \textit{atg-16.2} mutant animals. Interestingly, we noticed that L1 lethality varies in response to the amount of stress (such as temperature and handling conditions) during the hatching/L1 period of the \textit{atg-18} mutant strain (data not shown). We concluded that in \textit{atg-18(gk378)} or \textit{atg-16.2(ok3224)} autophagy mutant animals, the reduction in germline progenitor/stem cells leads to a reduction in brood size, and that susceptibility of \textit{atg-18} mutants to stress may result in L1 lethality.
3.4 Autophagy genes are important for the proper germline progenitor cell pool accumulation during development

To investigate possible mechanisms for the reduction in progenitor/stem cells in autophagy mutants, we analyzed whether (A) a decrease in the accumulation of progenitor cells occurs during larval gonad development and, or (B) if there is improper maintenance of the mitotic cell population during adulthood. To distinguish between these two possibilities, we evaluated the number of germ cells in the distal gonad of autophagy mutant animals (*atg-18, atg-16.2, and bec-1*), at different developmental (larval) stages. It has been shown that the *daf-2* gene is required for robust larval germline proliferation and that *daf-2* mutants have a delay in germ line development.
(Michaelson, Korta et al. 2010); thus, daf-2 mutants and wild-type animals were used as controls. Time course analysis of autophagy mutant animals showed that a progenitor/stem cells reduction occurs throughout the L4 larval stage in atg-18, atg-16.2 autophagy mutants, similar to bec-1 mutants (FIG. 3.4). In wild-type animals, the expansion of the mitotic progenitor pool population occurs during the L4 stage. In daf-2 mutants, the progenitor accumulation defect is already prominent in the L3 to L4 transition. However, in autophagy mutants, the number of germ cells was similar to that of wild-type animals during the L3/L4 transition stage and the mitotic germ cell population was significantly deficient only during the L4 stage. Interestingly, bec-1 mutants show defects at the early L4 stage, but atg-18 and atg-16.2 mutants display the lack of proliferation phenotype only by the mid L4 stages. It is unlikely that this difference is due to developmental timing, as we were very careful when determining the stage of the animals for this time-course analysis. Our data suggests that autophagy genes are required for the proliferation of germ cell progenitors that occurs during the L4 stage. However, we cannot exclude the possibility that a similar mechanism, required for germ cell accumulation during development, may be required for the maintenance of the germ line during adulthood.
3.5. Autophagy genes are not required for survival or the germ cell decision to proliferate or differentiate

Our results suggest that autophagy genes are necessary for the cell proliferation that occurs during germ line development. We considered three possible mechanisms for how autophagy could affect rapid cell proliferation. Autophagy could be required for (A) cell survival, (B) the balance between proliferation and differentiation, and/or (C) the cell cycle.

Figure 3.4. Autophagy genes are required for the proper accumulation of progenitor cell pool during germline development.

Time course analysis of the germ cell progenitor accumulation in the proliferative zone of gonads during germline development in wild type, bec-1(ok691), atg-16.2(ok3224), atg-18(gk378) and daf-2(e1370) mutant animals. Animals were grown at 15°C, switched to 20°C at the L3 larval stage and analyzed at the stated stage of development. bec-1(ok691) animals segregated from bec-1(ok691);ht1 heterozygous parents. Results are shown as the mean ± SEM (shown as error bars). Significance determined by two-tailed Student's T-test *** P≤0.001; number of analyzed gonads N≥15.
First, under normal physiological conditions germ cells undergo apoptosis in the late pachytene stage (Fox, Vought et al. 2011), and increased cell death can lead to the decline of the germ cell population. Hence, we analyzed whether autophagy genes are required for cell survival by determining if there was an increase in apoptotic cells in the gonads of autophagy mutant animals. We used animals carrying a functional CED-1::GFP transgene marker, which labels cells that engulf dying apoptotic cells. RNAi depletion of atg-18, bec-1, lgg-1, and vps-34 autophagy genes, did not cause improper accumulation of apoptotic cell corpses in the distal region of the germline (FIG.3.5A); moreover, there was no visible cellular debris in the distal gonad of young-adult animals. As previously reported, in 2-day-old autophagy gene depleted adults, an accumulation of apoptotic corpses was visible at the bend of the gonad, due to a delay in cell corpse degradation (Ruck, Attonito et al. 2011). However, we found that there were no apoptotic corpses at the L4 stage anywhere in the gonad, when the decrease in proliferation is already apparent, and no apoptotic corpses were observed in the distal end of the gonad at any stage. Thus, we concluded that the reduction of progenitor/stem cells number in young autophagy mutants is not due to a decrease in cell survival (or an increase in cell death).

Second, we investigated whether autophagy genes are required for the decision to proliferate or differentiate, so that their loss could influence the balance between proliferation and differentiation of germ cells. A precocious entry of germ cells into meiosis, could result in a decrease in the progenitor/stem cell pool. We took advantage of two single mutants, gld-1 and gld-3, which have defects in the transition from cell proliferation to differentiation (entry into meiosis), and analyzed whether loss of
**Figure 3.5.** Loss of autophagy genes does not affect germ cell survival or the decision to transition from mitosis to meiosis.

(A) Quantification of the number of apoptotic cell corpses at the gonad bend. CED-1::GFP is expressed in cells that engulf apoptotic nuclei. Animals were treated with RNAi against the autophagy genes `bec-1`, `atg-7`, `vps-34`, `lgg-1`, or `atg-18`, and the retromer genes `rme-8` or `snx-1`, and the number of dead engulfed cells was counted. Animals were raised at 20°C and analyzed as young adults. (B) Length of the mitotic region was measured in cell diameters in `gld-1(q485)` or `gld-3(q741)` mutant animals that were also RNAi depleted of `bec-1`, `vps-34`, `lgg-1`, `atg-7`, `atg-18`, or `lag-2` (as a positive control). Animals were grown at 15°C switched to 20°C at the L1 larval stage and analyzed as young adults. (A,B) Results are shown as the mean ± SEM (shown as error bars). Significance determined by two-tailed Student’s T-test *** P≤0.001; number of analyzed gonads N≥20. Empty vector (L4440) was used as a control.

**NOTE:** Strain used (A) MD701: `bcl39[y::h+]lim-7p::ced-1p::GFP+lin-15(+)]`. Expression of functional CED-1::GFP fusion protein in the sheath cells. (B) `gld-1(q485)` animals were segregated from the strain JK3025: `gld1(q485)i[hT2[bli-4(e937)] let?(q782)qIs48]III`, and `gld-3(q741)` animals were segregated from the strain JK 3345: `gld-3(q741)/mls14[dpy-10(e128)] II`. 
autophagy gene activity could enhance or suppress the transition phenotype of either mutant. *gld-1(q485)* mutants display a decrease in the length of the mitotic zone, due to an increase in differentiation, whereas *gld-3(q741)* mutants have an extension of the mitotic zone, due to a decrease in the number of differentiating nuclei that enter meiosis (Eckmann, Crittenden et al. 2004, Hansen, Wilson-Berry et al. 2004). To determine whether autophagy genes are required in the proliferation vs. differentiation switch, we analyzed the length of the mitotic region of *gld-1* or *gld-3* single mutants lacking autophagy gene activity (*bec-1, vps-34, lgg-1, atg-7* and *atg-18*) after RNAi depletion. However, the loss of *bec-1, vps-34, lgg-1, atg-7* and *atg-18* autophagy gene function had no significant change in the length of the proliferative zone in either *gld-1* or *gld-3* single mutants (FIG. 3.5B). In contrast, the loss of the GLP-1/Notch ligand LAG-2, by RNAi against *lag-2*, resulted in an enhanced phenotype of *gld-1*, and suppressed phenotype of *gld-3* mutants, in other words a positive control that reduced the mitotic region in either case. These data suggest that autophagy genes are not involved in the germ cell decision to transition of germ cells from mitosis to meiosis.

### 3.6. Autophagy gene activity is required for the cell cycle progression

Lastly, autophagy gene activity may be required for the progression of the cell cycle, and therefore, the lack of autophagy gene activity may result in stalling or a slowing down of the cell cycle. To investigate the progression of the cell cycle in autophagy gene mutants, we evaluated the number of cells in Mitotic-phase (actively
dividing, M-phase) and in DNA Synthesis-phase (S-phase), in the proliferating region of the gonads of \textit{atg-18}, \textit{atg-16.2}, and \textit{bec-1} homozygous mutant animals.

To determine if these mutations alter the frequency of germ cell divisions, we calculated the M-phase index, which measures a ratio of the mitotic cells in metaphase, or anaphase, to the total mitotic cell population. Compared to the wild-type control animals, \textit{bec-1}, \textit{atg-18}, and \textit{atg-16.2} autophagy mutant animals displayed a considerable decline in the number of actively dividing cells, as we noticed a significant reduction (P<0.001) of the M-phase index (FIG. 3.6A). Furthermore, we explored the S-phase index, by determining the ratio of the cells in S-phase, which are actively synthesizing new DNA and are EdU positive, relative to the total number of mitotic cells in the proliferative zone. The germline S-phase index in \textit{bec-1}, \textit{atg-18}, and \textit{atg-16.2} mutant animals was significantly reduced compared to that of wild-type animals (FIG.3.6B). The M-phase and S-phase index for \textit{atg-18} and \textit{atg-16.2} mutants were similar to those of \textit{bec-1} mutants. Taken together, a decrease in both M- and S-phase indices suggests that the progenitor/stem cells are cycling less frequently in autophagy mutants. It is worth noting that cell cycle arrest, which can be visualized by the presence of large nuclei, was never observed in any of the autophagy mutants.

3.7. Autophagy genes possibly control germline proliferation cell non-autonomously

Germline proliferation can be regulated cell non-autonomously, where signals originate from the surrounding somatic tissues, or cell autonomously where signals are
Figure 3.6. Autophagy genes have a role in cell cycle progression.

(A) Quantification of the M-phase index and (B) S-phase index in wild-type, atg-16.2 (ok3224), or atg-18(gk378) single mutant animals. (C) Quantification of the M-phase index in wild-type animals after treatment with RNAi against control (gfpI), the autophagy genes bec-1, vps-34, epg-8, atg-7, atg-12, lgg-1, atg-13, atg-18 or atg-9 and the lysosomal genes vps-16 or cup-5. For all (A - C), animals were raised at 20°C and analyzed as young adults. M-phase index was calculated as a ratio of α-pH3 positive cells (M-phase nuclei) to the total mitotic cell population. S-phase index was calculated as a ratio of EdU positive cells (S-phase nuclei) to the total mitotic cell population. Results shown as the mean ± SEM (shown as error bars). Significance determined by two-tailed Student’s T-test: * P≤0.05, *** P≤0.001; number of analyzed gonads N≥10.
produced within the germ line. To investigate the focus of action of autophagy genes, we utilized \textit{rrf-1} mutants that are mostly resistant to RNAi treatment in somatic tissues, and are sensitive to RNAi treatment in the germ line (Sijen, Fleenor et al. 2001). We found that RNAi depletion of \textit{bec-1, vps-34, lgg-1, atg-18, or lag-2} (as control) in wild-type animals significantly decreased the proliferation of germline progenitors, but the same RNAi treatment in \textit{rrf-1(ok589)} mutants had no effect on germline proliferation (FIG. 3.7). The same was true for the loss of \textit{LAG-2/DSL}, which encodes the ligand for GLP-1/Notch and is produced by the Distal Tip Cell. RNAi against \textit{lag-2/DSL} resulted in the expected, a decrease in proliferation when depleted in wild-type animals but not in \textit{rrf-1} germline RNAi sensitive mutants. Our results suggest that the requirement for autophagy signaling originates in somatic tissues. Interestingly, we note that RNAi depletion of \textit{rme-8, snx-1} retromer gene function by RNAi did not affect germline proliferation in wild type animals or \textit{rrf-1} mutants, which confirmed our previous findings. Thus, our results suggest that BEC-1, VPS-34, and ATG-18 function cell non-autonomously from surrounding somatic tissues to control germ cell proliferation.
3.8. Autophagy genes are required for the germcell overproliferation of \textit{glp-1} gain-of-function mutants

In \textit{C. elegans}, the GLP-1/Notch receptor pathway is required to keep germ cells in the mitotic undifferentiated state and inhibits differentiation and/or meiotic entry (Austin and Kimble 1987, Berry, Westlund et al. 1997). In the absence of \textit{glp-1} activity, only a few germ cells are produced, which enter meiosis precociously and differentiate as sperm (Austin and Kimble 1987). In contrast, constitutive \textit{glp-1} signaling results in germ cell overproliferation, and a Tumorous (Tum) phenotype, where cells divide continuously and never enter meiosis (Berry, Westlund et al. 1997). To understand if autophagy gene function is required for the Tum phenotype of \textit{glp-1(ar202)} mutants, we analyzed the
proliferation of the loss of autophagy gene function in glp-1(ar202) mutants, by RNAi or chromosomal mutations. glp-1(ar202) constitutive gain-of-function mutants, grown at the restrictive temperature, display a highly penetrant late-onset tumorous expansion of the distal mitotic zone (late-onset Tum), and an additional Proximal proliferation (Pro) phenotype in the germline (Pepper, Killian et al. 2003). In our experiments, we evaluated the late-onset Tum phenotype that is limited to the distal gonad, upon 12 hours of induction at the restrictive temperature. RNAi depletion of bec-1, vps-34, and atg-18 autophagy gene function in glp-1(ar202) mutants, resulted in a significant decrease of the stem/progenitor cells number in the distal gonad (FIG. 3.8 A, B). Similarly, double mutant animals for glp-1(gf) and autophagy genes, such as glp-1(ar202);bec-1(ok691), glp-1(ar202);atg-18(gk378) or atg-16.2(ok3224);glp-1(ar202), displayed a significant decrease in the number of progenitor/stem cells (FIG. 3.8 C). Thus, we concluded that autophagy gene activity is required for the excessive proliferation of the germ cells in glp-1 mutant animals.

3.9. ATG-18 and ATG-16.2 most likely control germ cell proliferation through the canonical DAF-2/IIR pathway

DAF-2/IIR signaling promotes germ cell proliferation through the canonical PI3K pathway, which requires DAF-18/PTEN to inhibit DAF-16/FOXO function (Michaelson, Korta et al. 2010). As it was discussed in Chapter 1, we showed that BEC-1/Beclin 1 functions in together with the DAF-2/IIR signaling pathway, and involves DAF-18/PTEN, but not DAF-16/FOXO to control stem cell proliferation (FIG 3.9). Here, we analyzed
**Figure 3.8.** Autophagy genes are required for the germline over proliferation phenotype of *glp-1(ar202)* mutant animals.

(A) Representative confocal images of DAPI stained gonads from *glp-1(ar202)* mutant animals RNAi depleted against *bec-1, vps-34, atg-18* or control *gfp* RNAi. * marks the distal end of the gonad. White line shows the end of the mitotic zone, and transition zone. Magnification 630X.

(B) Quantification of the number of mitotic nuclei in the mitotic zone from *glp-1(ar202)* after RNAi depletion of *bec-1, vps-34* or *atg-18* gene function.

(C) Quantification of the number of mitotic zone nuclei in the *glp-1(gf);atg-16.2, glp-1(gf);atg-18* or *glp-1(gf);bec-1* double mutants and *glp-1(gf), atg-16.2, atg-18* or *bec-1* single mutants. For all (A,B,C), animals were raised at 15°C and switched to 25°C as L4 larvae. Young adults were DAPI stained and analyzed. Results shown as the mean ± SEM (shown as error bars). Significance determined by two-tailed Student’s T-test: *** P≤0.001; number of analyzed gonads N≥30.
interactions between several other autophagy proteins and the DAF-2/IIR pathway, to
determine if they act similarly to bec-1. Interestingly, RNAi depletion of either daf-
18/PTEN or daf-16/FOXO in atg-18(gk378) autophagy mutants suppressed the
germline proliferation defect (FIG. 3.9 B, C). We observed a similar suppression of the
germline phenotype in atg-16.2(ok3224) mutant by RNAi depletion of daf-18/PTEN or
daf-16/FOXO (FIG. 3.9 B, C). This suggests genetic interactions between autophagy
genes (atg-18 and/or atg-16.2) with the canonical DAF-2/IIR pathway, through DAF-
18/PTEN and DAF-16/FOXO to control stem cell proliferation. This result stands in
contrast to what we found for BEC-1/Beclin 1, since the loss of bec-1 phenotype was
suppressed by RNAi depletion of daf-18, but not daf-16. However, this result has to be
further investigated with double mutant analysis. Moreover, RNAi depletion of daf-18, in
the atg-7(bp411) mutant background, did not suppress the proliferation phenotype of
atg-7 mutants, suggesing that ATG-7 affects proliferation of the germline progenitors
through a different pathway. However, this negative result in atg-7; daf-18 RNAi animals
should be further corroborated in atg-7; daf-18 double mutants.

The different interactions between ATG-7, BEC-1, ATG-18 and ATG-16.2
together with the different known pathways that control stem cell proliferation may be
attributed to the existence of multiple pathways, as for example an Atg5/Atg7-
dependent conventional pathway, and an alternative Atg5/Atg7-independent pathway. Mouse cells that lack Atg5 or Atg7 can still form autophagosomes/autolysosomes and
perform autophagy-mediated protein degradation (Nishida, Arakawa et al. 2009). Our
results demonstrate the potential for ATG-7 to act through a DAF-7/TGF-β signaling
Figure 3.9. Genetic analysis of interactions between autophagy genes and the DAF-2/IIIR signaling pathway.

(A) Quantification of the number of mitotic nuclei in wild type, *bec-1(ok691)*, *daf-2(e1370)* and *daf-2(e1370);bec-1(ok691)* double mutant animals. (B,C) Quantification of the number of mitotic cells in the mitotic zone of wild type, *atg-7(bp411)*, *atg-18(gk378)*, *atg-16.2(ok3224)*, *bec-1(ok691)*, *bec-1(ok700)* and *daf-2(e1370)* mutant animals treated with control (L4440) RNAi, *daf-18* RNAi and *daf-16* RNAi. (A-C) Animals were raised at 15°C, shifted to 20°C as L3 larvae and analyzed as young adults. Results are shown as mean ± SEM (shown as error bars). Significance determined by two-tailed Student’s T-test: * P ≤ 0.05 *** P ≤ 0.001. Number of analyzed gonads N ≥ 21.
pathway that promotes germline proliferation in a DAF-3 and DAF-5 dependent manner, and independently of the DAF-2/IR signaling pathway.

3.10. ATG-7 acts through DAF-7/TGFβ signaling pathway to promote germ cell proliferation

TGFβ signaling is another pathway that regulates germ cell proliferation (Park, Estevez et al. 2010, Dalfo, Michaelson et al. 2012) (FIG. 3.10 A). It controls the balance between proliferation and differentiation in response to environmental conditions, without affecting cell cycle progression (Narbonne and Roy 2006, Dalfo, Michaelson et al. 2012). The DAF-7/TGF-β ligand activates the DAF-4 (type II)/DAF-1 (type I) receptor complex, which phosphorylates the R-Smads, DAF-8 and DAF-14. This leads to negative regulation of a DAF-3/DAF-5 transcriptional repressor complex (Patterson, Koweek et al. 1997, da Graca, Zimmerman et al. 2004, Dalfo, Michaelson et al. 2012). To investigate whether autophagy genes act through the TGFβ signaling pathway, we investigated whether atg-7 or atg-18 genes require daf-3/CoSMAD or daf-5 to regulate germ cell proliferation. As previously discussed, we found that RNAi depletion of daf-7 gene function in bec-1 mutants enhanced the bec-1 mutant defects in germline proliferation, suggesting that DAF-7 and BEC-1 act in parallel pathways (FIG. 3.10 B). Similar results were observed upon RNAi depletion of cup-5, a lysosomal degradation gene, in daf-7 mutants, suggesting that bec-1 and cup-5 act in parallel pathways to the DAF-7/TGF-β signaling pathway. Surprisingly, RNAi depletion of atg-7 or atg-18 gene function, in the daf-7(e1372) mutant background, did not enhance the
Figure 3.10. Genetic analysis of possible interactions between autophagy genes and the TGFβ/DAF-7 signaling pathway

(A) Schematic representation of the TGFβ/DAF-7 signaling pathway. (B) Quantification of the number of mitotic cells in wild type, daf-7(e1372), daf-3(ok3610) or daf-5(e1386) animals, RNAi depleted of autophagy genes atg-7, atg-18, bec-1, or cup-5. Animals grown at 15°C and shifted to 20°C, as L3 larvae, and analyzed as young adults. Empty vector (L4440) was used as a control.

Results are shown as mean ± SEM (shown as error bars). Significance determined by two-tailed Student’s T-test: * P≤0.05, ** P≤0.01, *** P≤0.001. Number of analyzed gonads N≥12.
decreased proliferation phenotype of daf-7 single mutants (FIG. 3.10 B). Our results suggest that ATG-16.2, ATG-18 and DAF-7 act in the same pathway. More striking result was that mutations in daf-3 or daf-5 significantly suppressed the decreased in proliferation of atg-7 RNAi mutants (FIG. 3.10 B), indicating that DAF-3 and DAF-5 are required together with ATG-7, and that ATG-7 may act through the DAF-7/TGFβ to promote germ cell proliferation. Why this is the case, we really do not know at this time.

Interestingly, daf-3(ok3610) mutants depleted against atg-18, by RNAi, displayed more proliferation than the atg-18 RNAi depleted animals that were wild-type for daf-3. Thus, we note suppression of atg-18 RNAi by the loss of function mutation in daf-3. In contrast, no suppression of atg-18 RNAi depleted animals was observed in daf-5(e1368); atg-18(RNAi) animals. These data suggest that ATG-18 may partially interact with components of the DAF-7/TGFβ signaling pathway to promote germ cell proliferation. However, any negative results with RNAi have to be further investigated by the analysis of double mutants. Interestingly, during favorable conditions that support reproduction, DAF-7 signaling inhibits DAF-3 activity and permits appropriate fat deposition: however unfavorable environment conditions, inactivate DAF-7, and activate DAF-3, thereby resulting in increased fat storage levels (Greer, Perez et al. 2008). Thus, we cannot exclude the possibility that, similar to the regulation of fat deposition, ATG-18 interacts with DAF-3, but not DAF-5 to promote germ cell proliferation. Taken together, we can conclude that autophagy proteins, which function at different steps of the autophagy pathway, may interact with the DAF-7/TGF-β signaling pathway (as ATG-7), interact with some of its components (as ATG-18), or work in parallel (as BEC-1 and CUP-5) to promote germ cell proliferation.
3.11. Autophagy genes are necessary for the germline membrane organization

The germ cell nuclei of autophagy mutants are smaller/tighter compared to wild type (FIG. 3.1), when stained with DAPI. Interestingly, the variability in cell size might be due to distortions in the plasma membrane of autophagy mutants. It was shown in HeLa cells that during cell cycle progression, autophagy is inhibited during the early stages of mitosis by the phosphorylation of the Class III PtdIns3 kinase (phosphatidylinositol-3 kinase) Vps-34 at position Thr159, specifically by the mitotic cyclin-dependant kinase Cdk-1 (Furuya, Kim et al. 2010). Cdk-1 phosphorylation inhibits the formation of the Beclin-1/Vps-34 complex, which results in the inhibition of autophagy during mitosis. It was suggested that this is a normal event that occurs during mitosis. During cytokinesis, basal autophagy is no longer inhibited (Furuya, Kim et al. 2010), and a role for autophagy has been found in the midbody ring degradation (Pohl and Jentsch 2009, Thoresen, Pedersen et al. 2010, Isakson, Lystad et al. 2013). We propose that a signal or a recycled reagent provided by the basal degradation of an unknown substrate through autophagy is important for the membrane stability and this is required for the newly forming cleavage furrow during mitosis in the *C. elegans* germline. However, one important question that remains is whether this function requires autophagic degradation to occur in somatic tissues, or if autophagy is serving a non canonical function, such as secretion in germline development.

To address whether the germline defects we observed in autophagy mutants correlate with the disorganization of the plasma membrane, we used a
GFP::PH<sup>PLC1δ1</sup> reporter that expresses a fusion of GFP with a pleckstrin homology (PH) domain derived from mammalian PLC1 δ1. The PLC1δ1 PH domain binds with high affinity to a phosphoinositide lipid (PI<sub>4,5</sub>P<sub>2</sub>) that is generated on the plasma membrane (reviewed by (Hurley and Meyer 2001)) and labels the germline membranes in the gonad (Audhya, Hyndman et al. 2005). Top-focal plane images of GFP::PH<sup>PLC1δ1</sup> expression in wild type animals outlines equal-sized hexagons surrounding separate nuclei (FIG.3.11). In the germ line of animals fed with RNAi against bec-1, vps-34, atg-12, epg-8, atg-13, or atg-7 autophagy genes, the membranes are less pronounced/visible, often discontinued and forming random shapes, suggesting defects in membrane remodeling, formation, or maintenance. Thus, inactivation of autophagy genes function results in defective plasma membrane formation. Interestingly, atg-18(RNAi) animals appeared to have a thicker plasma membrane, which may suggest that improper lipid homeostasis on the plasma membrane interferes with normal cell cycle progression. Interestingly, we noticed that upon the depletion of all tested autophagy, retromer transport or lysosomal fusion genes (bec-1, vps-34, atg-12, epg-8, atg-13, lgg-1, atg-18, atg-9, atg-14, cup-5, arl-8, vps-16), mutant germ cells were able to form a cleavage furrow, even if the membranes appeared very faint (FIG.3.12). Since inactivation of autophagy and lysosomal required genes, results in plasma membrane organization defects, it is possible that disruption of any these processes may cause some indirect effect on the plasma membrane. We do not see a direct correlation between the plasma membrane phenotypes and cells arresting during cytokinesis. However, we cannot exclude the possibility that this membrane disruption can indirectly contribute to multiple defects such as those of cell cycle progression, and
the accumulation/maintenance of the proliferative/stem cell population in autophagy mutant animals.
Figure 3.11. Inactivation of autophagy genes by RNAi results in plasma membrane defects in the germ line.

Representative confocal images of wild-type worms that express the GFP::PH:\textsuperscript{PLC}\textsubscript{151} reporter, treated with RNAi against autophagy (bec-1, vps-34, epg-8, atg-7, atg-12, atg-13, lgg-1, atg-18, atg-9) and lysosomal genes (arl-8, cup-5 and vps-16). On the top focal plane of the distal region of the gonad, the wild-type cells have membranes with equal-sized hexagonal shape compartments. In RNAi depleted germ lines, the plasma membranes appeared disorganized and sometimes missing. Maximal projection of oocytes from wild-type animals, have organized oocytes, compartmentalized by “semi-transparent” membranes. In autophagy gene depleted animals, the plasma membranes and oocytes are distorted and the membrane is sometimes missing.
Figure 3.12. Loss of autophagy genes function does not prevent the formation of cleavage furrows in the germ line.

Representative confocal images of germ line cells from wild-type animals expressing the GFP::PH\textsuperscript{PLC151} reporter treated with RNAi against autophagy genes. The GFP::PH\textsuperscript{PLC151} reporter allows us to visualize the plasma membrane. In the red box, cells that have undergone cytokinesis are marked, arrows point to the newly forming membranes at the cleavage furrow. Empty vector (L4440) was used as a control.
Figure 3.13. Current model of autophagy genes promoting germ cell proliferation.
ATG-16.2 and ATG-18 promote germline proliferation cell non-autonomously through the canonical DAF-2/IIR signaling pathway. ATG-7 may promote proliferation cell non-autonomously through the DAF-7/TGFβ signaling pathway. Whether ATG-7 acting in the DTC or in/through other surrounding tissues still needs to be determined.
Chapter 4

Autophagy genes are required for normal lipid levels in *C. elegans* (Published paper: see attached p. 131)

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Acknowledgment: This is an Accepted Manuscript of an article published in Autophagy online [January 15, 2013], available online: http://www.tandfonline.com/doi/full/10.4161/auto.22930

Abstract:

Autophagy is a cellular catabolic process in which various cytosolic components are degraded. For example, autophagy can mediate lipolysis of neutral lipid droplets. In contrast, we here report that autophagy is required to facilitate normal levels of neutral lipids in *C. elegans*. Specifically, by using multiple methods to detect lipid droplets including CARS microscopy, we observed that mutants in the gene *bec-1* (VPS30/ATG6/BECN1), a key regulator of autophagy, failed to store substantial neutral lipids in their intestines during development. Moreover, loss of *bec-1* resulted in a decline in lipid levels in *daf-2* (Insulin/IGF-1 receptor (IIR) ortholog) mutants and in germline-less *glp-1*/Notch animals, both previously recognized to accumulate neutral lipids and have increased autophagy levels. Similarly, inhibition of additional autophagy genes, including *unc-51*/ULK1/ATG1 and *lgg-1*/ATG8/MAP1LC3A/LC3 during development, led to a reduction in lipid content. Importantly, the decrease in fat accumulation observed in animals with reduced autophagy did not appear to be due to a
change in food uptake or defecation. Taken together, these observations suggest a broader role for autophagy in lipid remodeling in *C. elegans*.

**Contribution to this paper**

In this work we analyzed the role of *bec-1* in determining fat levels in wild-type animals. To establish the role of BEC-1 in *C. elegans* lipid storage, I evaluated the effect of impaired *bec-1* function using homozygous *bec-1(ok691)* mutants on lipid accumulation. For the lipid level analysis, I used Oil-Red-O staining, a fixative-based dye that stains neutral lipids. I found a significant decrease in the Oil-Red-O stained neutral lipids in day 1 adult in *bec-1(ok691)* homozygous mutants compared with wild-type animals (Fig. 1A in attached paper). This analysis was also complemented by coherent anti-Stokes Raman (CARS) microscopy method (done by others) to quantitatively analyze the lipid storage, and we could conclude that loss of *bec-1* results significant decrease lipid droplet number, size of droplet and overall lipid content (Fig. 1B, C in attached paper).

Next, I assessed the role of *bec-1* in the fat accumulation phenotype of *daf-2* and *glp-1* loss-of-function mutants. The *daf-2/IIR* and germline-less *glp-1/Notch* mutants have been shown to display increased lipid storage capacity, suggesting possible crosstalk between autophagy, DAF-2/IIR and GLP-1/Notch signaling pathways. I found that compared to wild-type, 1 day adult animals that carry either *daf-2(e1370)* or *glp-1(e2141)* loss-of-function mutations, display an increase in lipid content as measured by Oil-Red-O staining (Fig. 1A in attached paper). Moreover, I investigated the role of BEC-1/Beclin1 in the lipid accumulation of *daf-2* and in *glp-1* mutants. I found that lack of the
bec-1 function in daf-2(e1370); bec-1(ok691) and glp-1(e2141); bec-1(ok691) double mutants decreased the neutral lipid accumulation caused by the daf-2(e1370) and glp-1(e2141) mutations. Taken together, my work contributed to establishing that BEC-1/Beclin 1 plays a critical role in lipid homeostasis, and helped to establish a link between lipid metabolism and autophagy.

Under food availability conditions, fat is released from the intestine to be taken up by developing oocytes in the gonad (Kimble and Sharrock 1983, Grant and Hirsh 1999). Interestingly, the extended lifespan of glp-1 mutant animals (that also have low fat level) requires the DAF-16/FOXO-regulated lipase, LIPL-4 and stearoyl-CoA desaturase, FAT-6, to convert stearic acid to oleic acid. When provided exogenous oleic acid, the longevity of glp-1 mutants lacking stearoyl-CoA desaturase activity can be restored (Wang, O'Rourke et al. 2008, Goudeau, Bellemin et al. 2011, Lapierre, Gelino et al. 2011). This led us to test the hypothesis that failure to utilize and load lipids in bec-1 mutant animals may be the cause for the decrease in germline proliferation. To investigate this hypothesis, we supplemented C.elegans diet with oleic acid, to potentially rescue the reduction of progenitor stem cells in bec-1 mutants. Despite the significant increase of fat content in bec-1 mutants fed oleic acid, determined by Oil-Red-O staining, we did not observe suppression of proliferation phenotype (Appendix FIG. A1). Our data corroborates previously published results by J. Watts lab, where dietary supplementation of only specific polyunsaturated fatty acids (PUFAs), dihomogamma-linolenic acid (DGLA), contribute to the membrane biology, and caused a dramatic effect on C. elegans germline proliferation. They found that supplementation with DGLA resulted in a striking dose-dependent sterility. Whereas,
supplementation with other related fatty acids, such as linoleic acid, linolenic acid, or
eicosapentaenoic acid had no effect on fertility (Brock, Browse et al. 2006, Watts and
Browse 2006). Taken together these observations suggest specificity and sensitivity of
the germline to utilize fats and lipids in the production of new germ cells and it would be
interesting to determine the precise relationship and possible specific requirements for
lipids on germline proliferation.
Chapter 5

5. Discussion and future directions

Autophagy is an evolutionarily conserved cellular recycling process important for organismal development, growth, and survival. During the autophagy process, *de novo* formed membrane engulfs cytoplasmic components, organelles, and/or long–lived proteins to form a double-membrane vesicle termed autophagosome (reviewed in (Mizushima 2007)). Autophagosomal contents are degraded upon the fusion with the lysosome by the lysosomal enzymes and recycled, to maintain crucial cellular metabolism and homeostasis. Autophagy is a highly conserved inducible and dynamic catabolic process. It responds rapidly to various hormonal and environmental cues, hence enables autophagy to drive the rapid cellular changes for proper cell differentiation, organismal development, immune defense, aging, and prevent various medical conditions such as neurodegeneration and cancer (reviewed in (Mizushima and Komatsu 2011)).

5.1. Role of BEC-1 regulated autophagy in cell proliferation

Autophagy proteins are evolutionary conserved from yeast to humans. Studies in yeast show that during autophagosome biogenesis, many autophagy proteins form functional complexes and act in the hierarchical manner. The process of autophagy involves different proteins at distinct steps: the BEC-1/VPS-34 complex is essential for vesicle nucleation, ATG-7, and ATG-16.2 are important during autophagosome vesicle...
formation, for LGG-1 or LGG-2 lipidation, and ATG-18 is involved in membrane shuttling (reviewed in (Xie and Klionsky 2007, Melendez and Neufeld 2008)) (FIG.1.1). The mammalian ortholog of BEC-1, Beclin1, was shown to act as a tumor suppressor (Liang, Jackson et al. 1999). A deficiency in Beclin1 or Atg7 leads to defects in the maintenance of immune progenitor cells in mice and results in severe depletion of their precursors (Arsov, Adebayo et al. 2011). Moreover, mice that lack Atg7, during T cell activation, have reduced proliferation, without a significant increase in apoptotic cell death (Hubbard, Valdor et al. 2010).

In C. elegans, our laboratory has demonstrated that BEC-1 is crucial for the process of autophagy, and endocytosis, and during development for embryogenesis, dauer development, cell corpse degradation, lipid homeostasis, and longevity (Melendez, Tallozcy et al. 2003, Lapierre, Gelino et al. 2011, Ruck, Attonito et al. 2011, Lapierre, Silvestrini et al. 2013). Furthermore, in mammalian tissue culture experiments, as well as in mouse mutants, multiple studies have shown that autophagy may suppress or promote tumor growth, depending on the type and metabolic state of the cell. Moreover, recent studies point to existing crosstalk between autophagy and cell cycle regulation; however, the exact mechanism for how autophagy may regulate the cell cycle progression is not clear. Given the evolutionary conservation of bec-1 and other autophagy genes from C. elegans to humans, understanding the molecular mechanisms by which autophagy modulates the proliferation of germ cells, can provide new important insights for anti-tumorigenic treatments.

In this work, we show that autophagy genes are required for the proliferation of germ cells in the C. elegans gonad. RNAi depletion of autophagy genes and several
lysosomal genes results in a reduction in the progenitor population pool, suggesting that the autophagy pathway, including lysosomal degradation, is necessary for the decision to proliferate or the maintenance of the stem/progenitor cell population in the germ line. Our results show that autophagy gene function is necessary for the cell proliferation during the expansion of germ cells that begins to occur in the L3-L4 larval stage. Interestingly, we noticed that lack of BEC-1, decreased the progenitor germ cell pool at the early L4 stage, whereas in animals that lack ATG-16.2 or ATG-18, the reduction in proliferation was observed during late L4 stage. Although the initial decline in proliferation of autophagy mutants is detected during germline development, the progenitor cell pool never reaches wild-type levels, which would suggest that autophagy is involved in the maintenance of the progenitor pool as well.

5.2. Crosstalk of the autophagy pathway and other signaling pathways to modulate germcell proliferation

The maintenance of the germline mitotic progenitor pool of cells relies on the GLP-1/Notch signaling from the Distal Tip Cell (Crittenden, Troemel et al. 1994) and is modulated by at least three signaling pathways: DAF-2/IIR (Michaelson, Korta et al. 2010), DAF-7/TGF-β (Dalfo, Michaelson et al. 2012) and LET-363/TOR (Pinkston, Garigan et al. 2006, Dalfo, Michaelson et al. 2012). In this project, we investigated whether autophagy genes act via GLP-1/Notch, DAF-2/IIR or DAF-7/TGF-β signaling pathways to affect germline proliferation. Our results show that ATG-16.2 and ATG-18 affect cell proliferation independently of the GLP-1/Notch and DAF-7/TGFβ pathways,
but through the canonical DAF-2/IIR pathway and require DAF-18/PTEN and DAF-16/FOXO. This is similar to the control of germ cell proliferation that exists during abundant food conditions (Dalfo, Michaelson et al. 2012). Similarly, BEC-1/BECN1 affects proliferation independently of GLP-1/Notch and DAF-7/TGFβ pathways. Interestingly, our results show that BEC-1/BECN1 interacts with DAF-2/IIR components, as daf-18/PTEN RNAi depletion suppressed the reduction of proliferation observed in bec-1 mutants. However, daf-16 RNAi depletion did not suppress the bec-1 phenotype, although it suppressed the atg-16.2 or atg-18 phenotypes. The negative result with RNAi depletion of daf-16 in bec-1 mutants will be corroborated further in daf-16; bec-1 double mutant that is being constructed. At this point, we do not understand why BEC-1 does not require DAF-16, however IIR signaling inhibits activity of another transcription factor, SKN-1/Nrf, which is directly phosphorylated by Akt/PKB and related kinases to promote cytoplasmic sequestration (Tullet, Hertweck et al. 2008). Given the fact that BEC-1 interacts with components of DAF-2/IIR pathway, we explored the possibility that BEC-1 is acting through SKN-1 to promote cell proliferation and we depleted skn-1 function by RNAi in wild-type and bec-1 mutant animals. Interestingly, lack of skn-1 gene function significantly suppressed lack of proliferation phenotype in bec-1 mutants, suggesting that BEC-1 promotes cell proliferation in the germline via SKN-1 transcription factor. Depletion of another transcription factor, efl-1/E2F4, which regulates G1/S cell cycle transition (DeGregori, Kowalik et al. 1995, Ceol and Horvitz 2001) did not suppress the proliferation defect of bec-1 mutants, suggesting that BEC-1 promotes proliferation independently from EFL-1/E2F4, and DAF-16/FOXO (FIG. 2.8). Furthermore, a fascinating new mechanism of germline cells-to-soma communication
has been proposed, which involves SKN-1/Nrf protein activation by fatty acid signals in germline-less animals (Steinbaugh, Narasimhan et al. 2015). This opens an interesting possibility for a role of autophagy in the direct or indirect regulation of the SKN-1 transcription factor activity.

Surprisingly, ATG-7 appears to affect germline proliferation independent of DAF-2/IIR pathway but in the same pathway with the DAF-7/TGF-β signaling pathway, in a DAF-3 and DAF-5 dependent manner. These results would argue that ATG-7 and BEC-1 possibly act in parallel pathways; if for example, the phenotype of atg-7 and bec-1 double mutants is enhanced, than either single mutant. These alternative modes of regulation seem reasonable since it was hypothesized that two different autophagy pathways exist in mammals: an Atg5/Atg7-dependent conventional pathway and an Atg5/Atg7-independent alternative pathway (Nishida, Arakawa et al. 2009). This opens up a possibility for several mechanisms for the regulation of cell proliferation by different autophagy pathways. In summary, we conclude that autophagy genes modulate germline proliferation at least through two different autophagy-signaling pathways, a BEC-1 dependent that interacts with DAF-2/IIR and an ATG-7 dependent that interacts with DAF-7/TGF-β.

BEC-1 interacts with other components of the DAF-1/IIR signaling pathway. We noticed that the loss of BEC-1/BECN1 has different effects on the proliferation of germ cells, depending on the background mutation existing in the cells. Similar to loss of bec-1 or daf-2 gene activity, loss of pdk-1 gene function causes significant reduction in the number of mitotic germline progenitor cells. However, we noted that in pdk-1 animals that were RNAi depleted against bec-1, the pdk-1(lf) mutant phenotype was
suppressed, and the number of germline progenitor cells was closer to wild type. We
did not observe such suppression of \textit{pdk-1(\text{lf})} by \textit{daf-2} RNAi, in fact \textit{pdk-1(\text{lf})} further
enhances germline proliferation defect caused by \textit{daf-2} RNAi. Thus, this may indicate
that there are differences in the mechanisms by which BEC-1 and DAF-2 control germ
cell proliferation.

The dual role of Beclin 1 in tumor development was shown in breast
CSCs/progenitor cells (Gong, Bauvy et al. 2013). This discrepancy in the mechanisms
of BEC-1 action can be possibly explained by overlapping requirements for specific
post-translational modifications of Beclin1 required for its autophagic and tumor
suppressing functions. For example, the Ser/Thr kinase death-associated protein
kinase (DAPK) that induces cell death, including autophagic cell death, functions as a
tumor-suppressor, and is deleted in many cancer types, induces autophagy through
Moreover, AMPK has also been shown to activate autophagy machinery components
by phosphorylation, including the upstream kinase, ULK1/UNC-51, and during glucose
starvation phosphorylates Beclin 1 at Ser residues S93 and S93 (Egan, Shackelford et
al. 2011, Kim, Kundu et al. 2011, Kim, Kim et al. 2013). During amino acid starvation-
induced autophagy, the stress-related kinases, members of the p38 mitogen-activated
protein kinase (MAPK) signaling pathway, MAPKAPK2 (MK2) and MAPKAPK3 (MK3),
have been shown to directly phosphorylate Beclin 1 at the S90 residue, leading to
activation of autophagy (Wei, An et al. 2015). MK2/MK3-dependent Beclin 1
phosphorylation (and starvation-induced autophagy) can be blocked in vivo by BCL2, a
negative regulator of Beclin 1 (Wei, An et al. 2015). Interestingly, a mutation on that
residue blocks the tumor-suppressor activity of Beclin 1 in human breast carcinoma (Wei, An et al. 2015). These examples demonstrate how the same stimuli can induce autophagy via different autophagic machinery components, in addition to the different environmental stress signals – such as glucose and/or amino acid starvation – dually activating autophagy and inhibiting tumorous cell proliferation, highlighting the importance of background mutations. While further studies are warranted to determine the precise role of Beclin 1 activation/phosphorylation by tumor-suppressor signaling molecules in mediating their tumor-suppressor function, it is notable that the overlapping requirement of specific post-translational modifications of Beclin 1 for its autophagy and tumor-suppressor activity may suggest a mechanistic link between these two functions, although more general effects on regulation of Vps34 activity in other trafficking events cannot be ruled out.

5.3. Cell non-autonomous role of autophagy in the cell cycle progression

A decrease in the proliferation of the germline progenitor pool could be due to lack of cell survival, precocious differentiation or a slowdown in the progression of the cell cycle. We considered all three possibilities for bec-1, as well as for other autophagy genes, such as atg-16.2, atg-18, and several others by RNAi. We established that BEC-1/Beclin1 or autophagy genes were not required for cell survival, or for the decision to transition from proliferation (mitosis) to differentiation (meiosis) in the germ cells. We found that BEC-1, ATG-16.2, and ATG-18 were all required for normal cell cycle progression. As these three proteins function in very different steps of the
autophagosomal biogenesis, we conclude that it is the BEC-1 autophagy function that is required for stem cell progenitor proliferation. This is further supported by the observation that retromer genes did not affect germ cell proliferation. However, it is still possible that other functions of autophagy genes are involved, for example, we have shown that in C. elegans, autophagy genes are required for lipid homeostasis (Lapierre et al., 2013). We tested this hypothesis with supplementation of oleic acid in the \textit{bec-1} mutants diet, and analyzing whether an increase in lipid synthesis could rescue the \textit{bec-1} lack of proliferation phenotype. We found this not to be the case (data on Appendix \textbf{FIG. A1}). Our data suggests that BEC-1/Beclin1 may be required for the G2/M phase progression. A remaining question is whether this requirement for BEC-1 is also cell non-autonomous. One hint that this may be the case, is our observation that the M-phase index was no longer decreased in animals that were \textit{bec-1} RNAi depleted in the \textit{rrf-1} strain, as shown in Chapter 2 (\textbf{FIG. 2.9 B}).

The role of IIR signaling has been also previously shown in the Drosophila germ line, where progenitor germ cell differentiation is regulated cell non-autonomously by somatic IIR signaling (Gancz and Gilboa 2013). Moreover, in our previous work, our laboratory found that BEC-1 is required for retrograde transport of MIG-14/Wntless cargo protein from endosomes to Golgi, ensuring the proper localization of the cargo protein (Ruck, Attonito et al. 2011). Interestingly, recent research shows that autophagy may have a partially divergent function in the secretion/expansion of the cytoplasmic constituents instead of their degradation (reviewed in (Ponpuak, Mandell et al. 2015)). Autophagy was shown to be required for the unconventional secretion of several cytosolic proteins that lack signal peptide sequences, such as Acb1 (acyl coenzyme A-
binding protein), interleukin (IL)-1β, and high mobility group B (HMGB)1 (Duran, Anjard et al. 2010, Duran, Pierre et al. 2010, Lee, Shin et al. 2010, Manjithaya, Anjard et al. 2010). In unconventional secretion, autophagosomes enclose the substrate and, instead of fusing with lysosomes for degradation, they fuse with the plasma membrane for secretion (Duran, Anjard et al. 2010, Manjithaya, Anjard et al. 2010, Dupont, Jiang et al. 2011). Additionally, it was shown that in colonic goblet cells, proteins involved in initiation and elongation of autophagosomes were required for efficient mucus secretion (Patel, Miyoshi et al. 2013). This new avenue of autophagy-dependent secretion may provide an interesting mechanism for the cell non-autonomous role of autophagy genes in germ cell proliferation. Our analysis may suggest the possibility that autophagy affects the expression/delivery of a particular ligand or ligands. It would be interesting to assess if autophagy is required for the function of two known C. elegans insulin-like ligands, ins-3 and ins-33, that promote germline proliferation through the DAF-2/IIR signaling in canonical PI3K pathway, inhibiting DAF-16/FOXO.

Another possible mechanism for autophagy in its cell non-autonomous regulation of cell proliferation would be that autophagy is required to internalize the signal that is coming from surrounding tissues. Such a phenomenon was implicated in the survival of dormant ovarian cancer cells. In this example, the tumor suppressor Aplasia Ras homolog member I (ARHI/DIRAS3) was shown to be downregulated in multiple malignancies, including ovarian cancer and its expression was shown to slow proliferation, inhibit motility, produce tumor dormancy, and induce autophagy (Lu, Yang et al. 2014). ARHI is required for autophagy-mediated cancer cell arrest and inhibits signaling through PI3K/Akt and Ras/MAP, by enhancing internalization and degradation
of the epidermal growth factor receptor (EGFR). Downregulation of PI3K/Akt and Ras/ERK signaling decreases the phosphorylation of FOXO3a, and mediates its nuclear translocation. Nuclear FOXO3a, induces ATG4 and MAP-LC3-I, and also the expression of Rab7. Thus, ARHI regulates autophagy in at least three different levels: it induces autophagy through the decrease in Akt/TOR signaling, enhances the expression of LC3 and ATG4 during membrane elongation, and upregulates Rab7 to enhance the fusion of autophagosomes and lysosomes. This example demonstrates how internalization of cell non-specific signals, such as growth factors, can regulate autophagy (Lu, Yang et al. 2014). We could propose that a similar mechanism is at play in the control of germ cell proliferation by the different autophagy genes, and this would explain why we see different interactions with the several pathways involved in germ cell proliferation.

5.4. Role of vesicular trafficking in the progenitor cell proliferation

Although, we show that BEC-1/Beclin1-dependent function and that of other autophagy genes are required for germ cell proliferation, we cannot exclude the possibility that an endocytic function of BEC-1 is a contributing factor. It may be that the bec-1 loss of function phenotype results from a combination of defects in multiple functions of BEC-1. For example, the disorganization of the germline plasma membrane observed in bec-1 mutants (FIG.2.2 C) may be due to another function of BEC-1, as this was not clearly observed in the loss of function phenotypes of other autophagy genes.
At this point, we do not know if the anatomical focus of BEC-1 for this phenotype is also cell non-autonomous.

In Drosophila, defects in two core components of the vesicle trafficking machinery, syntaxin Avalanche (Avl) and Rab5, resulted in the cellular accumulation of membrane proteins, including the Notch receptor, and caused epithelial cell polarity defects, coupled with overproliferation defects and tumors (Lu and Bilder 2005). This reveals a critical role for endocytic traffic in the control of both apico-basal polarity and cell proliferation. Since, our experiments show that lack of BEC-1 by RNAi resulted in drastic defects in membrane polarity, it would be interesting to analyze if this membrane defects are caused by the BEC-1 autophagic function or if it is due to a requirement for the endocytic function of BEC-1/Beclin1. However, these mechanisms are not mutually exclusive, since we observed that the lack of late endocytic or lysosomal genes (vps-16, arl-8) also required for germline proliferation (FIG. 3.1).

A recent study in Drosophila also demonstrated that the influence of autophagy on tissue overgrowth depends on the growth inducing stimulus and cell type (Perez, Das et al. 2015). Loss of autophagy gene function decreased tissue growth in a model of Ras mutant eye epithelial cells. However, the loss of autophagy gene function had the opposite effect on Notch induced overgrowth, as it significantly enhanced the growth of the eye epithelium. Interestingly, Hippo-triggered overgrowth indicated that autophagy does not influence the growth of the eye epithelium, but did influence the Hippo-pathway triggered overgrowth of glial cells (Perez, Das et al. 2015).
5.5. Role of secretory autophagy and unconventional protein secretion

An unconventional protein secretion function for autophagy genes was recently demonstrated, with several examples of alternative secretion of cytosolic cargo. For example, several leaderless cytosolic proteins, such as the inactive precursor of proinflammatory cytokine (IL-1β), which undergoes proteolytic activation (Rubartelli, Cozzolino et al. 1990, Schroder and Tschopp 2010), cytoskeletal protein (tubulin) and others, such annexin-I, have been reported to be part of the autophagy dependent secretome (Ohman, Teirila et al. 2014, Wang, Huang et al. 2014). Thus, we considered the possibility that lack of proliferation in autophagy mutants is caused by an alteration of conventional secretion. The conserved extracellular matrix protein, hemicentin (HIM-4) is secreted from skeletal muscle and gonadal leader cells and affects germ line development (Vogel and Hedgecock 2001). It was shown that mutations in him-4, affect the integrity of the rachis, increase formation of multinucleated cells and cause cytokinesis failure in the C. elegans gonad (Vogel and Hedgecock 2001, Vogel, Wagner et al. 2011). However, depletion of the major autophagy regulator BEC-1 did not affect the distribution of HIM-4::GFP fusion protein on the rachis lining in the mitotic region of the gonad (data on Appendix FIG. A.3), suggesting that BEC-1 is not involved in the secretion of HIM-4. However, this experiment did not fully eliminate the possibility that lack of proliferation in bec-1 autophagy mutants is not due to defects in conventional secretion.

Autophagy can provide an alternative trafficking route for proteins or ligands that are essential for the cellular proliferation process. It was shown that during oncogenic signaling, autophagy assists in trafficking of immature receptors to the plasma
membrane (Cleyrat, Darehshouri et al. 2014). At least two putative insulin-like ligands in
C. elegans, INS-3 (neuronal) and INS-33 (hypodermal), modulate germline proliferation
from somatic tissues (Michaelson, Korta et al. 2010); thus, it would be interesting to
investigate the possibility for the unconventional autophagy mechanism being involved
in insulin ligand modification to affect germline proliferation.

Interestingly, autophagy was shown to have other polarized sorting and plasma
membrane organelle biogenesis roles, where autophagy not only provides alternative
trafficking routes for integral membrane proteins to reach the plasma membrane and
carry out their functions, but it also controls biogenesis of complex domains and
organelles at the plasma membrane (Pampliega, Orhon et al. 2013). In the C. elegans
gonad, the asymmetrical distribution of phospholipids on the plasma membrane is
critical for maintaining cell integrity and physiology and for regulating intracellular
signaling and important cellular events, such as clearance of apoptotic cells (Darland-
Ransom, Wang et al. 2008). Since we see membrane polarization defects in the bec-1
mutants (FIG. 2.2) and our previous work showed defects in apoptotic cell corpse
clearance in autophagy mutants (Ruck, Attonito et al. 2011), it would be interesting to
further assess the role of autophagy proteins in the polarization of germline plasma
membrane.
5.6. Role of autophagy in the germcell proliferation during dietary restriction or starvation

Another interesting avenue of research to undertake would be to investigate how autophagy affects proliferation during dietary restriction or starvation. In this work, we showed that the depletion of BEC-1 by RNAi further reduced the germ cell proliferation defect in eat-2 mutants, dietary restricted animals (FIG. 2.6). The enhancement of germline phenotype in eat-2 mutants by bec-1 RNAi, suggests that BEC-1 acts independently from dietary restriction (DR) signaling. However, we do not know if other autophagy genes would act in a similar fashion. Moreover, recent work indicates that the TOR pathway is important for the C. elegans germline progenitor cell accumulation, and RSKS-1/S6K is a key mediator for the germline pool establishment in response to nutrient availability (Korta, Tuck et al. 2012). RSKS-1/S6K cell-autonomously promotes germ line cell cycle progression and inhibits differentiation in response to dietary restriction signaling (Korta, Tuck et al. 2012). Our work suggests that BEC-1-mediated autophagy act in a TOR independent mechanism, since BEC-1 and autophagy genes are required cell non-autonomously to regulate germ cell proliferation and cell cycle progression.

Interestingly, expression of BEC-1/Beclin 1 in 3 tissues: neuronal, muscle or epidermis, partially rescued the bec-1 mutant proliferation defects. Future experiments will determine if and which specific tissues may be required for the BEC-1/Beclin1 and other autophagy genes activity, and their function in germ cell proliferation. Given our current knowledge and understanding of the role of autophagy in cell proliferation, both in normal cells, during development and growth, or uncontrolled proliferation of cancer
stem cells, it is imperative to understand the role of various signaling pathways from surrounding tissues and cellular niches. This multifaceted approach to study how autophagy controls cell proliferation has a high potential to provide us with future mechanisms in the design of specific regulatory and therapeutic treatments for cancer.
Chapter 6

6. Experimental procedures

6.1. C. elegans strains

Standard procedures were used to maintain C. elegans strains as described by Brenner (1974). Temperature sensitive strains were maintained at 15 °C, their progeny were collected as synchronized L1s that hatch-off during a 2 h period (Pepper, Lo et al. 2003), and grown to the desired stage for analysis, unless noted otherwise. Strains used in this study were: wild-type N2 Bristol, VC517: bec-1(ok691)IV/ nT1[qls51]IV,V, QU3: bec-1(ok691)IV/nT1[qls51]IV,V, VC424: bec-1(ok700)IV/nT1[qls51]IV,V, JK3025: gld-1(q485)/hT2[bli-4(e937) let?(q782)qls48], JK 3345: gld-3(q741)/mln1[mls14 dpy-10(e128)]ll, VC893: atg-18(gk378)V, RB2372: atg-16.2(ok3224)ll, GC833: glp-1(ar202)lll, MD701: bcls39[lim-7p::ced-1p::GFP+lin-15(+)]V, OD58: unc-119(ed3) ll; ltl38 [pAA1; pie-1::GFP::PHPLC1resa + unc-119(+)], HZ1683: him-5(e1490) V; atg-2(bp576)X, HZ1684: atg-3(bp412)IV; him-5(e1490)V, HZ1686: bnl11; atg-7(bp511) V; him-5, HZ1687: atg-9(bp564)him-5(e1490)V, HZ1691: epg-8(bp521)I; him-5(e1490)V, GR1318: pdk-1(mg142gf)X, JT709: pdk-1(sa709lf)X, RB759: akt-1(ok525lf) V, VC204: akt-2(ok393lf)X, RB798: rift-1(ok589)I, NL3511: ppw-1(pk1425)I, XA3502: unc-119(ed3)Ill; qals3502[unc-119(+)+pie-1::YFP::Imn-1+pie-1::CFP::H2B], ET113: unc-119(ed3)lll; ekls2 [pie-1p::GFP::cyb-1+unc-119(+)], JH2194: unc-119(ed3)Ill; axls1571 [pie-1p::GFP::par-5(orf)::par-5 3'UTR+unc-119(+)], BS3392: gld-2(q497) gld-1(q485)/hT2 [dpy-18(h662)]ll; unc-32(e189) glp-1(q175)/hT2 [bli-4(e937)]lll. GC833: glp-1(ar202)Ill, CB4037: glp-1(e2141) ll, CB1372: daf-7(e1372)lll, RB22589:
Strains generated for this study:

QU20: glp-1(e2141); bec-1(ok691)IV/nT1[qIs51]IV,V, QU253: bec-1(ok691)IV/nT1[qIs51]IV,V; izEx6[pBEC::bec-1(+), pTG96[sur-5::GFP]], QU254: bec-1(ok691)IV; izEx6[pBEC::bec-1(+), pTG96[sur-5::GFP]], QU251: atg-16.2(ok3224)II; glp-1(ar202)III, QU251: glp-1(ar202)III; atg-18(gk378)V, QU25: glp-1(ar202)III; bec-1(ok691)IV/nT1[qIs51]IV,V, BT24: rhIs23 [GFP::him-4] III, EB2589: bec-1(ok691) IV/nT1[qIs51] IV,V; dzIsTi2 [pie-1p::bec-1::mCherry::tbb-2 3'UTR + NeoR], EB2590: bec-1(ok691) IV/nT1[qIs51] IV,V; dzIsTi3 [pie-1p::bec-1::mCherry::tbb-2 3'UTR + NeoR], EB2591: bec-1(ok691) IV/nT1[qIs51] IV,V; dzIsTi4 [pie-1p::bec-1::mCherry::tbb-2 3'UTR + NeoR]

6.2. RNA interference experiments (RNAi)

HT115 E. coli that express dsRNA for the corresponding target gene were obtained from the Ahringer and Vidal libraries (Kamath, Fraser et al. 2003, Rual, Ceron et al. 2004). Every clone was sequence verified and seeded on carbenicillin/IPTG plates. Parent animals were fed dsRNA expressing bacteria as L4, and F1 progeny were analyzed as young adults (with up to 2 eggs per gonad), unless noted otherwise. Bacteria with the empty RNAi expression vector L4440 or expressing dsRNA against gfp, gfp(RNAi), were used as negative controls.
Table 1. RNAi clones used in this study

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<th>Rev Primer Seq</th>
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</table>

6.3. Staining and Immunofluorescence

Germline phenotypes were analyzed using DAPI (Vectashield) staining on whole worms (Pepper et al., 2003) and dissected gonads (S.Crittenden and J.Kimble, Methods in molecular Bio). Gonads were extracted in phosphate-buffered saline solution (PBS) containing 0.25mM levamisole (R. Francis et al., 1994). Fixation was done 2% PFA for 10 min, permeabilized with 0.1% Triton X-100 in PBSB, blocked in PBSB for 30 min.

For the M- phase index determination, gonads were stained with anti-Phospho Histone H3 marker (Millipore) (1:500), at least for 4 hours, followed by 3 X 15 min
washes in PBSB and stained with a goat-anti-rabbit secondary antibody stain Alexa Fluor 488 or Alexa Fluor 564 (5μg/ml) (Life Technologies), for at least 2 hours, followed by 3 X 15 min washes in PBSB. As a final step, worms were DAPI stained (using Vectashield) and mounted on the 3% agarose slides for microscopy.

6.4. 5-ethyl-2-deoxyuridine (EdU) labeling

NGM plates were seeded with small amount of OP50 bacteria (50-100 μl) and grown overnight. Bacteria on NGM plates were killed by cross-linking (0.5J/sec for 10 min), and 1-12 h later, an EdU solution (stock 300 μM) that covers the entire lawn of killed bacteria was applied. After the EdU solution dried up, animals were placed on NGM plates and fed the dead bacteria plus EdU solution for 30 min followed by the immediate gonadal dissection. Gonads were extracted in 0.25μM levamisole, fixed using 3.7% PFA for 10min, permeabilized with 0.1% Triton X-100 in PBSB, blocked in PBSB for 30 min and processed using the Click-IT EdU reaction (Molecular Probes), according to the manufacturer’s protocol. Following the Click-IT reaction incubation, the gonads were antibody and DAPI stained before imaging.

6.6. Microscopy

For general worm handling, a Leica MZ16F stereo fluorescence scope was used. DIC microscopy of whole animals was performed using Nomarski Zeiss ApoTome microscope optics. To image extracted fluorescence immunostained and DAPI-stained
gonads (extracted and whole), a Leica TCS-SP5 laser-scanning confocal microscope was used. For the analysis of the S-phase and M-phase indices and localization profile of markers expressed in the germline, Z stacks were taken at 1.5 μm steps. Images on the confocal microscope were collected by a PMT (photomultiplier tube) detector and processed in 3-D projection mode, converted to TIFF format for further analysis.

6.7. Germline Proliferation Analysis

To determine the size of proliferative/stem cells population in the distal region of the gonad, we manually counted the number of nuclei in the proliferative zone, from the distal tip of the gonad to the transition zone, and included all the germ cells. The counting was done from Z-stacked images from whole animals or extracted gonads, processed in 3-D projection mode using Adobe Photoshop CS3. The mitotic index (M-phase index) was calculated as a ratio of phospho-Histone H3 positive cells in metaphase and anaphase, to the total number of nuclei in the proliferative zone (Maciejowski et al., 2006). The S-phase index was calculated as a ratio of the EdU positive cells, to the total number of mitotic cells. The presence in the cell of any EdU staining (on individual chromosomes or all chromosomes) was scored as an EdU positive cell.

6.8. Brood size experiment

Individual mid/late L4 stage animals were placed onto a plate at 20°C and allowed to self-fertilize. Mothers were transferred to new plates every 24 hours, until
they either stopped producing offspring or died. All the progeny were counted from all plates. After 24 h, unhatched eggs were counted for each plate. The progeny that segregated from each parent were counted, including unhatched eggs on all plates.

6.9 miniMos constructs

Transgene of interest (\textit{pie-1p::bec-1::mCherry::tbb-2}) was inserted into the miniMos vector (pCFJ910-neoR) by Gibson assembly. \textit{bec-1(ok691) IV/nT1 [qIs51]} (IV;V) strain was injected with the miniMos-based vector (pDC19) containing \textit{pie-1p::bec-1::mCherry::tbb-2 3'UTR} insert (10 ng/μl) and co-injection plasmids: pGH8–Prab-3:mCherry:unc-54UTR (20 ng/μl), pCFJ90–Pmyo-2:mCherry:unc-54UTR (5 ng/μl), pCFJ104–Pmyo-3:mCherry:unc-54UTR (20 ng/μl), pCFJ601–Peft-3:mos1 transposase:tbb-2UTR (100 ng/μl), pMA122–Phsp16.41:peel-1:tbb-2UTR (10 ng/μl). Three lines of \textit{bec-1(ok691) IV/nT1 [qIs51]} (IV;V); Is[\textit{pie-1p::bec-1::mCherry::tbb-2 3'UTR + NeoR}]

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<th>Genetic position</th>
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<th>Constructs</th>
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\textit{Table 2. miniMos constructs}
Appendix
Figure A.1. Supplementation by oleic acid did not suppress lack of germline proliferation in bec-1 mutant animals.
Quantification of the number of mitotic cells in wild type and bec-1(ok691) that were supplemented with oleic acid, EtOH was used as control. Animals were grown at 15°C and shifted to 20°C, as L3 larvae, and analyzed as young adults.
Results are shown as mean ± SEM (shown as error bars). Significance determined by two-tailed Students T-test: * P≤0.05, *** P≤0.001. Number of analyzed gonads N≥10.
Figure A.2. Expression of BEC-1 from the sheath cells does not rescue proliferation defect in \textit{bec-1} mutants.  
Quantitative analysis of \textit{bec-1}(\textit{ok691}) mutant animals, that express extrachromosomal BEC-1 from sheath cells under \textit{ced-1} promoter, compare to wild type and internal control \textit{bec-1}(\textit{ok691}) mutant animals lacking the array. Animals were grown at 20ºC and analyzed as young adults. Results reflect the average of at least three biological replicates shown as the mean ± SEM (shown as error bars). Significance determined by two-tailed Students T-test: * \(P \leq 0.05\), *** \(P \leq 0.001\); number of analyzed gonads \(N \geq 10\).
Figure A.3. BEC-1 does not affect the distribution of hemicentin rachis lining in the mitotic region of the gonad.
Representative confocal images of mid section of the gonad in wild-type animals treated with RNAi against control (L4440) and bec-1. Animals express GFP::HIM-4 to visualize hemicentin distribution on the plasma membrane. Magnification 630X.
Figure A.4. Inactivation of autophagy genes by RNAi does not disturb GLP-1/Notch receptor distribution on the plasma membrane of the germline.
Representative images of the wild-type and rrf-1 mutant animals stained with anti-GLP-1 antibody and DAPI (DNA stain). Animals were grown at 20°C and analyzed as young adults. Empty vector (L4440) was used as a control.
Figure A.5. Animals with depleted autophagy function are able to enter meiosis.
Representative images of the wild-type animals and animals with depleted bec-1, atg-18, lgg-1 and rme-8 function by RNAi stained with anti-CYE-1 (mitotic marker), anti-HIM-3 (meiotic marker) and DAPI (DNA stain) antibody. Animals were grown at 20ºC and analyzed as young adults.
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<th><strong>Mammalian gene</strong></th>
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<td>WIPI1, WIPI2, WIPI3, WIPI4</td>
<td>Localization of Atg2</td>
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<td>atg-2</td>
<td>ATG2</td>
<td>ATG2</td>
<td>ATG18-interracting protein</td>
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*Table 3. Conserved autophagy genes*
Acknowledgment: This is an Accepted Manuscript of an article published in Autophagy online [January 15, 2013], available online: http://www.tandfonline.com/doi/full/10.4161/auto.22930

**Autophagy genes are required for normal lipid levels in C. elegans**

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Key words: Autophagy, fat storage, lipid metabolism, intestine, Oil-Red-O staining, CARS microscopy, *C. elegans*

Running Title: Autophagy and lipid storage in *C. elegans*
ABSTRACT

Autophagy is a cellular catabolic process in which various cytosolic components are degraded. For example, autophagy can mediate lipolysis of neutral lipid droplets. In contrast, we here report that autophagy is required to facilitate normal levels of neutral lipids in C. elegans. Specifically, by using multiple methods to detect lipid droplets including CARS microscopy, we observed that mutants in the gene bec-1 (VPS30/ATG6/BECN1), a key regulator of autophagy, failed to store substantial neutral lipids in their intestines during development. Moreover, loss of bec-1 resulted in a decline in lipid levels in daf-2 (Insulin/IGF-1 receptor (IIR) ortholog) mutants and in germline-less glp-1/Notch animals, both previously recognized to accumulate neutral lipids and have increased autophagy levels. Similarly, inhibition of additional autophagy genes, including unc-51/ULK1/ATG1 and lgg-1/ATG8/MAP1LC3A/LC3 during development, led to a reduction in lipid content. Importantly, the decrease in fat accumulation observed in animals with reduced autophagy did not appear to be due to a change in food uptake or defecation. Taken together, these observations suggest a broader role for autophagy in lipid remodeling in C. elegans.
INTRODUCTION

Energy in the form of triglycerides (TGs) is required for all metazoans to maintain cellular homeostasis. Following nutrient starvation, a primary cellular response is the induction of macroautophagy, a process that degrades cellular components and recycles amino acids and other molecules critical for cellular survival. At the same time, nutrient starvation results in the mobilization of cellular lipid stores to supply free lipids for energy, thus pointing to regulatory and functional similarities between autophagy and lipid metabolism.

Recent studies have demonstrated links between autophagy and lipid metabolism in vertebrates. Originally, degradation of lipid droplets was thought to take place in the cytosol by resident lipases. However, selective macroautophagy, referred to as lipophagy, has recently been described to be required for the delivery of lipid droplets for lysosomal degradation. Specifically, inhibition of autophagy in cultured mouse hepatocytes, by knockdown of Atg5 or inhibition of autophagy by 3-methyladenine, results in a significant increase in TG storage in lipid droplets. Similar results have been reported using an in vivo mouse model, as autophagy mutant animals have fatty livers.

In contrast to the role of autophagy in hepatocytes, knockdown of Atg5 or Atg7 in 3T3 preadipocytes, results in decreased levels of adipocyte differentiation factors, failure of white adipocytes to differentiate, and consequently an inhibition of TG accumulation in these undifferentiated cells. A decrease in adipose tissue mass is similarly observed in an in vivo mouse model containing an adipose-specific deletion of Atg7. Therefore, autophagy can facilitate lipid breakdown in certain cell types such as the liver but may also enable lipid storage by affecting cell differentiation in adipose tissues.
While autophagy has been proposed to mediate lipolysis via a mechanism involving lipases localized to the autophagosome\textsuperscript{6,7} or the lysosome,\textsuperscript{8} the link between autophagy and lipid storage has so far only been described to occur with cell differentiation.\textsuperscript{4}

The genetics of lipid storage has recently become an area of intense study in the nematode \textit{Caenorhabditis elegans}.\textsuperscript{9-11} Recent studies indicate that modulating lipid metabolism is crucial for long-term survival in \textit{C. elegans}.\textsuperscript{12,13} \textit{C. elegans} do not have a specialized tissue to store fat, however the intestine and the hypodermis (epidermis) are both tissues where many aspects of the regulation of lipid storage appear to be conserved.\textsuperscript{10} Young larvae respond to starvation by entering an alternative developmental program resulting in a dauer animal, until favorable conditions are resumed.\textsuperscript{14} Entry into the dauer stage is associated with reduced insulin-like signaling, an increase in lipid storage, and metabolic changes that enable long-term survival in harsh environmental conditions. \textit{C. elegans} larvae with compromised insulin-like signaling (e.g., \textit{daf-2}/\textit{IIR} mutants) constitutively enter dauer stage, whereas mutant adults are long-lived.\textsuperscript{14} We have previously shown that many of the metabolic and physiological changes associated with the constitutive dauer phenotype of \textit{daf}-2 mutants do not occur in animals that lack autophagic activity.\textsuperscript{15} In particular, dauers, in which autophagic flux has been compromised, show reduced levels of stored neutral lipids raising the possibility that autophagy can directly facilitate lipid storage, at least in this specialized larva.\textsuperscript{15} We, and others, also have shown that autophagy is induced and required for mediating the increased lifespan of adults with compromised insulin-like signaling, suggesting that autophagy is part of the anti-aging effects observed in compromised Insulin/IGF-1 (IIS) signaling mutants.\textsuperscript{15-18}
Another signaling pathway recently linked to lipid metabolism is that of germline-less *glp-1*/*Notch* mutants. Similar to *daf-2* mutants, *glp-1* mutant animals also display an increase in lipid contents\(^1\)\(^9\),\(^2\)\(^0\) and increased autophagy levels.\(^6\) While lipid breakdown and autophagy have been linked in germline-less *glp-1* animals via the lipase LIPL-4,\(^6\) it is unknown whether autophagy plays a direct role in fat storage in *C. elegans* raised under favorable food conditions.

Here we showed that *bec-1*/VPS30/ATG6/BECN1 and other autophagy genes were required for lipid storage in normally developing *C. elegans*. Specifically, we found that mutants of the autophagy gene *bec-1* failed to accumulate substantial neutral lipids during development, producing extremely lean adult animals. Similarly, RNA inactivation of *bec-1*, *vps-34*, *lgg-1* and *unc-51* also significantly reduced fat content in adult animals. Moreover, both *daf-2* receptor mutants and germline-less *glp-1* animals failed to increase lipid levels during development when autophagy was impaired, consistent with a critical role for autophagy in lipid storage. Notably, mutants with compromised autophagy had normal pharyngeal pumping, food uptake and defecation during development. In addition, mutants with impaired retrograde transport contained normal levels of lipids, implying that the cellular process of autophagy plays a specific role in lipid remodeling. Taken together, these data suggest that the autophagy process plays a role in lipid storage in *C. elegans* under favorable food conditions. As the *C. elegans* intestine is a terminally differentiated tissue, we propose that the link between autophagy and fat storage might be of a more direct nature and may not inherently involve cell differentiation.
RESULTS

Role of bec-1 in determining fat levels in wild-type animals

To determine whether lipid storage is modulated by autophagy in C. elegans, we evaluated the effect of impairing autophagy on lipid content using a mutant of the autophagy gene bec-1. bec-1 is the C. elegans ortholog of yeast VPS30/ATG6 and mammalian BECN1/Beclin 1. We assayed homozygous bec-1(ok691) mutants, which have impaired autophagy.21,22 The bec-1 allele ok691 is a molecular null mutation, yet bec-1 homozygous mutants are rescued maternally and may reach adulthood, but are sterile and die as young adults.22 Considering a recent review on the limitations of various lipid analysis methods in C. elegans,23 we used Oil-Red-O staining for our initial analysis.24 Oil-Red-O staining is a fixative-based dye that stains lipids and correlates with TG mass measurements obtained using quantitative biochemical methods.20 When using this method, we found a profound decrease in the amount of Oil-Red-O stained neutral lipids in day 1 adult bec-1(ok691) homozygous mutants compared to wild-type animals (Fig. 1A).

To complement this analysis, we also used the more recent coherent anti-Stokes Raman (CARS) microscopy method to assess lipid levels in C. elegans.25 CARS microscopy allows for direct quantitative analysis of lipid storage without the use of labels. Using CARS microscopy, loss of bec-1 resulted in a very prominent decrease in lipid droplet number and overall lipid content (Fig. 1B and 1C). Looking at the distribution of lipid droplets in the intestine, a shift toward smaller lipid droplets was also apparent in the absence of bec-1 (Fig. S1). This shift in lipid droplet size was also apparent when we stained the bec-1(ok691) homozygous animals with Oil-Red-O (Fig.
The decrease in lipid levels observed in \textit{bec-1(ok691)} mutants was not simply a result of reduced feeding, as the rate of pharyngeal pumping was only slightly decreased in \textit{bec-1} mutant L4 larvae compared to wild type animals and defecation rates or BODIPY uptake were unaffected (\textbf{Fig. S2}). Similarly, \textit{bec-1(ok691)} mutant L4 larvae did not appear to be leaner due to increased energy expenditure as they did not show noticeably increased activity levels compared to wild-type animals (data not shown). Collectively, these results imply that \textit{bec-1/BECN1} may play a direct role in facilitating lipid accumulation in developing \textit{C. elegans}. While our assays showed a decrease in lipid levels after inactivation of \textit{bec-1/BECN1}, they did not allow us to determine whether the decrease in lipid content results from an increase in lipid breakdown, a lack of lipid biosynthesis, or defective recycling and storage of lipids.

\textbf{Role of \textit{bec-1} in the fat accumulation phenotype of \textit{daf-2} and in \textit{glp-1} loss-of-function mutants}

To further investigate a role for autophagy in lipid accumulation, we next examined two mutants known to display increased lipid storage capacity: the Insulin/IGF-1-like receptor \textit{daf-2}\textsuperscript{10,26} and germline-less \textit{glp-1}/Notch mutants.\textsuperscript{20} These mutants also show increased autophagy levels raising the possibility that the autophagy and lipid phenotypes may be interconnected in these animals.\textsuperscript{6,15}

Consistent with previous reports,\textsuperscript{10,20,26} we found that day 1 adult animals carrying either \textit{daf-2(e1370)} or \textit{glp-1(e2141)} loss of function mutations, displayed an increase in lipid content as measured by Oil-Red-O staining, compared to wild-type animals (\textbf{Fig. 1A}). Further analysis of \textit{daf-2(e1370)} or \textit{glp-1(e2141)} mutants by CARS
microscopy confirmed that lipid content in the intestinal cells was significantly increased in either single mutant (Fig. 1B and 1C). Consistent with these observations, intestinal lipid droplets in either glp-1 or daf-2 mutant worms tended to be larger than those found in wild-type animals (Fig. S1). Overall, the distribution of lipid droplets was predominantly intestinal, but a layer of small lipid droplets was detectable in the hypodermis of these animals (data not shown).

We next introduced the bec-1(ok691) mutation into daf-2 and glp-1 loss-of-function mutants to investigate the role of bec-1/BECN1 in lipid accumulation in these mutant backgrounds. We found that daf-2(e1370); bec-1(ok691) and glp-1(e2141); bec-1(ok691) double mutants no longer showed an increase in Oil-Red-O staining compared to single daf-2(e1370) and glp-1(e2141) mutants, respectively (Fig. 1A). Similarly, we found that day 1 adult daf-2(e1370); bec-1(ok691) and glp-1(e2141); bec-1(ok691) double mutants displayed a reduction in the number of lipid droplets, the size of the droplets, as well as a decrease in the lipid contents when analyzed by CARS microscopy (Fig. 1B and 1C).

Gene inactivation by feeding bacteria expressing dsRNA against bec-1/BECN1 also decreased normal fat contents in a wild-type animals and drastically reduced the accumulation of lipids normally observed in daf-2(e1370) and glp-1(e2141) mutants (Fig. 2 and 3). Similar results were observed with a different glp-1 loss of function allele, bn18 (Fig. S6A), as well as an additional daf-2 allele, m41 (Fig. S7), which also induces accumulation of lipids. We found that food uptake or defecation in glp-1 or daf-2 mutants remained unchanged after RNAi against bec-1/BECN1 (Fig. S4-S6). Taken together, our analysis of daf-2 and glp-1 animals confirmed that bec-1/BECN1 plays a
critical role in fat homeostasis, and supported a link between lipid metabolism and autophagy in these mutants, as observed in wild-type animals.

**Role of other autophagy and retromer genes in lipid accumulation**

In addition to its role in autophagy, BEC-1/BECN1 has been shown to function in a complex with the Class III phosphatidylinositol-3-kinase (PtdIns3K) VPS-34, the functional ortholog of yeast Vps34, in *C. elegans* retrograde transport from endosome to Golgi. To determine whether the autophagy process could be a determining factor in the reduced lipid levels observed in *bec-1/BECN1* mutants, we inhibited other genes with effects in different steps of the autophagy process. To do this, we fed wild-type N2 animals, from hatching and throughout development, bacteria expressing dsRNA against *bec-1/BECN1*, *vps-34/VPS34/PIK3C3*, *unc-51/ATG1/ULK1*, and *lgg-1/ATG8/MAP1LC3A (LC3)*. Analysis in yeast and in mammalian cells have shown that the Atg1/ULK1 protein kinase acts in the induction of autophagy, Vps34/PIK3C3 acts together with Vps30/Atg6/BECN1 at the nucleation step, and Atg8/LC3 is part of the protein conjugation system for vesicle completion. Knockdown of autophagy genes was previously shown to either cause abnormal daf-2 dauers or to limit the formation of GFP::LGG-1 puncta, a commonly used assay to assess autophagy in *C. elegans*. Indeed, elevated GFP::LGG-1 puncta was associated with the lack of daf-2/IGF-1 receptor activity and the lack of a germline. Like *bec-1* deletion mutants, RNAi against *vps-34/VPS34/PIK3C3*, *unc-51/ATG1/ULK1*, and *lgg-1/ATG8/LC3* resulted in a decrease in lipid contents in the intestine of day 1 wild-type animals (**Fig. 2A, 2C and 3A**). More importantly, wild-type N2 animals treated with RNAi against *bec-1/BECN1*,
vps-34/VPS34/PIK3C3, unc-51/ATG1/ULK1, and lgg-1/ATG8/LC3, showed no decrease in food uptake or defecation, and yet these animals also displayed a decrease in lipid levels (Fig. 2, 3, and S3). Similarly, a decrease in lipid levels was observed in day 1 old daf-2(e1370) mutants (Fig. 2A, 2D) following RNAi-mediated knockdown of bec-1/BECN1 and vps-34/VPS34/PIK3C3 and in glp-1(e2141) animals, following RNAi against bec-1/BECN1, vps-34/VPS34/PIK3C3, unc-51/ATG1/ULK1, and lgg-1/ATG8/LC3 (Fig. 3A, 3D). A decrease in lipid content was also observed in glp-1(bn18) mutants after RNAi against bec-1/BECN1, vps-34/VPS34/PIK3C3, unc-51/ATG1/ULK1, and lgg-1/ATG8/LC3 (Fig. S6). Importantly, no significant change was observed in food uptake or defecation for daf-2 or glp-1 mutant animals after RNAi treatment against bec-1/BECN1, vps-34/VPS34/PIK3C3, unc-51/ATG1/ULK1, and lgg-1/ATG8/LC3 (Fig. S3-S6). Quantification of Oil-Red-O staining confirmed a significant reduction in overall lipid content as a result of RNAi against several autophagy genes in daf-2(e1370) or glp-1(e2141) animals (Fig. 2D and 3D). Taken together, these observations indicated that autophagy is required for adequate lipid content in C. elegans, suggesting a more dynamic role for autophagy in lipid homeostasis.

As we have previously shown that bec-1/BECN1 and vps-34/VPS34/PIK3C3 act in the retrograde transport from endosome to Golgi,\textsuperscript{22} we decided to further investigate the role of retromer genes in lipid accumulation. Since we did not observe a decrease when we stained wild-type N2, daf-2(e1370) or glp-1(e2141) animals that had been treated with RNAi against rme-8/RME-8 or vps-35/VPS35 (Fig. 2B and 3B), we also analyzed rme-8(b1023) mutants by CARS microscopy (Fig. S8). Interestingly, although rme-8 and vps-35 mutants strongly affect retromer function,\textsuperscript{29-31} these strains did not
show a reduction in lipid content when compared to control animals (Fig. S8), and again had no significant change in food uptake or defecation (Fig. S9). Thus, these data suggest that lipid accumulation does not depend on retrograde transport. Instead, our findings point to a novel requirement for the autophagy process regulating lipid levels, opening the possibility that lipid homeostasis is tightly linked to the autophagy/lysosomal pathway.

**DISCUSSION**

Autophagy has recently been linked to lipid metabolism, for instance by mediating cell differentiation of adipocytes to ensure lipid storage.\(^4\),\(^32\) Here, our studies suggest that the regulation of lipid homeostasis in *C. elegans* requires functional autophagy. Specifically, we observed that the loss of *bec-1/BECN1* was sufficient to significantly impair lipid accumulation in the animal’s terminally differentiated intestinal cells. Likewise, knockdown of other autophagy genes working in different steps of the autophagy process,\(^27\) such as *vps-34/VPS34/PIK3C3*, *unc-51/ATG1/ULK1* and *lgg-1/ATG8/LC3* led to a decrease in lipid content. Importantly, the reduced lipid content was not due to reduced pharyngeal pumping, nutrient absorption or defecation. It remained unclear, however, whether the decrease in lipid content resulted from an increase in lipid breakdown, a lack in lipid biosynthesis, or defective recycling or storage of lipids. While future experiments are needed to differentiate between these possibilities, one hint that the decrease in lipid accumulation may not be due to an increase in the breakdown of lipids, is that *glp-1* animals that have been fed RNAi against autophagy genes display reduced lipase activity.\(^6\) Another possibility is an
increased rate in the uptake of lipids by the oocyte, however, knockdown of bec-1/BECN1 reduces the uptake of the yolk protein VIT-2::GFP into oocytes.\textsuperscript{21} Thus the decrease in lipid levels, in the intestine, is not likely due to an increased rate of uptake by the growing oocytes. As some autophagy genes, including bec-1/BECN1, play additional roles in endocytosis,\textsuperscript{22} and this process has been linked to lipid storage,\textsuperscript{33} it is possible that impairing both autophagy and endocytosis contribute to the decrease in lipid levels. However, we found that inactivation of retromer genes did not decrease the accumulation of lipids. Since inhibition of the autophagy process at different steps gives similar results, and inhibition of retromer genes did not have a significant effect, we suggest that autophagy is critical for lipid accumulation in \textit{C. elegans}, although we have not formally excluded that bec-1/BECN1, and the other tested genes, serve non-autophagic functions in lipid homeostasis.

These novel observations add insights into earlier research on the link between autophagy and lipid homeostasis. Previous \textit{in vitro} studies using a preadipocyte mouse cell line have shown that inhibition of autophagy results in a decrease in the accumulation of triglycerides (TGs) that occurs during the differentiation process into adipocytes. In \textit{Atg7} knockout mice, the effects of the loss of autophagy on adipocyte differentiation are more complex, in addition to the decrease in white adipose mass, the tissue displayed features of brown adipocytes.\textsuperscript{4,5} These two types of adipocytes have different functions; white adipocytes store large amount of TG for breakdown, whereas brown adipocytes have a higher number of mitochondria and an increase in the rate of fatty acid ß-oxidation. While showing a role for autophagy in lipid accumulation, these mammalian studies did not address whether the decrease in lipid storage observed in
autophagy-deficient animals could be secondary to a failure to differentiate adipocytes, and instead represent a direct effect on lipid storage. Since *C. elegans* do not formally have a white or brown adipocyte equivalent, we could not address whether autophagy has a similar effect in the intestine of these invertebrate animals. However, we observed that inhibition of autophagy in a fully differentiated tissue impaired fat levels while not affecting the food intake or energy expenditure of the animal. Future experiments should address how autophagy mediates these effects on lipid metabolism, for instance by regulating mitochondria number and the rate of fatty acid ß-oxidation, as observed in mammalian systems. Similarly, it will be interesting to address whether autophagy regulates lipid metabolism in differentiated adult adipocytes.

While our experiments provided evidence that autophagy was required for lipid accumulation, autophagy is typically viewed as a catabolic process. Thus, an apparent question brought up by these results is whether active degradation of cellular components by autophagy is necessary for lipid storage, biosynthesis or remodeling via the same process. Our data is consistent with a possible, albeit controversial, mechanism in which lipid storage may be driven by the ability of cells to rapidly recycle material, such as certain fatty acids via autophagy. One alternate possibility is that by-products of lipid breakdown resulting from autophagy may be essential for storing lipids by providing raw materials needed (e.g., building blocks for membrane formation) or by indirectly stimulating lipogenesis via signaling. Another possibility is that proper mitochondrial function in lipid metabolism relies on a continual influx of fatty acids from autophagy-related lipolysis, or autophagy flux could be important for the function of a
regulator of lipid storage or biosynthesis. Future experiments are needed to address how autophagy may modulate lipid accumulation in *C. elegans*.

The apparent benefit for cells to dynamically recycle fatty acids between processes of lipogenesis and lipolysis are still to be fully investigated. However, we note that several healthy, long-lived longevity mutants, such as *daf-2/IIR* and *glp-1/Notch* mutants, display autophagy-dependent increases in both intestinal lipolysis activity\(^6\) and lipid contents.\(^{10,20,26}\) This is also the case in dauer larvae, in which fine-tuning of lipid partitioning suggests that particular types of lipid breakdown or levels influence the capacity of worms to survive under unfavorable conditions.\(^{13}\) To provide a benefit to the organism, however, it appears that concomitant lipogenesis may be required to prevent lipid store depletion, as seen in several *daf-2* strains.\(^{26}\) We previously published that the lack of *bec-1/BECN1* activity decreased the accumulation of lipids that occurs in *daf-2* dauers.\(^{15}\) Whereas it is unclear whether the effects on lipid homeostasis in *daf-2* mutant dauers and *daf-2* mutant long-lived adults are mediated by identical mechanisms, our studies show that autophagy plays a role in both. In conclusion, since lipid breakdown and lipogenesis may coexist in the same tissue in *C. elegans*, we propose that dynamic lipid remodeling via autophagy plays an important role not only during animal development but also to ensure survival during adulthood by guaranteeing optimal lipid partitioning and energy metabolism.
ACKNOWLEDGMENTS

We thank all Hansen and Meléndez lab members for helpful discussions and comments on the manuscript. We thank Lana Tolen and Zahava Rubel in the Meléndez lab for their initial studies on the role of bec-1 in lipid storage. We thank Drs. Cathy Savage Dunn and Hannes Bülow for comments on the manuscript, and Dr. Nathalia Holtzman for advice on image processing and quantification.

This work was also supported by an NIH/UCSD grant to LL (grant number P50 AG005131-29), a grant from the Nevada INBRE Program of the National Center for Research Resources to TTL, an R01 grant from the National Institute of Aging to MH (grant number R01 AG038664), and a National Science Foundation Research Initiation Award and a National institute of Aging supplement award to AM. MH and AM are both Ellison Medical Foundation New Scholars in Aging.
MATERIAL AND METHODS

Strains

The following strains were used in this study: N2 (wild-type/WT), CF1903: *glp-1*(e2141), GC888: *glp-1*(bn18), and the following strains were obtained from the *Caenorhabditis Genetics Center*: CB1370: *daf-2*(e1370), DR1564: *daf-2*(m41), and VC517: *bec-1*(ok691), KN555: *vps-35*(hu68), and DH1206: *rme-8*(b1023).

The following strains were made for this study: QU20: *glp-1*(e2141); *bec-1*(ok691)/nT1, QU36: *daf-2*(e1370); *bec-1*(ok691)/nT1.

Abbreviations of genes, gene products and compounds used in this study

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Compounds

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<tr>
<td>C12 BODIPY</td>
<td>(D3823, Invitrogen) used to determine food uptake in vivo</td>
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<tr>
<td>Sodium Azide</td>
<td>(S227, Fisher Scientific) used to paralyze animals for imaging</td>
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Experimental conditions

Animals were raised on NGM agar plates containing standard *E. coli* food source OP50 at permissive temperature 15°C and changed to the restrictive temperature of 25°C, as L4 larvae. Animals were assayed 1 day later as 1 day-old adults. The same population
was assayed for pharyngeal pumping, defecation rates, BODIPY uptake and stained with Oil-Red-O.

**Pharyngeal Pumping Rates**
Pharyngeal pumping rates were measured in 1 day-old adults under a dissecting scope in 20 second intervals and pumping rate (pumps/minute) were determined in at least 10 animals per trial for each of two trials analyzed. Prior to measurements taken, animals were incubated at room temperature (22°C) for 1 hour. ANOVA comparisons (performed with the software GraphPad Prism 5.0) were made using control animals as stated.

**Defecation Rates**
Defecation rates were determined by measuring the average of 2 consecutive cycles\(^{34}\). Prior to measurements taken, animals were incubated at room temperature (22°C) for 1 hour. At least 10 animals were analyzed for each trial and the values represent the combined average of 2 trials. ANOVA comparisons (performed with the software GraphPad Prism 5.0) were made using control animals as stated.

**BODIPY C12 Uptake Assay**
1 day-old adults were placed on 2cm NGM OP50 *E. coli* seed with 100ul of 5uM C1-BODIPY-C12 (D3823, Invitrogen) diluted in phosphate buffer saline (PBS).\(^{33}\) Animals were fed BODIPY for 1 hour at room temperature (22°C), collected and washed with 1X phosphate buffered saline (PBS), mounted and immobilized on 2% agarose pads in 5ul
M9 buffer containing 25 mM sodium azide. Using a magnification of 160X, fluorescent images were taken using AxioCam Zeiss Imager A1. Fluorescence was quantified using Image J software. On average, 30 worms per trial were quantified (the values represent the combined average of 3 trials). ANOVA comparisons (performed with the software GraphPad Prism 5.0) were made using control animals as stated.

**Oil-Red-O lipid staining and quantification**

Lipid levels were analyzed using Oil-Red-O staining of fixed animals as previously described. Quantification of Oil-Red-O staining was performed as previously described. On average 50 animals were quantified for each trial (values represent the combined data from 3 trials), using Image J software, representing a total population of 100 animals.

**CARS microscopy and lipid quantification**

Coherent anti-Stokes Raman Scattering (CARS) microscopy was performed using the following parameters: the signal (924.2 nm) and idler (1255 nm) outputs of an optical parametric oscillator (OPO, Levante Emerald, Berlin, Germany) were used as the pump and Stokes beams, respectively, to produce a frequency difference of 2851 cm\(^{-1}\). The OPO was synchronously pumped by the second harmonic output (532 nm) of a mode-locked Nd:YVO4 laser (HighQ Laser, Austria). The pump and Stokes beams were inherently synchronized and collinearly overlapped at the exit port of the OPO. The laser beams were passed through a laser scanner (C1plus, Nikon, Melville, NY) and focused with a 60x IR objective into the sample. The combined laser power was
attenuated with neutral density filters to 66 mW at sample. Epi-reflected signal was directed into a multi-channel detector, spectrally separated with dichroic mirrors, selected with bandpass filters (Semrock, Lake Forest, IL), and detected with red-sensitive photomultiplier tubes (R10699, Hamamatsu, Japan). Bandpass filters for autofluorescence and CARS signal were 510/42 nm and 736/128 nm, respectively.

Images were acquired at 10 seconds per frame and presented as 3-D stacks of approximately 30 frames taken at 1-micron increment along the vertical axis. Image analysis was performed post-acquisition using NIH ImageJ software. Quantification was performed on 4 stacks (3 µm apart) per animal encompassing the intestine cells. ANOVA statistical analyses of lipid droplet number, size and lipid content were performed using the software GraphPad Prism 5.0. Wild-type and daf-2(e1370) mutants were raised at the permissive temperature, while glp-1(e2141) animals were raised at the restrictive temperature of 25°C.

RNAi treatments

To circumvent defects that occur during embryonic development, L1 larvae were collected as they hatched and transferred to plates seeded with HT115 E. coli that express dsRNA for the corresponding target gene. Bacteria expressing specific dsRNA were obtained from the Ahringer and the Vidal libraries. Only healthy individuals were assayed following RNAi treatment.
REFERENCES


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FIGURE LEGENDS

Figure 1. Loss of bec-1 impaired lipid storage in C. elegans.

(A) Representative images of fixed one day-old adult animals stained with Oil-Red-O with the following genotypes: N2 wild-type (WT), glp-1(e2141), daf-2(e1370), and bec-1(ok691), glp-1(e2141);bec-1(ok691) and daf-2(e1370); bec-1(ok691) (Magnification, 160-fold). This experiment was repeated three times with similar results.

(B) CARS micrographs of lipid droplets in one day-old adult N2 wild-type (WT), and glp-1(e2141), daf-2(e1370), and bec-1(ok691) single mutant animals, followed by the glp-1(e2141); bec-1(ok691) and daf-2(e1370); bec-1(ok691) double mutant animals (Magnification 240-fold). This experiment was repeated once with similar results.

(C) Quantification of lipid droplet number, area size and corresponding lipid content of day 1 adult wild-type (WT) N2 (1), glp-1(e2141) (2), daf-2(e1370) (3), bec-1(ok691) (4), glp-1(e2141); bec-1(ok691) (5) and daf-2(e1370); bec-1(ok691) (6) animals. n=6 for each strain (*P<0.05, **P<0.0001, ANOVA using the control strain wild-type N2). See also Fig. S1 for analysis of lipid drop distribution, and material and methods section for how animals were raised during development in these experiments.

Figure 2. Inhibition of autophagy genes reduced lipid content in wild-type animals and in daf-2(e1370) mutants.

(A) Wild-type N2 (WT) and daf-2(e1370) animals were fed control bacteria or bacteria expressing dsRNA against bec-1/BECN1, vps-34/VPS34/PIK3C3, unc-51/ATG1/ULK1, and lgg-1/ATG8/LC3, and in (B) we show wild-type N2 (WT) and daf-2(e1370) animals fed control bacteria or RNAi against the retromer genes rme-8/RME8, and vps-
35/VPS35. All animals were fed from L1 larvae and throughout larval development. Animals were raised at the permissive temperature and shifted as L4 larvae to the restrictive temperature of 25°C. Animals were fixed and lipid accumulation was determined by staining with Oil-Red-O (Magnification, 160-fold). This experiment was repeated twice with similar results.

(C) Quantification of Oil-Red-O staining of wild-type N2 animals after RNAi treatment against autophagy and retromer genes (n=30 for all treatments). Data (average of three experiments) are identical to data shown in 2C and shown for comparison only (*P<0.05, **P<0.01, ANOVA using the control strain wild-type N2 animals fed L4440 control or target gene RNAi bacteria).

(D) Quantification of Oil-Red-O staining (n=30) of daf-2(e1370) animals after RNAi treatment against autophagy and retromer genes. Data represent the average of three experiments (*P<0.05, **P<0.01, ANOVA comparison using the control strain wild-type daf-2(e1370) animals fed L4440 RNAi bacteria).

**Figure 3. Autophagy genes knockdown decreased lipid content in wild-type animals and in glp-1(e2141) mutants.**

(A) Representative images of wild-type N2 (WT) and germline-less glp-1(e2141) animals fed control bacteria or bacteria expressing dsRNA against autophagy genes bec-1/BECN1, vps-34/VPS34/PIK3C3, unc-51/ATG1/ULK1, and lgg-1/ATG8/LC3. This experiment was repeated twice with similar similar results.

(B) Representative images of wild-type N2 (WT) and germline-less glp-1(e2141) animals fed RNAi against control or retromer genes rme-8/RME-8, and vps-35/VPS35.
Animals were treated with RNAi from embryogenesis throughout larval development. Animals were raised at the permissive temperature and shifted as L4 larvae to the restrictive temperature of 25°C and harvested as 1 day-old adults. Animals were fixed and lipid accumulation was determined by staining with Oil-Red-O (Magnification, 160-fold). This experiment was repeated twice with similar results.

(C) Quantification of Oil-Red-O staining of wild-type N2 animals after RNAi treatment against autophagy and retromer genes (n=30 for all treatments). Data represent the average of three experiments (*P<0.05, **P<0.01, ANOVA using the control strain wild-type N2 animals fed L4440 control or target gene RNAi bacteria).

(D) Quantification of Oil-Red-O staining of glp-1(e2141) animals fed dsRNA expressing bacteria to autophagy or retromer genes. (n=30 for each RNAi experiment). The average of three experiments is shown (*P<0.05, **P<0.01, ANOVA using the strain glp-1(e2141) animals fed L4440 RNAi bacteria).
Figure 1

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A

WT (N2)  
bec-1(ok691)

glp-1(e2141)  
bec-1(ok691)
daf-2(e1370)  
daf-2(e1370); bec-1(ok691)

B

WT  
glp-1  
daf-2  
bec-1  
glp-1; bec-1  
daf-2; bec-1

C

# of droplets

Avg. droplet area (μm²)

Lipid content (AU)

1  2  3  4  5  6

0  100  200  300  400

0  1  2

700  600  500  400  300  200  100

**  *
Figure 2

A

RNAi: Control  
bec-1  vps-34  lgg-1  unc-51

WT (N2)  

daf-2(e1370)

B

RNAi: Control  
rme-8  vps-35

WT (N2)  

daf-2(e1370)

C

Quantification of Oil-Red-O Staining in WT (N2) Animals

D

Quantification of Oil-Red-O Staining in daf-2 (e1370) Mutants
Figure 3

A

RNAi: Control  \( \text{bec-1} \)  \( \text{vps-34} \)  \( \text{lgg-1} \)  \( \text{unc-51} \)

WT (N2)

\( \text{glp-1(e2141)} \)

B

RNAi: Control  \( \text{rme-8} \)  \( \text{vps-35} \)

WT (N2)

\( \text{glp-1(e2141)} \)

C

Quantification of Oil-Red-O Staining in WT (N2) Animals

D

Quantification of Oil-Red-O Staining in \( \text{glp-1(e2141)} \) Animals
Figure S1. Analysis of lipid droplet distribution from CARS microscopy.
Lipid droplet area for N2 wild-type (WT), glp-1(e2141), daf-2(e1370), bec-1(ok691), glp-1(e2141); bec-1(ok691) and daf-2(e1370); bec-1(ok691) animals was plotted. Each bar represents a single worm. Areas were quantified with ImageJ software and approximated to the nearest whole number for presentation purposes.
Figure S2. *bec-1* mutants have a slight decrease in pharyngeal pumping but have no significant change in BODIPY uptake or defecation rates.

(A) Quantification of pharyngeal pumping rates (n=20), (B) defecation rates (n=10) and (C) BODIPY uptake (n=20) for *bec-1* mutants that segregate from the heterozygous parent. Data shown is the average of two trials (*P*<0.05, Student’s t-test comparison using the control strain wild-type N2 animals). The error bars on all graphs represent +/- SEM.
Figure S3. Knockdown of autophagy by RNAi has no significant effect on food uptake, or pharyngeal, and defecation rates.

(A) Quantification of pharyngeal pumping rates of wild-type N2 animals after RNAi against autophagy and retromer genes (n=10 for each RNAi experiment), (B) defecation rates (n=5 for each RNAi experiment) and (C) food uptake as measured by BODIPY staining (n=10 for each RNAi experiment). As previously reported,34-36 wild-type animals have pharyngeal pumping rates of ~250 pumps/minute (Fig S3A), and defecation cycles of ~55 seconds (Fig. S3B). Wild-type N2 (WT) animals treated with RNAi against the autophagy genes: bec-1, vps-34, unc-51, lgg-1, or the retromer genes: rme-8 or vps-35, displayed no significant change in pharyngeal pumping, defecation rates and BODIPY uptake when compared to control RNAi animals. Data shown is the average of two experiments (*P<0.05, **P<0.01, ANOVA comparison using wild-type N2 animals fed RNAi bacteria with control empty vector (L4440). The error bars on all graphs represent +/- SEM.
Figure S4. Knockdown of autophagy but not retromer genes reduces the lipid content of daf-2(e1370) animals without affecting food intake.

Quantification of (A) pharyngeal pumping rates (n=10 for each RNAi experiment), (B) defecation rates (n=5 for each RNAi experiment) and (C) food uptake as measured by BODIPY staining (n=10 for each RNAi experiment) for daf-2(e1370) animals fed control RNAi bacteria or RNAi against autophagy and retromer genes. daf-2(e1370) mutants display a decrease in the rate of pharyngeal pumping (200 pumps/min) when compared to wild-type N2 (250 pumps/min) animals (Fig. S4A), as has been previously reported for class II daf-2 alleles.37 Knockdown of autophagy or retromer genes in daf-2(e1370) mutants had no significant effect on pharyngeal pumping, defecation or BODIPY uptake when compared to daf-2(e1370) animals fed the control dsRNA. Data shown is the average of two experiments (*P<0.05, **P<0.01, ANOVA comparison using daf-2(e1370) animals fed RNAi bacteria with control empty vector (L4440). The error bars on all graphs represent +/- SEM.
Figure S5. Knockdown of autophagy but not retromer genes reduces fat accumulation of glp-1(e2141) animals without affecting food intake.

Quantification of (A) pharyngeal pumping rates (n=10 for each RNAi experiment), (B) defecation rates (n=5 for each RNAi experiment) and (C) food uptake as measured by BODIPY staining (n=10 for each RNAi experiment) of glp-1(e2141) animals after RNAi treatment against control, autophagy and retromer genes. glp-1(e2141) mutant animals displayed similar pharyngeal pumping, defecation and BODIPY uptake to wild-type N2 animals, and there was no significant change in glp-1(e2141) mutants after feeding RNAi against autophagy or retromer genes. The average of two experiments is shown (*P<0.05, **P<0.01, ANOVA comparison using the glp-1(e2141) animals fed RNAi bacteria with control empty vector (L4440). The error bars on all graphs represent +/- SEM.
**Figure S6. Autophagy genes knockdown decreases lipid content in glp-1(bn18) mutants.**

(A) Representative images of wild-type N2 and glp-1(bn18) animals fed dsRNA against autophagy and retromer genes and (B) quantification of Oil-Red-Staining (n=10 for each experiment). (C) Quantification of pharyngeal pumping rates (n=10 for each experiment), (D) defecation rates (n=5 for each experiment) and (E) food uptake as measured by BODIPY staining (n=10 for each experiment). Experiments were repeated with similar results (*P<0.05, **P<0.01, ANOVA comparison using the glp-1(bn18) animals fed RNAi bacteria with control empty vector (L4440). The error bars on all graphs represent +/- SEM.
Figure S7. Autophagy gene knockdown decreases lipid content in *daf-2(m41)* mutants.
Representative images of wild-type N2 and *daf-2(m41)* animals fed dsRNA against autophagy genes during development.
Suppl. Figure 8

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Figure S8. Loss of autophagy but not retromer gene activity affects lipid accumulation. (A) CARS micrographs of lipid droplets in day 1 adult WT, rme-8(b1023), unc-51(e369) animals (Magnification 240-fold). (B) Quantification of lipid droplet number, area size and corresponding lipid content of day 1 adult wild-type (WT) N2, rme-8 (b1023), unc-51(e369) animals; n=6 for each strain (*P<0.05, **P<0.0001, ANOVA comparison using the control N2 strain). (C) Representative photographs of Oil-Red-O staining and (D) quantification of the Oil-Red-O staining for (WT) N2, rme-8(b1023), vps-35(hu68), unc-51(e369) animals. Experiments were repeated at least two times with similar results (*P<0.05, **P<0.01, ANOVA comparison using wild-type N2 animals). The error bars on all graphs represent +/- SEM.
Figure S9. Retromer mutants rme-8 and vps-35, and autophagy unc-51 mutants exhibit normal feeding and defecation rates.

(A) Quantification of pharyngeal pumping rates (n=15 for each experiment), (B) defecation rates (n=10 for each experiment) and (C) food uptake as measured by BODIPY staining (n=30) for (WT) N2, rme-8(b1023), vps-35 (hu68), and unc-51(e369) animals. The average of two experiments is shown (*P<0.05, **P<0.01, ANOVA comparison using wild-type N2 animals). The error bars on all graphs represent +/- SEM.
Bibliography:


