The Recycling GTPase, RAB-10, Regulates Autophagy Flux in Caenorhabditis elegans

Nicholas J. Palmisano
The Graduate Center, City University of New York

How does access to this work benefit you? Let us know!
Follow this and additional works at: http://academicworks.cuny.edu/gc_etds
Part of the Biology Commons, Cell Biology Commons, and the Genetics Commons

Recommended Citation
http://academicworks.cuny.edu/gc_etds/2008

This Dissertation is brought to you by CUNY Academic Works. It has been accepted for inclusion in All Graduate Works by Year: Dissertations, Theses, and Capstone Projects by an authorized administrator of CUNY Academic Works. For more information, please contact deposit@gc.cuny.edu.
The recycling GTPase, RAB-10, regulates autophagy flux

in Caenorhabditis elegans

by

Nicholas Joseph Palmisano

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

2017
The recycling GTPase, RAB-10, regulates autophagy flux in Caenorhabditis elegans

by

Nicholas Joseph Palmisano

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Dr. Alicia Meléndez
Chair of Examining Committee

Dr. Laurel Eckhardt
Executive Officer

Supervisory Committee:

Dr. Cathy Savage-Dunn (Queens College, CUNY)

Dr. Zahra Zakeri (Queens College, CUNY)

Dr. Chris Li (City College, CUNY)

Dr. Hannes Buelow (Albert Einstein College of Medicine)

THE CITY UNIVERSITY OF NEW YORK
ABSTRACT

The recycling GTPase, RAB-10, regulates autophagy flux in Caenorhabditis elegans

by

Nicholas J. Palmisano

Advisor: Dr. Alicia Meléndez

Autophagy and endocytosis are two cellular pathways that are vital to cell growth and homeostasis. Autophagy is a dynamic and catabolic process involving the formation of a double-membrane vesicle called the autophagosome, which engulfs long-lived proteins and damaged organelles. Endocytosis involves the uptake of extracellular material into the cell through the formation of intracellular vesicles termed endosomes. Although both endocytosis and autophagy are interconnected processes, the extent to which endocytic proteins and/or compartments contribute to autophagy, and how these endocytic components do so, is still unknown. To improve our understanding of the connections that exist between autophagy and endocytosis, we conducted an RNAi screen for endocytic genes that altered GFP::LGG-1 expression and blocked tissue remodeling in daf-2/IIIR mutants. The goal of this screen was to identify new or previously characterized endocytic genes that function in autophagy, further elucidate the mechanism by which these genes control autophagy, and enhance our understanding as to how distantly related processes are interconnected. daf-2 encodes the insulin-like receptor (IIR) and daf-2/IIIR loss of function mutants have elevated levels of autophagy, measured by an increase in puncta labeled with the autophagosome reporter, GFP::LGG-1. RNAi-mediated knockdown of autophagy genes in daf-2/IIIR mutants results in the formation of GFP::LGG-1-positive aggregates, exemplifying defects in autophagosome formation. Thus, using daf-2/IIIR loss of function mutants, we screened for endocytic genes that when depleted by RNAi, altered GFP::LGG-1 expression.
One of the genes identified in our screen was the small GTPase, RAB-10, a regulator of endocytic trafficking from yeast to mammals. Our results show that \textit{rab-10} loss of function alters the localization pattern of the autophagy reporter, GFP::LGG-1 in wild type and \textit{daf-2(e1370)} mutant seam cells. We show that the changes in the localization of the GFP::LGG-1 reporter in \textit{rab-10} mutants is due to defects in autophagy flux. Supporting defective flux due to \textit{rab-10} loss, we find that lysosomal inhibition does not further disrupt GFP::LGG-1 localization in \textit{rab-10} mutants. Moreover, autophagosomes fail to fuse with lysosomes in \textit{daf-2} mutants treated with \textit{rab-10} RNAi, and a loss of \textit{rab-10} function results in the accumulation of the autophagy cargo adaptor protein, SQST-1::GFP, in \textit{daf-2(e1370)} mutants. We determined that RAB-10 is required for the localization of GFP::ATG-9 to punctate structures/foci, which may represent the unique vesicle, called the “ATG-9 reservoir”. Lastly, we found that the GTPase cycling ability of RAB-10 is required for its role in autophagy. Future studies will be needed to determine the exact mechanism by which RAB-10 regulates autophagy flux. In conclusion, our data support a role for \textit{rab-10} in promoting autophagy flux and adds to the field of autophagy by reinforcing the importance of RAB GTPases in the regulation of autophagy function.
Acknowledgements

The completion of the work presented in this thesis would not be possible without the help, guidance, and support from my friends, family, and colleagues. I would like to express my deepest gratitude to my mentor, Dr. Alicia Meléndez. Her expertise, guidance, and support have allowed me to progress through the years and become an independent scientist, for which I am very grateful. I would like to thank Dr. Cathy Savage-Dunn for introducing me to C. elegans, and I am grateful for her enthusiasm, advice, and support throughout the years. I would like to thank the members of my committee, Dr. Hannes Buelow, Dr. Zahra Zakeri, and Dr. Chris Li, for their insight, critical feedback, and guidance.

I would like to give sincere thanks to the members of the Biology department at Queens college, and to my fellow graduate students. I thank Uday Madaan, James Clark, Corinna Singleman, Sana Khan, Sushma Teegala, Xenia Freilich, Travis Davis, Genevieve Feldmann for their support and friendship throughout the years. I am forever grateful to the past and current students in the Meléndez lab: Brenda Gonzalez, David Jimenez, Lev Starikov, Sara Wong, Hannah Hong, Feng Lin, Natalie Rosario, Ariel Gold, Gabriel Shechter, Minah Hong, and Matt Wysocki. Most importantly, I am forever thankful to two of the bravest and strongest women I have ever encountered in my life thus far, Kristina Ames and Melissa Silvestrini. Your friendship and direction have been invaluable over the years, and look forward to continuing our friendship in the years to come.

I am extremely blessed and lucky to have the support of my family and friends throughout my graduate career. To my friend Dave, and all my cousins for their constant support throughout the years. To my girlfriend, Katie, whose friendship and love has provided me with strength necessary to complete this thesis. To my brother, Chris, and his partner, Stephanie. Your patience, friendship, understanding, and love have gotten me through all the rough times during graduate school, and I will forever be grateful for that. Lastly, to my parents, for their support and love; without you, none of this would be possible, literally.
Table of Contents

Title page i
Copyright page ii
Approval page iii
Abstract iv
Acknowledgements vi
List of figures xi
List of tables xiii

Chapter 1

1. Introduction 1

1.1 C. elegans as a model organism 1

1.1.1 C. elegans growth and life cycle 1
1.1.2 Hypodermal/Epidermal seam cells 2
1.1.3 Intestinal epithelial cells 2
1.1.4 C. elegans dauer larvae 3
1.1.5 Regulation of dauer development 4

1.1.5.1 The DAF-11/Guanylyl cyclase pathway 5
1.1.5.2 The DAF-7/TGF-β signaling pathway 6
1.1.5.3 The DAF-2/Insulin-like signaling pathway 6
1.1.5.4 The DAF-9/Steroid hormone pathway 8

1.2 Autophagy: Process of self-cannibalism 9

1.2.1 Types of autophagy 9
1.2.2 Importance of autophagy 9
1.2.3 Overview of autophagy 10

1.2.3.1 Regulation of autophagy via TOR signaling 11
1.2.3.2 Autophagy induction 15
1.2.3.3 Recruitment and recycling of Atg9/ATG9/ATG-9 17
1.2.3.4 Vesicle nucleation 19
1.2.3.5 Vesicle elongation 21
1.2.3.6 Cargo recognition 25
1.2.3.7 Lysosomal Fusion 27
1.2.4 The role of amphisomes in autophagy function 30

1.3 Membrane Trafficking 32
1.3.1 Overview of membrane trafficking 32
1.3.2 Cell Polarization 33
1.3.3 RAB GTPase regulation and cycling 34
1.3.4 The Endocytic pathway 36
1.3.4.1 Early endosomes/Sorting endosomes 38
1.3.4.2 Late endosomes/Multivesicular bodies 38
1.3.4.3 The Lysosome/Vacuole 39
1.3.4.4 Recycling endosomes 40
1.3.4.5 Endosome-to-Golgi trafficking: Roles of the Retromer complex 41
1.3.4.6 Golgi-to-Plasma Membrane trafficking: Roles of the Exocyst complex 43

1.4 Phagocytosis 45

1.5 The role of Sec4/RAB10/RAB-10 in membrane trafficking 47

1.6 Thesis rationale 56

Chapter 2

2. The recycling endosome protein RAB-10 promotes autophagic flux and localization of the transmembrane protein ATG-9 59

2.1. Abstract 60
2.2. Introduction 61
2.3. Results: 63
2.3.1. Identifying the small GTPase, rab-10, as a mediator of autophagy function 63
2.3.2. RAB-10 is required for proper localization of GFP::LGG-1 in hypodermal seam cells 65
2.3.3. RAB-10 is required to maintain basal levels of autophagy 67
2.3.4. RAB-10 is required for autophagy flux in C. elegans 67
2.3.5. RAB-10 loss results in an enlargement of SQST-1::GFP foci 71
2.3.6. RAB-10 is required for trafficking of GFP::ATG-9 71
2.3.7. GTPase cycling of RAB-10 is required for GFP::ATG-9 traffic in intestinal cells 75

2.4. Discussion 78

2.5. Materials and Methods 82
   2.5.1. C. elegans strains 82
   2.5.2. Construction of ATG-9 transgenic strains 83
   2.5.3. Microscopy and Image analysis 84
   2.5.4. RNA interference 85
   2.5.5. Chloroquine treatment 85

2.6. Acknowledgements 86

Chapter 3

3. The Atg6/Vps30/Beclin 1 ortholog BEC-1 mediates endocytic retrograde transport in addition to autophagy in C. elegans (Attached manuscript found in Appendix B) 87

3.1. Abstract 87
3.2. Contribution to this paper 87

Chapter 4

4. Discussion and future directions 89
4.1. RAB-10 promotes autophagy flux in C. elegans 89
4.2. RAB-10 GTPase cycling is required for autophagy activity 90
4.3. RAB-10 is required for the localization of ATG-9 to punctate structures 91
4.4. Elucidating the mechanism by which RAB-10, and other endocytic genes, regulate autophagy activity in C. elegans (Discussion on work described in Appendix A) 92
4.4.1 RAB-10 functions in a distinct step of the autophagy pathway

4.4.2 Identification of additional endocytic genes that regulate autophagy activity

Chapter 5

5. Materials and Methods

5.1. C. elegans strains

5.2. Construction of ATG-9 transgenic strains

5.3. Microscopy and Image analysis

5.4. RNA interference

5.5. Chloroquine treatment

Appendix A

A. Additional support for RAB-10 promoting autophagy in C. elegans

A1. RNAi screen to identify endocytic genes with a role in autophagy

A2. GFP::RAB-10 expression levels decrease during autophagy induced conditions

A3. unc-51 and atg-7 are epistatic to rab-10 in the autophagy pathway

A4. The GTPase cycling activity of RAB-10 is required for autophagy

A5. Identification of RAB-10 effector proteins involved in autophagy

A6. RAB-10 promotes the degradation of SQST-1::GFP foci in the pharynx and intestine

A7. Elucidating the mechanism by which RAB-10 promotes autophagy flux

Appendix B

B. The Atg6/Vps30/Beclin1 ortholog BEC-1 mediates endocytic retrograde transport in addition to autophagy in C. elegans

(Journal article published in “Autophagy”, Taylor and Francis Group, DOI: 10.4161/auto.7.4.14391)
Appendix C

C. Chapter 16: Detection of Autophagy in Caenorhabditis elegans

References 168

List of figures

Figure 1.1: Schematic representation of autophagy C. elegans 11

Figure 1.2: Regulation of GTPase cycling 35

Figure 1.3: Schematic representation of membrane trafficking in C. elegans intestinal epithelia 37

Figure 2.1: Endocytic genes isolated in candidate gene RNAi screen 64

Figure 2.2: RAB-10 loss alters the localization of GFP::LGG-1 foci in daf-2(e1370) dauer and non-dauer seam cells 66

Figure 2.3: Loss of rab-10 increases the number of GFP::LGG-1-positive foci in hypodermal seam cells of wild type animals 68

Figure 2.4: RAB-10 is required for proper autophagic flux 70

Figure 2.5: RAB-10 is required to maintain the number and size of SQST-1/p62::GFP positive foci in daf-2(e1370) mutants 72

Figure 2.6: RAB-10 controls the size of GFP::ATG-9-positive foci in daf-2 intestinal cells 74

Figure 2.7: RAB-10 is required for the proper localization of mCherry::LGG-1 3 in intestinal cells 76

Figure 2.8: Proper GTP hydrolysis of RAB-10 is required for the recruitment of ATG-9 to vesicles in intestinal cells 77

Figure 2.9: ATG-9 does not colocalize with RAB-10-positive endosomes 79

Figure 2.10: Loss of rab-10 does not alter the localization of ATG-9 to LGG-1-positive autophagosomes 80

Figure A1: Changes in SQST-1::GFP localization and alae formation in daf-2(e1370) mutants treated with endocytic RNAi screen candidates 104

Figure A2: GFP::RAB-10 expression is reduced during dauer formation and increased during reduced autophagy activity 108
Figure A3: unc-51 and atg-7 are epistatic to rab-10 in the autophagy pathway

Figure A4: RAB-10 GTPase Activity is needed to rescue the altered localization of mCherry::LGG-1 in daf-2 mutants

Figure A5: RAB-10 GTPase cycling is needed to rescue the altered localization of GFP::ATG-9 in intestinal cells

Figure A6: Depletion of RAB-10 effector proteins does not result in enlarged GFP::LGG-1 foci in daf-2 dauer seam cells

Figure A7: Depletion and loss of the RAB-10 effector proteins, SEC-15 and TBC-2, alters GFP::LGG-1 expression in daf-2(e1370) mutants

Figure A8: Loss of rab-10 blocks the degradation of the SQST-1::GFP cargo adaptor protein in the pharynx of L3 animals

Figure A9: Depletion of rab-10 enhances the formation of enlarged SQST-1::GFP foci in rpl-43(bp399) mutants

Figure A10: ATG-9 does not localize to RAB-5-positive early endosomes

Figure A11: Loss of rab-10 does not change the colocalization between ATG-9 and RAB-5-positive early endosomes in 1-day old adults

Figure A12: ATG-9 does not localize to RAB-7-positive late endosomes

Figure B1: bec-1 gene, protein, mutations and phenotypes

Figure B2: bec-1 mutants display defects in endocytosis

Figure B3: bec-1 mutants display a defect in retrograde transport

Figure B4: BEC-1 colocalizes with RME-8

Figure B5: bec-1 mutants display lack of cell corpse clearance

Figure B6: Morphology of recycling endosomes is not affected in bec-1 mutants

Figure B7: Degradation of CAV-1::GFP occurs in bec-1 mutants

Figure B8: Lack of bec-1 and vps-34 affect the localization of MIG-14::GFP

Figure B9: RNAi to the lysosomal biogenesis cup-5 increases MIG-14::GFP to nearly wild-type levels in the bec-1 mutant animals

Figure B10: RNAi against vps-34 decreases the number of RME-8::GFP positive puncta

Figure B11: The number clathrin GFP::CHC-1 positive endosomes is not affected in animals treated with RNAi against bec-1 or vps-34

Figure B12: bec-1 loss of function increases the number and the size of GFP::SNX-1 positive puncta

Figure C1: Autophagy in C. elegans

Figure C2: GFP::LGG-1 expression in hypodermal seam cells of daf-2(e1370) mutants
List of tables

Table 2.1: Strains used in this study 82
Table 5.1: Strains used in this study 98

Table A1: RNAi clones identified to alter GFP::LGG-1 and SQST-1::GFP localization in daf-2(e1370) dauer 105

Table B1: Lack of zygotic bec-1 results in adult lethality 160
Table B2: Lack of maternal and zygotic bec-1 results in embryonic lethality 160
Table B3: bec-1 mutants accumulate aberrant vacuoles 160

Table C1: C. elegans autophagy genes 178
Table C2: Fluorescent reporters for monitoring Autophagy in C. elegans 184
Chapter 1

1. Introduction

1.1. *C. elegans* as a model organism

1.1.1. *C. elegans* growth and life cycle

*Caenorhabditis elegans* (*C. elegans*) is a microscopic free-living nematode, which is easily cultivated on agar petri dishes or in liquid culture with *Escherichia coli* (*E. coli*) as a food source (Brenner 1974; Lewis and Fleming 1995). These animals exist in two sexes, males and self-fertilizing hermaphrodites, and both sexes contain five pairs of autosomes; with hermaphrodites containing two X chromosomes and males containing only one X chromosome (Nigon and Dougherty 1949; Brenner 1974). Males are extremely rare in most populations, and arise at a frequency of 0.1% due to non-disjunction occurring in the hermaphrodite germ line (Nigon and Dougherty 1949; Brenner 1974).

*C. elegans* develop through four larval stages, L1-L4, before becoming a mature egg laying adult, with temperature affecting the rate of development (Byerly et al. 1976). Under conditions of stress, such as high temperatures or crowding, *C. elegans* develop into a developmentally arrested L3 larval stage referred to as dauer diapause, and can remain in this stage for several months until normal growth conditions resume (Cassada and Russell 1975a; Albert et al. 1981; Golden and Riddle 1984). *C. elegans* have a rapid life cycle of 3 days, from the egg to an egg-laying adult, at 20°C, and can be grown at temperatures ranging from 15°C to 25°C (Brenner 1974; Byerly et al. 1976). Wild type animals have a mean life span of ~20 days at 20°C and have brood sizes of ~300 progeny (Johnson and Wood 1982).

*C. elegans* is a simple organism, however, it contains many complex tissues and organs such as a pharynx, intestine, gonad, nervous system, body wall muscle, and epidermis/hypodermis. A hermaphrodite contains 959 somatic nuclei, and all cell lineages from a single-cell embryo to a multicellular adult have been mapped (Sulston et al. 1983; White 1988). *C. elegans* is a transparent nematode, which allows for easy visualization of all tissue types, and real-time visualization of fluorescent-tagged reporter proteins expressed in various tissues of interest. Additionally, *C. elegans* was the first multicellular organism to have its entire genome sequenced, allowing for the identification of evolutionarily conserved genes across many species, and the isolation of mutations for gene study (Consortium 1998).
1.1.2. Hypodermal/Epidermal seam cells

Seam cells are lateral hypodermal/epidermal cells organized as a single row on each side of *C. elegans* (White 1988). They are responsible for the formation of several neuronal cell lineages, such as neurons of the lumbar ganglia and tail spike neurons (Sulston and Horvitz 1977). Additionally, seam cells secrete cuticle-specific collagen proteins necessary for cuticle formation, and the formation of cuticular ridges called alae, which are found only on L1, dauer, and adult animals (Sulston and Horvitz 1977; Singh and Sulston 1978; Thein et al. 2003; Sapio et al. 2005). Because seam cells are responsible for the formation of alae, alae are positioned directly above seam cells (Singh and Sulston 1978).

After hatching, L1 larvae contain 10 seam cells (H0-H2, V1-V6, and T) on each side of their body, which are embedded in the hyp7 syncytium, part of the hypodermis/epidermis (White 1988). Seam cells are connected to the hypodermis by small adherens junctions situated along their apical membrane, and by small gap junctions located on their lateral membranes (Altun and Hall 2009b). Prior to each larval molt, all seam cells, except for H0, divide in a stem cell-like manner, where the anterior daughter fuses with the hyp7 hypodermal syncytium and the posterior daughter continues to divide in a stem cell-like fashion until the L4/adult molt. At the L4/adult molt, seam cells terminally differentiate and fuse with one another to form a syncytium of 16 nuclei (Sulston and Horvitz 1977; Singh and Sulston 1978).

Seam cells are polarized epithelial cells that display both apical-basal and anterior-posterior polarity (Wildwater et al. 2011; Yamamoto et al. 2011). These cells are elongated in the anterior-posterior direction, and their apical face lies in direct contact with the cuticle, while the basolateral face lies in contact with the body cavity/pseudocoelomic space, and the hyp7 hypodermal syncytium (White 1988). Prior to molting and dauer formation, these cells display large amounts of golgi bodies, endocytic vesicles, and autophagosomes (White 1988; Meléndez et al. 2003). Seam cells, along with the hypodermis, go through significant remodeling when undergoing dauer formation (Singh and Sulston 1978; White 1988; Meléndez et al. 2003).

1.1.3. Intestinal epithelial cells

The intestine is one of the largest organs in the *C. elegans* body plan, and is responsible for digestion, absorption of nutrients, and the synthesis and storage of macromolecules, such as yolk proteins.
and lipids (Kimble and Sharrock 1983; White 1988; Ashrafi et al. 2003; Altun and Hall 2009a). The *C. elegans* intestine consists of a tube of 20 polarized epithelial cells, arranged as a sequence of nine intestinal rings. Each intestinal ring consists of two bilaterally symmetrical cells, with the exception of the first intestinal ring, which consists of four cells (Deppe et al. 1978; Sulston et al. 1983; Leung et al. 1999). These cells are mononucleated after hatching; however, at the start of the L1/L2 molt, all intestinal nuclei, except for the 6 most anterior nuclei, divide and become binucleate, due to a lack in the corresponding cell division (Hedgecock and White 1985; Altun and Hall 2009a).

Every intestinal cell has an apical surface that consists of microvilli facing the lumen, and a basolateral surface that is covered by a basal lamina facing the body cavity (White 1988; Leung et al. 1999; Altun and Hall 2009a). Neighboring cells are linked to one another by adherens junctions located on their apical membranes, and gap junctions located on their lateral membranes (Leung et al. 1999; Altun and Hall 2009a). These junctions are not only important for the exchange of material between cells, they are also important for ensuring the separation of apical and basolateral domains (White 1988; Leung et al. 1999; Labouesse 2006).

Intestinal cells are large in size and have an extensive endomembrane system consisting of the endoplasmic reticulum (ER), Golgi bodies, mitochondria, “autofluorescent” lipofuscin gut granules, endosomes, lysosomes, and autophagosomes (Clokey and Jacobson 1986; Borgonie et al. 1995; Chen et al. 2006; Altun and Hall 2009a; Sato et al. 2014a). These vesicles can be observed, and studied in the intestine, using fluorescent reporters linked to vesicular proteins that localize to specific endocytic compartments, such as fluorescently tagged Ypt51/RAB5/RAB-5 labeling early endosomes (Chen et al. 2006).

1.1.4. *C. elegans* dauer larvae

Dauer formation relies heavily on three environmental stimuli: food depletion, crowding, and high temperatures (>25°C) (Golden and Riddle 1982; Golden and Riddle 1984). Prior to the first larval molt, if environmental conditions are not favorable for reproductive growth, a developmental decision is made causing animals to develop into dauer larvae (Cassada and Russell 1975a; Golden and Riddle 1984). Under harsh environmental conditions, late L1/early L2 larvae progressively develop into a pre-dauer or
L2d larval stage (Golden and Riddle 1984). If environmental conditions support reproductive growth, L2d larvae can continue through normal development; however, if conditions remain harsh, L2d larvae develop into dauer larvae (Golden and Riddle 1984). Dauer larvae have morphological features that distinguish them from their L3/L2d counterparts, which include a thickened cuticle, cuticular alae, body constriction and elongation, a buccal plug, increased lipid stores, arrested gonad development, and resistance to detergent (Cassada and Russell 1975a; Popham and Webster 1979; Golden and Riddle 1984; Vowels and Thomas 1992). With regard to body constriction and elongation, anatomical distinctions between dauer and L3 larvae are greatly evident (Popham and Webster 1979). Dauer intestinal cells are reduced in volume, contain less cytoplasmic content and a reduced intestinal lumen, and contain electron dense endosomes, yolk granules, large lipid droplets, and small autophagosomes (Popham and Webster 1979; Wolkow and Hall 2013). Additionally, in dauer animals, seam cells shrink in volume, resulting in body constriction and alae production (Singh and Sulston 1978; White 1988). This shrinking of cell volume may be attributed to the increased number of vesicular structures, such as autophagosomes, found in these cells during dauer formation (Singh and Sulston 1978; White 1988; Meléndez et al. 2003). Indeed, compared to L3 larvae, dauer larvae display high levels of autophagy activity in both seam cells and intestinal cells (Meléndez et al. 2003; Hansen et al. 2008a; Chapin et al. 2015). Demonstrating the importance of autophagy during dauer formation, RNAi-mediated depletion of multiple autophagy genes (see below) results in abnormal dauer formation and reduced longevity, in daf-2(e1370)/Insulin-like receptor (IIR) temperature sensitive mutants, which enter dauer when grown at the restrictive temperature (see below) (Meléndez et al. 2003; Toth et al. 2008; Kwon et al. 2010).

The morphological features of dauer animals allows them to survive harsh environmental conditions for up to 60 days, compared to the 20-day lifespan of growing animals (Klass and Hirsh 1976). Once environmental conditions improve, dauer larvae exit diapause and enter into the L4 larval stage to resume normal development (Cassada and Russell 1975a).

1.1.5. Regulation of dauer development

The external environment is the main regulator of dauer formation. Animals constitutively secrete dauer pheromone, which consists of several ascaroside molecules (Jeong et al. 2005; Butcher et al.
Dauer pheromone is synthesized by DAF-22, a thiolase involved in peroxisomal beta-oxidation (Golden and Riddle 1985; Butcher et al. 2009). High temperature, and crowding result in higher levels of pheromone in the environment, which promotes dauer formation (Golden and Riddle 1982; Golden and Riddle 1984). Pheromone sensation is mediated by G-protein coupled receptors expressed in chemosensory neurons (amphid neurons), such as ASI, ASJ, and ASK, which regulate the transcription of genes that encode components of the guanylyl cyclase pathway, TGF-β signaling pathway, Insulin-like/IGF-1 (IIR) receptor signaling pathway, and the steroid hormone pathway (Hu 2007; Kim et al. 2009; McGrath et al. 2011; Park et al. 2012b). These signaling pathways work together to regulate growth and development, and mutations in any of these pathways can result in a dauer constitutive phenotype (Daf-c) or a dauer defective phenotype (Daf-d) (Riddle et al. 1981; Estevez et al. 1993; Thomas et al. 1993; Gottlieb and Ruvkun 1994; Ren 1996; Schackwitz 1996; Patterson et al. 1997b; Birnby et al. 2000; Inoue and Thomas 2000a; da Graca et al. 2004; Fielenbach and Antebi 2008).

1.1.5.1. The DAF-11/Guanylyl cyclase pathway

The main regulator of the guanylyl cyclase pathway is *daf-11*, which encodes a transmembrane guanylyl cyclase that converts GTP to cGMP, and is expressed in chemosensory neurons (Birnby et al. 2000). cGMP levels act as a signal that determines whether animals enter into dauer or continue through developmental growth (Schackwitz et al. 1996; Birnby et al. 2000). In chemosensory neurons, cGMP produced by DAF-11 acts on the cGMP gated ion channel, composed of two subunits, TAX-2 and TAX-4, which transduce signals to maintain normal development (Birnby et al. 2000; Hu 2007; Fielenbach and Antebi 2008). *daf-11, tax-2,* and *tax-4* loss of function mutants have defects in chemosensation and display a Daf-c phenotype (Thomas et al. 1993; Vowels and Thomas 1994; Coburn et al. 1998; Birnby et al. 2000). High pheromone levels negatively regulate cGMP production by partly antagonizing DAF-11, resulting in a signaling cascade that initiates dauer formation (Schackwitz et al. 1996; Birnby et al. 2000). DAF-7/TGF-β and DAF-2/insulin-like receptor (IIR) signaling pathways act parallel or downstream of DAF-11, since the gene expression of *daf-7,* and the insulin-like peptide, *daf-28,* is reduced in *daf-11* mutants (Thomas et al. 1993; Murakami et al. 2001; Li et al. 2003).
1.1.5.2. The DAF-7/TGF-β signaling pathway

The homolog of TGF-β in C. elegans is DAF-7, a BMP-like ligand that is expressed solely in the ASI chemosensory neuron (Ren 1996). During favorable conditions, DAF-7 expression promotes normal larval development, while during unfavorable conditions, DAF-7 inhibition promotes dauer entry (Ren 1996). DAF-7 binds to the type I and II receptor kinases, DAF-1 and DAF-4, respectively, which lead to activation of the R-Smads, DAF-8 and DAF-14 (Georgi et al. 1990; Estevez et al. 1993; Inoue and Thomas 2000b). DAF-8 and DAF-14 then inhibit a protein complex consisting of DAF-3 and DAF-5 (Thomas et al. 1993; Patterson et al. 1997b). DAF-3 encodes a Co-Smad, while DAF-5 encodes an ortholog of the SKI/SNO oncogene, and together, both act in the nucleus to promote dauer induction (Patterson et al. 1997b; da Graca et al. 2004). Mutations in daf-7, daf-1, daf-4, daf-8, and daf-14 result in a Daf-c phenotype, while mutations in daf-3 and daf-5 result in a Daf-d phenotype (Riddle et al. 1981; Swanson and Riddle 1981; Albert 1997). Interestingly, although DAF-7 is expressed solely in ASI neurons, most other components of the TGF-β pathway appear to be widely expressed, suggesting that DAF-7 is secreted from ASI neurons into neighboring cells to regulate dauer formation (Ren 1996; Patterson et al. 1997b; Inoue and Thomas 2000b; Fielenbach and Antebi 2008). The DAF-7/TGF-β pathway is highly interconnected with the DAF-2/IIR pathway, and lies upstream of the DAF-9/Steroid hormone pathway (see below) (Thomas et al. 1993; Gottlieb and Ruvkun 1994; Albert 1997). Some of the downstream targets of the TGF-β pathway include insulin-like ligands and hormone synthesis genes (Liu et al. 2004). daf-7, daf-8, and daf-14 loss of function (LOF) mutants exhibit downregulation of insulin-like agonists, and an upregulation of insulin-like antagonists (Pierce et al. 2001; Liu et al. 2004; Shaw et al. 2007; Park et al. 2012a). Moreover, daf-7 mutants display nuclear translocation of the Forkhead/FOXO transcription factor, DAF-16, which is a major downstream regulator of the insulin signaling pathway (see below) (Ogg et al. 1997; Lee et al. 2001). In addition, daf-7, daf-8, and daf-14 LOF mutants show reduced expression of the hormone synthesis gene, DAF-9, and upregulation of the nuclear hormone receptor, DAF-12 (see below) (Liu et al. 2004). Therefore, TGF-β converges on other signaling pathways to regulate dauer formation.

1.1.5.3. The DAF-2/Insulin-like signaling pathway
Insulin signaling in *C. elegans* is controlled by the actions of the sole insulin-like receptor (IIR), DAF-2 (Fig. 1.1) (Kimura et al. 1997). *C. elegans* is predicted to have 40 insulin-like peptides (ILPs), which bind to DAF-2 as either agonists or antagonists of the signaling pathway, and therefore exhibit both functional redundancy and diversity (Pierce et al. 2001). ILPs are mainly expressed in amphid chemosensory neurons, but are also expressed in intestinal cells, and/or muscle cells, while DAF-2 is expressed in neurons, XXX neuroendocrine cells, the hypodermis, the intestine, and muscle (Kimura et al. 2011). ILPs are secreted to regulate developmental growth, or dauer formation, by binding to DAF-2 expressed in other tissues (Pierce et al. 2001; Fileenbach and Antebi 2008; Matsunaga et al. 2016).

Under favorable conditions, secreted ILP agonists bind to DAF-2, and result in the activation of the class I PI3-kinase, AGE-1 (Morris et al. 1996). Activated AGE-1 then converts phosphatidylinositol 3,4-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3), which activates the AKT/PKB kinase, PDK-1 (Morris et al. 1996; Paradis et al. 1999). PDK-1 then activates the serine/threonine kinase, AKT-1/2, which in turn inhibits the FOXO transcription factor, DAF-16 (Fig. 1.1) (Ogg et al. 1997; Paradis and Ruvkun 1998; Lee et al. 2001). During normal growth conditions, DAF-16 is mainly cytoplasmic; however, during unfavorable environmental conditions, such as reduced insulin signaling, DAF-16 is localized to nuclei (Paradis and Ruvkun 1998; Henderson and Johnson 2001; Lee et al. 2001; Lin et al. 2001a; Kwon et al. 2010). In the nucleus, DAF-16 regulates the transcription of stress response/tolerance genes, such as those important for oxidative stress resistance, thermotolerance, antimicrobial defense, and dauer formation (Honda and Honda 1999; Libina et al. 2003; Murphy et al. 2003; Ookuma et al. 2003; Kwon et al. 2010; Furuhashi and Sakamoto 2016).

*daf-2* LOF mutants, *pdk-1* LOF mutants, *age-1* null mutants, and *akt-1*; *akt-2* double null mutants all have a Daf-c and longevity phenotype, both of which are suppressed by DAF-16/Daf-d null mutations (Kenyon et al. 1993; Thomas et al. 1993; Gottlieb and Ruvkun 1994; Dorman et al. 1995; Morris et al. 1996; Albert 1997; Lin et al. 1997; Paradis et al. 1999; Toth et al. 2008; Kwon et al. 2010). In addition, *daf-2* IIR pathway mutants exhibit elevated levels of autophagy, which is required for their Daf-c and longevity phenotypes (Meléndez et al. 2003; Hansen et al. 2008a). The elevated levels of autophagy observed in *daf-2* LOF mutants depend on DAF-16 activity and reduced LET-363/TOR activity (Fig. 1.1) (see “Regulation of autophagy via TOR signaling” below) (Jia et al. 2009; Guo et al. 2014c).
Parallel to DAF-16 inhibition, DAF-2/IIR signaling negatively regulates the bZIP transcription factor, SKN-1 (Tullet et al. 2008). SKN-1 expression is mainly localized to chemosensory neurons and the intestine (An and Blackwell 2003). Similar to DAF-16, SKN-1 is cytoplasmic during growth conditions, yet nuclear during times of stress, where it regulates the expression of collagen genes, and the transcription of stress response genes, some in common with DAF-16 (An and Blackwell 2003; An et al. 2005; Tullet et al. 2008; Robida-Stubbs et al. 2012; Ewald et al. 2015). Although SKN-1 does not regulate genes important for dauer morphogenesis, it does negatively regulate ILP agonists of DAF-2, such as DAF-28, and also plays a role in the activation of autophagy during nutrient poor conditions (see “Regulation of autophagy via TOR signaling” below) (Tullet et al. 2008; Okuyama et al. 2010; Mosbech et al. 2013; Palikaras et al. 2015). In all, the DAF-2/IIR signaling pathways initiate a cascade of events that regulates genes important for protection and survival during times of stress.

1.1.5.4. The DAF-9/Steroid hormone pathway

The steroid hormone pathway is regulated by the actions of \textit{daf-9} and \textit{daf-12} (Antebi et al. 1998; Antebi et al. 2000; Jia et al. 2002). \textit{daf-9} encodes a cytochrome P450/steroidogenic hydroxylase, and is expressed in the hypodermis/epidermis and neuroendocrine cells, while \textit{daf-12} encodes a nuclear hormone receptor, and is ubiquitously expressed (Antebi et al. 2000; Jia et al. 2002; Gerisch and Antebi 2004; Fielenbach and Antebi 2008). DAF-9 receives input from DAF-7/TGF-β and DAF-2/Insulin-like (IIR) signaling pathways to regulate dauer formation (Gerisch et al. 2001; Jia et al. 2002; Gerisch and Antebi 2004). DAF-9 acts upstream of DAF-12, and synthesizes dafachronic acid molecules (DA) (Gerisch et al. 2001; Jia et al. 2002; Gerisch and Antebi 2004). DA acts as a ligand for DAF-12, where DA binding results in reproductive growth, while a lack of DA binding promotes dauer formation (Antebi et al. 2000; Motola et al. 2006). \textit{daf-9} mutants have a Daf-c phenotype that requires functional \textit{daf-12}, while \textit{daf-12} mutants can either be Daf-c or Daf-d, depending on the type of mutation (Antebi et al. 1998; Antebi et al. 2000; Fielenbach and Antebi 2008). Daf-d alleles of \textit{daf-12} suppress all known Daf-c alleles in upstream pathways, highlighting the importance of \textit{daf-12} in integrating the upstream signaling pathways that regulate the decision to maintain larval growth, or initiate dauer formation (Antebi et al. 1998; Antebi et al. 2000; Fielenbach and Antebi 2008). Downstream targets of DAF-12 include heterochronic genes and microRNAs.
that control developmental timing and larval progression during favorable conditions, and promote dauer formation during unfavorable conditions (Hammell et al. 2009; Tennessen et al. 2010; Hochbaum et al. 2011).

1.2. Autophagy: Process of self-cannibalism

1.2.1. Types of autophagy

Autophagy, a process of “self-cannibalism”, was first described in mammals through morphological studies of rat liver cells (DeDuve 1963; Deter et al. 1967). Autophagy is an evolutionarily conserved stress response pathway in eukaryotic cells that leads to the bulk degradation and recycling of cellular components through the lysosomal pathway (Deter et al. 1967; Levine and Klionsky 2004; Meijer et al. 2007; Mizushima 2007; Melendez and Neufeld 2008). Three main forms of autophagy exist in eukaryotes: chaperone-mediated autophagy, microautophagy, and macroautophagy (DeDuve and Wattiaux 1966; Deter et al. 1967; Neff et al. 1981; Ahlberg et al. 1982; Backer et al. 1983; Cuervo and Dice 2000). All three forms contribute to the lysosomal degradation of cellular material; however, each form is regulated differently.

Chaperone-mediated autophagy involves the selective recognition of cytosolic proteins containing a KFERQ amino acid motif (Backer et al. 1983; Backer and Dice 1986). Chaperone complexes localized in the cytosol bind to substrates and mediate their transport across the lysosomal membrane (Chiang et al. 1989; Dice 1990; Cuervo and Dice 1996).

Microautophagy involves the direct engulfment of target molecules and/or cytoplasm at the lysosomal surface through the invagination of the lysosomal membrane (Ahlberg et al. 1982; Mortimore et al. 1988; Sattler and Mayer 2000; Uttenweiler et al. 2005).

Finally, macroautophagy, commonly referred to as “autophagy”, involves the de-novo formation of a double-membrane vesicle called the autophagosome, which engulfs cargo, such as polyubiquitinated proteins or damaged mitochondria, and delivers it to the lysosome for degradation and recycling (Deter et al. 1967; Arstila and Trump 1968; Levine and Klionsky 2004; Mizushima 2007; Melendez and Neufeld 2008).

1.2.2. Importance of autophagy
Autophagy is widely known for its pro-survival role during stress conditions, in addition to its role in overall cellular homeostasis, as it is required for the maintenance of amino acid pools and other macromolecules during starvation (Mortimore and Schworer 1977; Kuma et al. 2004; Komatsu et al. 2005; Onodera and Ohsumi 2005; Singh et al. 2009; Yang et al. 2010; Kim et al. 2011; Fader et al. 2012; Suraweera et al. 2012; Karsli-Uzunbas et al. 2014). Although autophagy was first described in mammals, most of the core autophagy genes that regulate autophagosome formation were discovered in *Saccharomyces cerevisiae*, by screening for mutations that decreased survival during starvation, as well as mutations that disrupted the cytoplasm-to-vacuole targeting (cvt) process, a selective form of autophagy found in yeast (*Saccharomyces cerevisiae* will be referred to as yeast throughout the document) (Tsukada and Ohsumi 1993a; M. Thumm 1994; Harding et al. 1995; Harding 1996; Hutchins and Klionsky 2001; Klionsky et al. 2003). Highlighting the importance of autophagy across species, *S. cerevisiae*, *C. elegans*, and *M. musculus*, display a variety of developmental defects, and in some cases lethality, when containing mutations in autophagy genes, and exposed to stress (Tsukada and Ohsumi 1993a; Meléndez et al. 2003; Yue et al. 2003; Kuma et al. 2004; Scott et al. 2004; Boya et al. 2005; Komatsu et al. 2005; Juhasz et al. 2007). In humans and mammals, impaired autophagy is associated with the development of cancers, such as breast, prostate, and lung cancers, as well as neurodegenerative disorders, such as Alzheimer’s and Parkinson’s disease (Yue et al. 2003; Nixon et al. 2005; Yu et al. 2005; Hara et al. 2006; Komatsu et al. 2006; Marino et al. 2007; Martinez-Vicente et al. 2010; Liu et al. 2013a; Sun et al. 2016). In summary, autophagy acts as a quality control system to maintain cellular homeostasis and function (Elmore et al. 2001; Kuma et al. 2004; Hara et al. 2006; Juhasz et al. 2007; Khan et al. 2008; Lu et al. 2014).

1.2.3. Overview of autophagy

As described below, the majority of our understanding of autophagy in *C. elegans* comes from studies done in yeast and mammals. Autophagy occurs at low/basal levels during normal growth conditions; however, it is significantly upregulated in response to stress, such as nutrient deprivation, increased temperature, hypoxia, and the accumulation of damaged organelles and/or defective long-lived proteins (Meléndez et al. 2003; Levine and Klionsky 2004). These stressors lead to the initiation of the autophagy process, which is divided into distinct steps: induction, vesicle nucleation, vesicle elongation, and lysosomal
fusion and degradation (Suzuki et al. 2001; Mizushima 2007; Xie and Klionsky 2007; Nakatogawa et al. 2009b). In an evolutionarily conserved manner, each step of autophagy is regulated by specific sets of proteins, which coordinate with one another to form an autophagosome (Fig. 1.1) (see below).

Figure 1.1: Schematic representation of autophagy C. elegans. Autophagy is regulated by the DAF-2/IIR signaling pathway (Meléndez et al., 2003). During favorable growth conditions, DAF-2/IIR signaling inactivates DAF-16, leading to LET-363/TOR activation and autophagy inhibition (Jia et al., 2004; Noda et al., 1998; Ogg et al., 1997). In times of stress, such as starvation, DAF-2/IIR signaling is inactivated, and autophagy is induced, by the activation of the UNC-51 induction complex and recruitment of the integral membrane protein, ATG-9, from ATG-9-reservoirs (Kamada et al., 2000; Noda et al., 2000; Sekito et al., 2009). Membrane nucleation requires the PI3KC3 complex, consisting of VPS-34 and BEC-1, which recruits the LGG-1 and ATG-16 conjugation complexes needed for the expansion of the isolation membrane and formation of a double membrane autophagosome (Kametaka et al., 1998; Kirisako et al., 1998; Kihara et al., 2001; Kuma et al., 2002). ATG-7 is an E1-like activating enzyme that synthesizes both conjugation complexes (Kim et al., 1999; Tanida et al., 2001). In the process of expansion, cargo bound to an adaptor protein (SQST-1) is engulfed and the autophagosome is delivered to the lysosome. The autophagosome then fuses with the lysosome, in a manner dependent on RAB-7, and the autophagic contents are degraded and recycled by the cell (Schimmoller and Riezman., 1993).

1.2.3.1. Regulation of autophagy via TOR signaling
One of the main regulators of autophagy is the evolutionarily conserved protein kinase, TOR (Target of Rapamycin) (Fig. 1.1) (Heitman et al. 1991; Cafferkey et al. 1993; Sabatini et al. 1994). TOR is a molecular sensor of nutrient levels in the cell that receives input from insulin, amino acids, glucose, reactive oxygen species, and DNA damage (Barbet et al. 1996; Di Como and Arndt 1996; Rohde et al. 2001; Fingar and Blenis 2004). Via these signals, TOR regulates cell growth, protein synthesis, and autophagy (Fingar and Blenis 2004; Sarbassov et al. 2005).

One of the mechanisms by which TOR determines the nutrient status of the cell is by localizing to the vacuolar/lysosomal membrane (Reinke et al. 2004; Sturgill et al. 2008). The vacuole/lysosome contains various types of nutrient transporters on its surface that export macromolecules into the cell (Maguire et al. 1983; Town et al. 1998; Russnak et al. 2001; Gao et al. 2005; Shimazu et al. 2005; Yang et al. 2006; Liu et al. 2012a; Rebsamen and Superti-Furga 2016). The translocation of TOR, to and from the lysosome, allows the cell to sense and respond to differing nutrient levels, either activating or repressing cell growth (Rohde et al. 2001; Fingar and Blenis 2004; Peli-Gulli et al. 2015; Kira et al. 2016). TOR localization to lysosomes depends on the evolutionarily conserved Rag GTPases, Grt1/RAGA/B and Grt2/RAGC/D (Kim et al. 2008a; Sancak et al. 2008; Bar-Peled et al. 2012). In the presence of amino acids, these Rag GTPases recruit TOR to the vacuolar/lysosomal membrane, where it becomes activated and inhibits autophagy (see Autophagy induction below) (Peli-Gulli et al. 2015). Upon nutrient deprivation, TOR is inactivated and dissociates from the vacuolar/lysosomal membrane, which allows for the induction of autophagy (Sancak et al. 2008; Bar-Peled et al. 2012). As part of a positive feedback loop, prolonged autophagy activity replenishes diminished nutrients, which results in the reactivation of TOR, and re-inhibition of autophagy (Yu et al. 2010).

Two TOR genes exist in yeast, TOR1 and TOR2, and both were identified in a genetic screen that suppressed the antiproliferative effects of rapamycin (Heitman et al. 1991; Cafferkey et al. 1993). It was shortly discovered thereafter that rapamycin treatment in yeast resulted in the induction of autophagy, even in the presence of nutrients (Noda and Ohsumi 1998). Subsequent studies showed that Tor1 negatively regulates the Atg1 protein complex during nutrient rich conditions (refer to Autophagy induction) (Kamada et al. 2000; Hosokawa et al. 2009; Jung et al. 2009; Kamada et al. 2010). Tor1 also regulates autophagy at the transcriptional level (Beck and Hall 1999; Cardenas et al. 1999; Chan et al. 2001; Natarajan et al. 2010).
2001; Valenzuela et al. 2001). Tor1 negatively regulates Gln3 and Gcn4, which are two transcription factors that increase the transcription of autophagy genes during starvation conditions (Chan et al. 2001; Natarajan et al. 2001; Valenzuela et al. 2001). Additionally, Tor1 interacts with the Snf1/AMPK signaling pathway, which senses the energy status of the cell (Wang et al. 2001b; Hardie et al. 2003; Orlova et al. 2006). Snf1 is a positive regulator of autophagy that is inhibited by Tor1 during nutrient rich conditions (Wang et al. 2001b; Orlova et al. 2006). It is hypothesized that Snf1 positively regulates autophagy activity by acting on Atg1, but the mechanism remains unclear (Wang et al. 2001b; Orlova et al. 2006).

Evidence for the regulation of autophagy by mTOR (mammalian/mechanistic TOR) came from studies in rat hepatocytes, which showed that conditions that inhibit autophagy also activate the ribosomal protein S6, a protein positively regulated by mTOR (Blommaart et al. 1995). A single TOR gene exists in mammals, whose gene product can be found in two protein complexes, mTORC1 and mTORC2 (Kim et al. 2002; Sarbassov et al. 2004). mTORC1 contains the protein subunit, RAPTOR, while mTORC2 contains the protein subunit, RICTOR (Kim et al. 2002; Sarbassov et al. 2004). Both complexes are implicated in the regulation of cell growth and autophagy; however, there is a greater understanding for the regulation of autophagy via mTORC1 (Laplante and Sabatini 2009; Kim and Guan 2015). mTORC1 is regulated by the class I PI3K signaling pathway, which responds to growth factors, such as insulin (Foukas et al. 2006; Hawkins et al. 2006). In the presence of growth factors and nutrients, PI3K signaling leads to the activation of the serine/threonine kinase, AKT (also called protein kinase B) (Peng et al. 2003; Hawkins et al. 2006; Vander Haar et al. 2007). AKT positively regulates mTORC1 activity, which inhibits the ULK1/2 autophagy induction complex (refer to Autophagy induction below) (Inoki et al. 2002; Zhang et al. 2003; Long et al. 2005). During stress conditions, such as reduced insulin signaling or dietary restriction, AKT is inactivated, which activates the AMP-activated protein kinase (AMPK), a serine/threonine kinase that senses intracellular ATP levels (Vander Haar et al. 2007; Kim et al. 2011). Activated AMPK inhibits mTORC1 activity, and positively regulates autophagy (refer to Autophagy induction below) (Hosokawa et al. 2009; Jung et al. 2009; Egan et al. 2011; Kim et al. 2011; Tian et al. 2015). Additionally, mTORC1 inactivation regulates the transcription of autophagy genes, via the nuclear translocation of the transcription factor, TFEB, a regulator of lysosomal genes and autophagy genes (Settembre et al. 2011; Martina et al. 2012).
LET-363 is the *C. elegans* ortholog of mTOR and Tor1/2, and is found in two complexes, CeTORC1 and CeTORC2 (Long et al. 2002; Robida-Stubbs et al. 2012). Animals deficient in LET-363 activity, or containing a mutation in the RAPTOR ortholog, *daf-15*, arrest as dauer-like larvae (incomplete dauers), have elevated levels of autophagy, and display a longevity phenotype, which is dependent on autophagy (Long et al. 2002; Vellai et al. 2003; Jia et al. 2004; Toth et al. 2008; Robida-Stubbs et al. 2012).

As described above, the transcription factors, DAF-16 and SKN-1, are major downstream targets of the IIR pathway (Gottlieb and Ruvkun 1994; Ogg et al. 1997; Lee et al. 2001; Oh et al. 2006). Upon reduced insulin levels, DAF-16 and SKN-1 enter the nucleus to control the transcription of stress response genes (Ogg et al. 1997; Lee et al. 2001; Lee et al. 2003b). One of the genes that is negatively regulated by DAF-16 activity is *DAF-15*, which upregulates autophagy (Jia et al. 2004; Hansen et al. 2008a; Guo et al. 2014c). Concurrently, SKN-1 in the nucleus not only regulates the expression of stress response genes, it negatively regulates insulin-like agonists, which can further reduce insulin signaling and promote autophagy (Okuyama et al. 2010). Moreover, SKN-1 is required for increased mitophagy as a result of reduced insulin signaling (Palikaras et al. 2015). Therefore, reduced insulin signaling leads to a cascade of events that inhibit LET-363 activity, upregulate DAF-16 and SKN-1, and lead to autophagy induction (Hansen et al. 2008a; Guo et al. 2014c). Interestingly, LET-363/CeTORC1 was shown to negatively regulate both DAF-16 and SKN-1 activity, suggesting that a negative feedback loop exists between LET-363, DAF-16, and SKN-1 (Jia et al. 2004; Robida-Stubbs et al. 2012).

LET-363 is also part of the signaling pathways that regulate dietary restriction (DR), which partially overlaps with the IIR pathway (Dunn et al. 1997; Iser and Wolkow 2007; Narasimhan et al. 2009). DR animals exhibit reduced pharyngeal pumping, have reduced brood sizes, display a longevity phenotype, and have elevated levels of autophagy, at least partially due to reduced LET-363 activity (Panowski et al. 2007; Hansen et al. 2008a; Sheaffer et al. 2008; Honjoh et al. 2009). There are various forms of DR, each of which induces autophagy via distinct mechanisms.

Animals with a LOF mutation in the nicotinic acetylcholine receptor, *eat-2*, have reduced pharyngeal pumping and are dietary restricted (Avery 1993; Lakowski and Hekimi 1998; Hansen et al. 2008a). *eat-2* LOF mutants exhibit a longevity phenotype that is dependent on both autophagy and reduced LET-363 activity (Hansen et al. 2007; Jia and Levine 2007; Hansen et al. 2008a). However, the autophagic
response elicited by eat-2 is mediated by the FOXA transcription factor, PHA-4 (refer to section "The role of Sec4/RAB10/RAB-10 in membrane trafficking") (Jia and Levine 2007; Panowski et al. 2007; Hansen et al. 2008a; Sheaffer et al. 2008). During normal growth conditions, LET-363/CeTORC1 negatively regulates PHA-4 signaling, while reduced LET-363/CeTORC1 activity increases the PHA-4-dependent transcription of stress response genes as a result of DR (Sheaffer et al. 2008).

Mutations in two ceramide synthase genes, hyl-1 and lagr-1, results in a DR phenotype, increased longevity, and elevated levels of autophagy, dependent on DAF-16 and SKN-1 (Mosbech et al. 2013). Lastly, a specific form of DR, where the bacterial food source is diluted on agar plates (sDR), results in a longevity phenotype that is dependent on the activities of DAF-16, and AAK-2, an ortholog of the catalytic subunit of Snf1/AMPK (Greer et al. 2007). In sDR, AAK-2 directly phosphorylates and activates DAF-16, which leads to the activation of stress response genes (Greer et al. 2007). AAK-2 functions downstream and/or in parallel to IIR, and both DAF-16 and AAK-2 regulate autophagy induction due to reduced IIR (Apfeld et al. 2004; Tullet et al. 2014).

In summary, reduced nutrient uptake elicits a response that converges on LET-363, which differentially induces the activities of PHA-4, DAF-16, and SKN-1. In general, DAF-16, SKN-1, and PHA-4 are required for the elevated levels of autophagy, in addition to other phenotypes, associated with reduced LET-363/TORC1 activity as a result of DR or reduced IIR (Robida-Stubbs et al. 2012).

Overall, TOR is an evolutionarily conserved central molecular switch that promotes or inhibits autophagy depending on environmental conditions, such as reduced insulin signaling or DR. Nutrient replete conditions lead to the downregulation of TOR activity, resulting in the upregulation of the transcription factors that upregulate autophagy genes and other stress response pathways.

1.2.3.2. Autophagy induction

In *S. cerevisiae*, autophagy induction requires activation of the serine/threonine kinase, Atg1, along with Atg13 (Matsuura et al. 1997; Kamada et al. 2000; Kamada et al. 2010). Under nutrient rich conditions, TORC1 hyperphosphorylates Atg13, preventing its association with Atg1, thereby inactivating the autophagy pathway (Kamada et al. 2000; Kamada et al. 2010). Under nutrient poor conditions, TORC1 becomes inactivated, leading to the dephosphorylation of Atg13 and its association with Atg1, which results
in the activation of Atg1 kinase activity (Funakoshi et al. 1997) (Cheong et al. 2008). The Atg1-Atg13 complex then interacts with Atg17, Atg29, and Atg31, autophagy proteins specific for starvation-induced autophagy. Together, these proteins form a ternary complex (Atg1 induction complex) important for the formation of the pre-autophagosomal structure (PAS) or isolation membrane (IM) (Cheong et al. 2008; Kawamata et al. 2008).

In mammals, UNC-51-like kinases 1 and 2 (ULK1 and ULK2) are orthologous to yeast Atg1 and C. elegans UNC-51 (Kuroyanagi et al. 1998). Irrespective of nutrient conditions, ULK1/2 interacts with ATG13, FIP200 (mammalian ortholog of Atg17), and ATG101, to form an ULK1/2 induction complex (Hosokawa et al. 2009; Mercer et al. 2009). Similar to that found in yeast, under nutrient rich conditions, mTORC1 binds to and inhibits the ULK1/2 complex by phosphorylating ULK1/2 and ATG13 (Hosokawa et al. 2009; Jung et al. 2009). Under nutrient poor conditions or rapamycin treatment, mTORC1 dissociates from the ULK1/2 complex, and through a series of phosphorylation events, the ULK1/2 complex localizes to the site of autophagosome formation (Ganley et al. 2009; Hosokawa et al. 2009; Mercer et al. 2009; Longatti et al. 2012). At the IM, ULK1/2 leads to the phosphorylation of the PI3KC3 complex, which leads to vesicle nucleation (see below) (Russell et al. 2013). ULK1/2 is also regulated by the evolutionarily conserved energy sensor AMPK (Kim et al. 2011; Tian et al. 2015). In response to nutrient limited conditions, AMPK directly phosphorylates ULK1/2 and mTORC1, which activates ULK1/2 and inhibits mTORC1 (Kim et al. 2011).

In C. elegans, only three members of the ULK1/Atg1 induction complex have been characterized: UNC-51 (Atg1/ULK1 ortholog), EPG-1 (Atg13/ATG13 ortholog), and EPG-9 (ATG101 ortholog) (Fig. 1.1) (Ogura et al. 1994; Tian et al. 2009a; Liang et al. 2012). UNC-51 was discovered as a neuronal specific protein important for axon elongation, and mutations in unc-51 result in an uncoordinated phenotype (McIntire et al. 1992; Ogura et al. 1994). UNC-51 is essential for autophagy, as evident by the formation of enlarged fluorescently labeled LGG-1/Atg8/LC3 foci in unc-51 mutant embryos or dauer larvae (Meléndez et al. 2003; Tian et al. 2009a; Liang et al. 2012). UNC-51 also plays a role in maintaining overall body size, and maintaining lipid content in intestinal cells (Aladzsity et al. 2007; Lapierre et al. 2013b). Furthermore, loss of unc-51 results in defects in dauer morphogenesis, and reduces the longevity phenotype of glp-1/Notch germline-less mutants, which require autophagy for longevity (Meléndez et al. 2003; Lapierre et al.
2011). EPG-1 and EPG-9 were isolated in forward genetic screens used to identify genes important for the degradation of autophagy specific cargo, such as germline P granules, which are germ cell specific protein aggregates (Schisa et al. 2001; Tian et al. 2009a; Liang et al. 2012). EPG-9 directly interacts with EPG-1, and defects in either epg-1 or epg-9 result in the formation of enlarged fluorescently labeled LGG-1/LC3 foci, or SQST-1 foci, in embryos or larvae (Meléndez et al. 2003; Tian et al. 2009a; Liang et al. 2012; Guo et al. 2014c).

1.2.3.3. Recruitment and recycling of Atg9/ATG9/ATG-9

Atg9 is the sole transmembrane (six-pass) protein involved in autophagosome formation, and is responsible for providing a source of membrane to the developing autophagosome (Noda et al. 2000; Yamada et al. 2005; Suzuki et al. 2007; Yamamoto et al. 2012). As such, autophagosome formation requires the constant cycling of Atg9 from membrane-bound organelles to the PAS, and vice versa. The organelles that contribute membrane to the developing autophagosome derive from the endoplasmic reticulum (ER), Golgi bodies, endosomes, mitochondria, and the plasma membrane (Hayashi-Nishino et al. 2009; Hailey et al. 2010; Mari et al. 2010b; Ravikumar et al. 2010).

In yeast, Atg9 is localized to the trans-Golgi network, mitochondria, recycling endosomes, and unique vesicles of unknown origin, sometimes referred to in the literature as “Atg9 reservoirs” (Fig. 1.1) (Reggiori et al. 2005; Mari et al. 2010b; Mari and Reggiori 2010; Ohashi and Munro 2010; Yamamoto et al. 2012; Imai et al. 2016). Trafficking of Atg9 to the PAS begins with the formation of Atg9 vesicular structures/tubules near the Golgi body, which require the SNARE proteins Sso1/2 and Sec9 (Nair et al. 2011). From Atg9 tubules, Atg9-positive vesicles form and are brought toward the PAS by the Rab GTPase, Sec4, and its guanine exchange factor, Sec2 (Geng et al. 2010). En route to the PAS, the Atg1 induction complex intercepts the Atg9 vesicles and acts as a protein scaffold for autophagosome formation (Suzuki et al. 2007; Sekito et al. 2009; Suzuki et al. 2015; Rao et al. 2016).

Once Atg9 is delivered to the PAS, it is recycled back to peripheral vesicles, which requires the Atg1 induction complex, and the Atg2-Atg18 protein complex (Reggiori et al. 2004a; Obara et al. 2008; Papinski et al. 2014). The phosphorylation of Atg9 by Atg1 allows for the localization of the Atg18-Atg2 complex to the PAS, and the subsequent recycling of Atg9 back to peripheral vesicles (Reggiori et al. 2011).
2004a; Obara et al. 2008; Sekito et al. 2009; Papinski et al. 2014). The proper localization of Atg18 to the
PAS requires phosphatidylinositol-3-phosphate (PI3P) (Obara et al. 2008). Atg18 also regulates the
recruitment of Atg16 and Atg8 to the PAS, in a PI3P dependent manner (Nair et al. 2010).

In mammals, during nutrient rich conditions, ATG9 is found to localize to the trans-Golgi network,
endoplasmic reticulum exit sites, early endosomes, late endosomes, and recycling endosomes (Young et
from the endomembrane system to the PAS, which depends on ULK1 and the Class III VPS34 nucleation
complex (Young et al. 2006; He et al. 2013; Takahashi et al. 2014). Moreover, ATG9 is found in clathrin-
occluded vesicles, where it eventually localizes to RAB11-positive recycling endosomes (Puri et al. 2013).
Interestingly, ATG9 does not incorporate itself into autophagosomes, suggesting that ATG9 delivers donor
membrane transiently, and is quickly recycled back to endocytic vesicles (Orsi et al. 2012). ATG9 traffic to
and from the PAS is also dependent on several GTPase activating proteins (GAPs), such as TBC1D5 and
TBC1D14 (Longatti et al. 2012; Popovic and Dikic 2014). TBC1D14 is a GAP for RAB11, and a negative
regulator of autophagy. During starvation conditions, TBC1D14 dissociates from RAB11-positive recycling
endosomes, allowing them to contribute to autophagosome formation (Longatti et al. 2012). TBC1D5, on
the other hand, is a positive regulator of autophagy that promotes autophagosome formation by directing
ATG9 trafficking to the PAS (Popovic and Dikic 2014).

The mechanism of ATG9 recycling back to the endomembrane system is still not fully understood
in mammals. WIPI1/2 and ATG2A/B are orthologs of yeast Atg18 and Atg2, respectively (Proikas-Cezanne
et al. 2004; Polson et al. 2010; Velikkakath et al. 2012). Similar to yeast, WIPI2 directly binds and recruits
the ATG16L1 conjugation complex to the PAS, in a PI3P-dependent manner, which results in LC3 lipidation
(Polson et al. 2010; Dooley et al. 2014). Depletion of WIPI2 results in the accumulation of ATG9 to immature
omegasomes (ER derived autophagosomes), suggesting that WIPI2 is required for ATG9 removal from
autophagosomes, and not trafficking to autophagosomes (Orsi et al. 2012). However, depletion of ATG9
diminishes WIPI2 puncta formation, suggesting that ATG9 and WIPI2 may regulate one another in a
positive feedback manner (Orsi et al. 2012). A role of ATG2A/B in ATG9 dynamics has not been elucidated;
however, ATG2A/B is required for proper autophagosome formation (Velikkakath et al. 2012).
In *C. elegans*, *atg-9*, *atg-18*, and *atg-2* are orthologs of Atg9/ATG9, Atg18/WIPI1/2, and Atg2/ATG2A/B, respectively (Meléndez et al. 2003; Schaheen et al. 2006; Lu et al. 2011b). Mutations in *atg-9*, *atg-18*, or *atg-2*, result in defects in autophagosome formation and degradation of autophagy-specific cargo, such as SQST-1 or P granules (Zhang et al. 2009; Lu et al. 2011b). Moreover, mutations in autophagy genes that participate in the early steps of autophagosome formation, such as *unc-51*, *epg-1*, *atg-18*, or *atg-2*, lead to the formation of enlarged ATG-9::GFP foci, supporting the evolutionarily conserved role of these genes in the regulation of ATG-9 dynamics (Lu et al. 2011b; Lin et al. 2013).

### 1.2.3.4. Vesicle nucleation

Vesicle nucleation requires activity of the class III PI3K (PI3KC3) complex, whose function is to produce phosphatidylinositol 3-phosphate (PI3P), a key regulator of autophagy (Polson et al. 2010; Taguchi-Atarashi et al. 2010; He et al. 2013; Dooley et al. 2014; Wu et al. 2014). In *S. cerevisiae*, the core machinery of the PI3KC3 complex consists of Vps34, Atg6/Vps30, and Vps15 (Stack et al. 1993). Vps34 is the sole class III PI3-kinase in yeast, and synthesizes PI3P via phosphorylation of phosphatidylinositol (Schu et al. 1993). Vps15, a serine/threonine kinase, is essential for the phosphorylation and activation of Vps34 (Herman et al. 1991; Stack et al. 1993). Atg6/Vps30 acts as a scaffold and binds to additional proteins to regulate the activity of the core PI3KC3 complex. Through binding with Atg6/Vps30, the core complex can interact with either Atg14 or Vps38, and form distinct subcomplexes, (Kametaka et al. 1998; Kihara et al. 2001). Atg14 confers specificity toward autophagy in complex I, while Vps38 confers specificity toward vacuolar protein sorting (endocytic trafficking to the vacuole) in complex II (Kametaka et al. 1998; Kihara et al. 2001). During autophagy induced conditions, Atg14 is responsible for the localization of complex I to the PAS, resulting in PI3P production, and further recruitment of autophagy proteins to the developing autophagosome (Kametaka et al. 1998; Obara et al. 2006). One of these proteins includes Atg18, which recycles Atg9 and recruits autophagy proteins needed for autophagosome elongation (Nair et al. 2010) (see below).

Vesicle nucleation in mammals is more intricate and highly regulated compared to *S. cerevisiae*; however, there are some similarities. Similar to yeast, the core PI3KC3 machinery in mammals includes VPS34 (Vps34 ortholog), p150 (Vps15 ortholog), and BECLIN1/BECN1 (Atg6 ortholog) (Volinia et al. 1995).
Moreover, BECLIN1 forms distinct complexes with ATG14L/BARKOR (ortholog of Atg14) and UVRAG (ortholog of Vps38) (Itakura et al. 2008). However, distinct from yeast, both the ATG14L/BARKOR-containing and UVRAG-containing PI3K complexes regulate autophagy and endocytosis (Morris et al. 2015). Furthermore, additional regulators of the PI3KC3 complex exist in mammals, which are not found in yeast. These additional regulators include: BAX interacting factor-1 (BIF-1), RUN domain and cysteine rich domain containing BECLIN1-interacting protein (RUBICON), and Activating molecule in BECLIN1-regulated autophagy 1 (AMBRA1), all of which directly bind to BECLIN1 (Fimia et al. 2007; Takahashi et al. 2007; Matsunaga et al. 2009). BECLIN1 itself was discovered as a binding partner of the anti-apoptotic protein, B-cell lymphoma 2 (BCL-2), which negatively regulates autophagy (Itakura et al. 2008; Matsunaga et al. 2009; Zhong et al. 2009).

Under normal conditions, AMBRA1, a WD40 domain containing protein, inhibits autophagy by tethering the PI3KC3 complex to the dynein motor complex bound to microtubules (Fimia et al. 2007; Di Bartolomeo et al. 2010). Once autophagy is induced, AMBRA1 dissociates from the dynein complex in an ULK1-dependent manner, resulting in the translocation of the PI3KC3 complex to the PAS (Di Bartolomeo et al. 2010; Strappazzon et al. 2011; Nazio et al. 2013). At the PAS, ATG14L regulates the attachment of the PI3KC3 complex to membrane enriched with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) and PI3P, via its Barkor autophagosome targeting sequence (BATS) domain (Fan et al. 2011b; Tan et al. 2016). ATG14L then phosphorylates BECLIN1, leading to activation of the PI3KC3 complex, the production of PI3P, and the recruitment of proteins needed for autophagosome enclosure (see below) (Polson et al. 2010; Fogel et al. 2013; Dooley et al. 2014). Recently, ATG14L has been shown to regulate lysosomal fusion events with autophagosomes and endosomes, via its interaction with SNARE proteins (Kim et al. 2012; Diao et al. 2015).

UVRAG functions in endosome maturation events, competes with ATG14L for interaction with the PI3KC3 complex, and plays an important role in both autophagosome formation and maturation, through its interactions with BIF-1 and the C-Vps/HOPS complex (RAB7 guanine exchange factor), respectively (Wurmser et al. 2000; Liang et al. 2006; Takahashi et al. 2007; Itakura et al. 2008; Liang et al. 2008a; Li et al. 2012b). During normal conditions, UVRAG is localized to late endosomes, and to the ER, in a PI3P dependent manner (Itakura et al. 2008; He et al. 2013). Upon autophagy induction, UVRAG interacts with
both BECLIN 1 and BIF-1, which together, facilitate the trafficking of ATG9 vesicles to the PAS (Takahashi et al. 2007; He et al. 2013; Takahashi et al. 2014). Interestingly, independent of BECLIN1 binding, UVRAG interacts with components of the C-Vps/HOPS complex, which is important for both autophagosome and endosome fusion with the lysosome (Liang et al. 2008a; Sun et al. 2010; Kramer and Ungermann 2011). The autophagy and endocytic promoting effects of the UVRAG-containing PI3KC3 complex can be repelled by the actions of RUBICON, which sequesters UVRAG from the C-Vps/HOPS complex, blocking RAB7 activation (Sun et al. 2010). In addition, RUBICON inhibits autophagosome maturation by decreasing the kinase activity of VPS34 (Zhong et al. 2009; Sun et al. 2011a).

The components of the core PI3KC3 complex found in yeast and mammals, also exist in C. elegans. bec-1, vps-15, and vps-34 were identified based on sequence similarities to their mammalian and yeast counterparts (Roggo et al. 2002; Meléndez et al. 2003). As in yeast and mammals, BEC-1 physically associates with VPS-34, and together, both are important for PI3P production, normal autophagy function, and dauer morphogenesis (Fig. 1.1) (Roggo et al. 2002; Meléndez et al. 2003; Takacs-Vellai et al. 2005; Hansen et al. 2008a; Zhao et al. 2009b; Ruck et al. 2011). Additionally, BEC-1 physically interacts with CED-9, the C. elegans ortholog of BCL-2 (Takacs-Vellai et al. 2005). Both BEC-1 and VPS-34 are necessary for the longevity phenotype associated with glp-1/Notch germline-less mutants and eat-2 dietary restricted animals, both of which require autophagy for longevity (Jia and Levine 2007; Hansen et al. 2008a). Separate from their autophagy functions, BEC-1 and VPS-34 facilitate the retromer-dependent endocytic trafficking of the Wnt sorting receptor, MIG-14/Wntless (refer to “Endosome-to-Golig trafficking” below) (Ruck et al. 2011). Additionally, BEC-1, VPS-34, and other autophagy proteins, function in the phagocytic degradation of apoptotic cell corpses (refer to “Phagocytosis” section and chapter 3) (Takacs-Vellai et al. 2005; Ruck et al. 2011; Huang et al. 2012; Li et al. 2012a). Lastly, BEC-1 and VPS-34 function in lipid homeostasis, since bec-1 and vps-34 mutants show significantly reduced lipid levels in intestinal cells (Lapierre et al. 2013b). EPG-8, the C. elegans ortholog of Atg14/ATG14L/BARKOR, is required for the degradation of autophagy substrates, and binds to BEC-1 through its coiled coil domain (Zhang 2011). An ortholog of Vps38/UVRAG has not yet been identified in C. elegans.

1.2.3.5. Vesicle elongation
Enclosure of the autophagosome depends on the actions of the Atg8 and Atg12 ubiquitin-like conjugation systems (Geng and Klionsky 2008). Both Atg12 and Atg8 are classified as ubiquitin-like proteins due to their structural similarities to ubiquitin (Hanada and Ohsumi 2005; Suzuki et al. 2005; Matsushita et al. 2007; Kumeta et al. 2010). In yeast, Atg12 is conjugated to Atg5, in a reaction dependent on the E1-like activating enzyme, Atg7, and the E2-like conjugating enzyme, Atg10 (Mizushima et al. 1998a; Kim 1999; Shintani et al. 1999; Kuma et al. 2002; Hanada et al. 2007; Matsushita et al. 2007). Interestingly, Atg12-Atg5 conjugation does not require an E3-like ubiquitin ligase, since Atg10 can directly mediate the conjugation reaction (Yamaguchi et al. 2012). Atg5 interacts with the small alpha helical protein, Atg16, which self-associates to form a coiled-coil dimer (Matsushita et al. 2007; Fujioka et al. 2010). Each monomer of Atg16 interacts with an Atg5-Atg12 conjugate, resulting in formation of an Atg5-Atg12-Atg16 heterohexameric complex (Atg16 complex), which localizes to the PAS, and acts as an E3-like enzyme for the Atg8-Phosphatidylethanolamine (PE) conjugation reaction complex (Kuma et al. 2002; Hanada et al. 2007; Romanov et al. 2012).

The conjugation of Atg8 to PE requires the cleavage of the carboxy terminus of Atg8, by the cysteine protease Atg4, exposing a glycine residue (Kirisako et al. 2000). This cleavage event results in the formation of a cytosolic form of Atg8, known as Atg8-I (Kabeya et al. 2000; Kirisako et al. 2000). Atg8-I is then processed by Atg7, which in addition to the Atg5-Atg12 conjugation reaction, acts as the E1-like activating enzyme for Atg8 lipidation (Ichimura et al. 2000). Atg3 is the E2-like conjugating enzyme for the Atg8 conjugation reaction that is activated by the Atg16 complex, which enhances the transfer of the glycine residue of Atg8, from Atg3 to PE (Kirisako et al. 1999; Ichimura et al. 2000; Hanada et al. 2007; Sakoh-Nakatogawa et al. 2013). This results in the lipidated/membrane bound form of Atg8 (Atg8-PE), which associates with the inner and outer membrane of the autophagosome (Kirisako et al. 1999; Kirisako et al. 2000). On the outer membrane of the autophagosome, Atg8 is deconjugated and recycled by Atg4, a process important for autophagosome biogenesis and maturation (Kirisako et al. 2000; Nair et al. 2012; Nakatogawa et al. 2012a; Yu et al. 2012). Atg8 functions in membrane expansion and autophagosome size, inferred from its ability to induce the hemifusion of membranes labeled with PI3P (Nakatogawa et al. 2007; Xie et al. 2008). Additionally, Atg8 also serves as an additional regulator of protein recruitment to the autophagosome, by binding to proteins that contain an Atg8-family interacting motif (AIM), such as Atg1,
Atg3, or Atg7 (Behrends et al. 2010; Noda et al. 2010; Nakatogawa et al. 2012b). AIM motifs are also located on cargo adaptor proteins, such as Atg19 in the cytoplasm-to-vacuole targeting (cvt) pathway, or Atg32 in mitophagy, which attach to cargo destined for degradation (Shintani et al. 2002; Chang and Huang 2007; Noda et al. 2008; Noda et al. 2010; Kondo-Okamoto et al. 2012).

In mammals, the ATG5-ATG12-ATG16L1 (ATG16L1 complex) and LC3-II (Atg8-PE ortholog) conjugation systems operate in the same manner as in yeast, with slight differences. Both complexes are important for isolation membrane expansion, and are interdependent on one another for autophagosome formation (Tanida et al. 2001; Tanida et al. 2002a; Mizushima et al. 2003; Fujita et al. 2008). As with yeast, formation of the ATG16L1 complex is dependent ATG7/Atg7 and ATG10/Atg10 (Tanida et al. 2001; Nemoto et al. 2003). The ATG16L1 complex, localized at the PAS, acts as an E3-like enzyme for LC3 conjugation, and determines the sites of LC3 lipidation on the autophagosome, through its interaction with ATG3 (Mizushima et al. 1998b; Mizushima et al. 2003; Fujita et al. 2008). Interestingly, ATG16L1 is found in clathrin-coated vesicles distinct from ATG9 clathrin-coated vesicles, which eventually localize to RAB11-positive recycling endosomes containing ATG9 (Puri et al. 2013). It is postulated that recycling endosomes positive for ATG9 and ATG16L1 contribute to autophagosome formation (Puri et al. 2013).

In mammals, there are six Atg8 homologs that are divided into two subfamilies. The MAP1LC3 (microtubule-associated protein 1 light chain-3) subfamily consists of LC3A, LC3B and LC3C, while the GABARAP (GABA\textsubscript{A} receptor-associated protein) subfamily, consists of GABARAP, GABARAPL1, and GABARAPL2/GATE-16 (Xin et al. 2001; He et al. 2003b; Weidberg et al. 2010; Wild et al. 2014). LC3 was the first mammalian ortholog of Atg8 identified, and was isolated as a binding partner of the microtubule-associated protein 1 (MAP1) (Kuznetsov and Gelfand 1987; Mann and Hammarback 1994). Thereafter, based on sequence similarity to LC3B, other members of the LC3 and GABARAP subfamilies were identified (Wang et al. 1999; Sagiv et al. 2000; He et al. 2003b). All members of the Atg8 superfamily associate with autophagosomes; however, each member has a different role in autophagosome biogenesis (He et al. 2003b; Kabeya et al. 2004; Weidberg et al. 2010; Weidberg et al. 2011). Members of the LC3 subfamily appear to regulate autophagosome elongation and enclosure, while members of the GABARAP subfamily regulate ULK1 recruitment to the PAS, and autophagosome-lysosome fusion events, although
LC3B has been shown to be involved in fusion as well (Weidberg et al. 2010; Alemu et al. 2012; Wang et al. 2015a; Schaaf et al. 2016; McEwan et al. 2015; Wang et al. 2016b).

LC3B, hereafter called LC3, is the most widely studied homolog of the Atg8 superfamily in mammals (Wild et al. 2014). The conjugation of LC3 to PE is mechanistically similar to yeast, and requires the same set of proteins. ATG4B cleaves LC3 at its carboxy terminus and exposes a glycine residue, forming LC3-I. Through the actions of ATG7, ATG3, and the ATG16L complex, the exposed glycine residue of LC3-I is then covalently conjugated to PE, forming LC3-II (Kabeya et al. 2000; Tanida et al. 2001; Tanida et al. 2002b; Hemelaar et al. 2003; Mizushima et al. 2003; Tanida et al. 2004). LC3-II then associates with the inner and outer membrane of the autophagosome, where it is eventually recycled from the outer membrane, by ATG4B (Kabeya et al. 2000; Kabeya et al. 2004). Moreover, LC3 interacts with proteins that contain an LC3-interacting region (LIR) motif that serves the same function as the AIM motif (Pankiv et al. 2007; Behrends et al. 2010; Alemu et al. 2012; Birgisdottir et al. 2013; Wild et al. 2014). One of the proteins that contains a LIR motif, is the adaptor protein, p62/SQSTM1, which binds to polyubiquitinated proteins and LC3 (see “Cargo recognition” below) (Pankiv et al. 2007).

All members of the two conjugation complexes have been identified in C. elegans (Meléndez et al. 2003; Tian et al. 2010; Wu et al. 2012; Zhang et al. 2013). LGG-1 and LGG-2 are two orthologs of Atg8/LC3, which like in yeast and mammals, are found in cytosolic and membrane bound forms, bind to proteins that contain a LIR/AIM motif, and function in distinct steps of the autophagy pathway (Alberti et al. 2010b; Manil-Segalen et al. 2014b; Wu et al. 2015). LGG-1 functions in autophagosome formation, whereas both LGG-1 and LGG-2 function in autophagosome-lysosome fusion (see below) (Manil-Segalen et al. 2014b; Wu et al. 2015). LGG-1 acts upstream of LGG-2, and is required for the recruitment of LGG-2 to autophagosomes, allowing for autophagosome fusion to occur (Manil-Segalen et al. 2014b; Wu et al. 2015). Null mutations in \textit{lgg-1} cause a highly penetrant lethality phenotype; however, \textit{lgg-1} or \textit{lgg-2} null mutant embryos and L1 larvae that do not display lethality accumulate fluorescently labeled P granules and SQST-1-positive aggregates (Zhang et al. 2009; Sato and Sato 2011; Wu et al. 2015). Additionally, RNAi-mediated depletion of \textit{lgg-1} or \textit{lgg-2} results in the formation of abnormal dauer larvae (Meléndez et al. 2003; Alberti et al. 2010b).
Embryos null for \textit{atg-3}, \textit{atg-5}, \textit{atg-7}, and \textit{atg-10} accumulate P granules, and display a reduction in the number of LGG-1 labeled foci, confirming their importance in autophagy and LGG-1 conjugation (Zhang et al. 2009; Tian et al. 2010; Zhang et al. 2013). Interestingly, a null allele of \textit{atg-7} results in the accumulation of GFP::LGG-1 foci in L3 or dauer larvae, suggesting LGG-1 does accumulate over time in \textit{atg-7} mutants (N. Palmisano, unpublished results). Additionally, RNAi inactivation of \textit{atg-7} results in defective dauer morphogenesis, and reduces the long lifespan of dietary restricted \textit{eat-2} mutants and \textit{daf-2}/IIR mutants (Meléndez et al. 2003; Hars et al. 2007a; Jia and Levine 2007). Two orthologs for \textit{Atg4/ATG4} and \textit{Atg16/ATG16L1} are found in \textit{C. elegans}, which are \textit{atg-4.1/2} and \textit{atg-16.1/2}, respectively (Wu et al. 2012; Zhang et al. 2013). \textit{atg-4.1} plays a more significant role in autophagy than \textit{atg-4.2}, since \textit{atg-4.1} mutants, but not \textit{atg-4.2} mutants, accumulate pre-processed LGG-1, fluorescently labeled P granules, and SQST-1::GFP-positive aggregates (Wu et al. 2012). Both ATG-16.1 and ATG-16.2 have partially redundant, yet distinct functions in autophagy (Zhang et al. 2013). Mutations in \textit{atg-16.2} result in stronger defects in autophagy, compared to mutations in \textit{atg-16.1} (Zhang et al. 2013). Additionally, ATG-16.2 interacts with ATG-5, and mutations in \textit{atg-5} or \textit{atg-16.2} severely effect LGG-1::GFP puncta/foci formation. (Zhang et al. 2013).

1.2.3.6. Cargo recognition

The process of autophagy was once considered to be mainly non-selective, removing non-specific protein aggregates and organelles from the cell. Evidence has accumulated suggesting that autophagy is actually a very selective process, with specific forms of autophagy, such as aggrephagy, pexophagy, mitophagy, etc (Johansen and Lamark 2011; Rogov et al. 2014). This selectivity depends on molecular tags that bind to cargo destined for degradation, and also depends on the adaptor proteins that bind to both the tag and the autophagosome used to transport the cargo (Kirkin et al. 2009b; Rogov et al. 2014). Common to all autophagy adaptor proteins is their ability to link cargo to the autophagosome by binding to Atg8/LC3 (Kanki et al. 2009; Kondo-Okamoto et al. 2012; Motley et al. 2012; Lu et al. 2014).

In yeast, several types of cargo adaptor proteins exist that recognize specific types of cargo. The cytoplasm-to-vacuole targeting (\textit{cvt}) pathway is a selective form of autophagy found in yeast that delivers prApe1 (precursor Aminopeptidase 1) and Ams1 (α-mannosidase 1) to the vacuole (Klionsky et al. 1992;
Klionsky and Ohsumi 1999; Hutchins and Klionsky 2001; Scott et al. 2001). The cargo adaptor protein for prApe1 and Ams1 is Atg19 (Scott et al. 2001). Atg19 binds to prApe1 and Ams1, and links them to the autophagosome by binding to Atg8 (Shintani et al. 2002; Chang and Huang 2007; Noda et al. 2008; Suzuki et al. 2010; Sawa-Makarska et al. 2014). Yeast mitophagy and pexophagy require the adaptor proteins, Atg32 and Atg36, respectively (Kanki et al. 2009; Motley et al. 2012). In pexophagy, Atg36 recognizes peroxisomes by binding to the peroxisomal membrane protein, Pex3, while in mitophagy, Atg32 is localized to the mitochondrial outer membrane (Kanki et al. 2009; Motley et al. 2012). In aggrephagy, polyubiquitinated proteins are recognized by the cargo adaptor protein Cue5, which binds to both K48-linked, and K63-linked ubiquitin chains (Lu et al. 2014). Interaction between the cargo adaptor protein and Atg8 is dependent on AIM motifs, located on the cargo adaptor protein (Shintani et al. 2002; Chang and Huang 2007; Noda et al. 2008; Noda et al. 2010; Kondo-Okamoto et al. 2012).

In mammals, the recognition of autophagic cargo operates in a similar fashion as in yeast. Most selective forms of autophagy found in mammals utilize ubiquitin as a molecular tag for autophagosome recognition (see below) (Rogov et al. 2014). There are many autophagy adaptor proteins in mammals, such as HDAC6, NBR1, NDP52, and OPTN/Optineurin; however, the most well-studied adaptor protein is p62/SQSTM1 (Kawaguchi et al. 2003; Kirkin et al. 2009a). p62/SQSTM1 was originally identified as a 62-kDa protein that binds to the src homology 2 (SH2) domain of p56lck, a tyrosine kinase found in T-cells (Park et al. 1995; Joung et al. 1996). Important for autophagy function, p62/SQSTM1 possesses a ubiquitin-binding UBA domain, which binds to K48-linked and K63-linked ubiquitin chains (Vadlamudi et al. 1996; Long et al. 2008; Wooten et al. 2008; Tan et al. 2014). Cytoplasmic protein inclusions, such as Lewy bodies, neurofibrillary tangles, and Huntington aggregates are commonly polyubiquitinated and bound to p62/SQSTM1 (Stumptner et al. 1999; Kuusisto et al. 2001; Zatloukal et al. 2002b; Nagaoka et al. 2004; Bjorkoy et al. 2005; Kim et al. 2008b). p62/SQSTM1 does not only play a role in aggrephagy, it is also participates in the autophagic degradation of polyubiquitinated mitochondria in mitophagy (Ding et al. 2010; Narendra et al. 2014; Matsumoto et al. 2015). Using its LIR motif, p62/SQSTM1 directly interacts with the LC3 and GABARAP protein family members, and mediates the degradation of itself and polyubiquitinated cargo (Bjorkoy et al. 2005; Pankiv et al. 2007).
SQST-1 is the *C. elegans* ortholog of p62/SQSTM1 (Tian et al. 2010). Although a direct interaction between SQST-1 and ubiquitin has not been investigated in *C. elegans*, evidence exists showing that SQST-1 is functionally similar to p62/SQSTM1. SQST-1 colocalizes with LGG-1, contains a LIR motif, and closely associates with accumulated germline P granules (Tian et al. 2010; Lu et al. 2011b; Lin et al. 2013). Additionally, SQST-1 itself is degraded by autophagy, and mutations in various autophagy genes, such as *lgg-1* or *atg-9*, result in the accumulation of SQST-1::GFP aggregates in embryos and larvae (Zhang et al. 2009; Lu et al. 2011b; Wu et al. 2015). Additional adaptor proteins found in *C. elegans*, include EPG-2 and SEPA-1 (Zhang et al. 2009; Tian et al. 2010). Both EPG-2 and SEPA-1 directly bind to embryonic P granules to facilitate their autophagic degradation (Zhang et al. 2009; Tian et al. 2010). Additionally, both EPG-2 and SEPA-1 bind to LGG-1 via their respective LIR motifs (Wu et al. 2015). In all, the autophagic degradation of cargo depends largely on adaptor proteins that bind to both cargo and the autophagosome.

### 1.2.3.7 Lysosomal Fusion

The final destination for autophagosomes is the lysosome/vacuole, where lysosomal enzymes degrade autophagic cargo (DeDuve 1963; Bainton 1981; Li and Kane 2009). In general, autophagosomes are delivered to endosomes/lysosomes along microtubules and actin filaments (Kochl et al. 2006; Xie et al. 2010). In addition to its degradative role, the lysosome plays a central role in the release and recycling of macromolecules produced from the degradation process (Sokol et al. 1988; Town et al. 1998; Yang et al. 2006). On their membranes, lysosomes contain various transporters, such as amino acid transporters, which allow digested molecules to enter into the cytosol (Maguire et al. 1983; Town et al. 1998; Russnak et al. 2001; Yang et al. 2006; Liu et al. 2012a; Rebsamen and Superti-Furga 2016). Additionally, lysosomes can release digested material by fusing with the plasma membrane, in a process called lysosomal exocytosis (Reddy et al. 2001; Rao et al. 2004; Medina et al. 2011; Samie and Xu 2014; Vieira 2016). Highlighting the importance of the lysosome, aberrant lysosomal function results in defects in cell homeostasis, which can contribute to lysosomal storage diseases, such as Niemann-Pick disease or Mucolipidosis Type IV (Sokol et al. 1988; Pryor et al. 2006). The mechanism of autophagosome fusion with the vacuole/lysosome is still not fully understood; however, fusion events occur in four general steps: 1. Priming, 2. Tethering, 3. Docking, and 4. Fusion (Wickner 2010).
Knowledge of autophagosome fusion with the vacuole comes from general vesicular/membrane trafficking studies, and involves several key proteins, such as SNARE proteins, the Ypt7 GTPase, and the Homotypic fusion and protein sorting (HOPS) complex (Mayer et al. 1996; Mayer and Wickner 1997; Fischer von Mollard and Stevens 1999; Ishihara et al. 2001; Laage and Ungermann 2001; Kweon et al. 2003; Xu et al. 2010). Vacuole priming is the preparation/activation of the vacuole for vesicle fusion events (Wickner 2010). By default, SNARE proteins on vesicles, including the vacuole and autophagosome, are in a cis-conformation and are unable to bind to SNAREs on opposing vesicles (Ungermann et al. 1998). In an ATP-dependent manner, Sec18, a SNARE binding protein, disrupts the cis-SNARE complex, leading to the formation of “free” SNAREs needed for vesicle tethering (see below) (Mayer et al. 1996; Ishihara et al. 2001). On both the vacuole and autophagosome, the GTPase Ypt7 recruits the HOPS complex, which is a guanine exchange factor (GEF) for Ypt7, and is critical for vesicle tethering (Mayer and Wickner 1997; Wurmser et al. 2000; Hickey et al. 2009; Xu et al. 2010; Kramer and Ungermann 2011; Liu et al. 2016). Once the vesicles come into close contact, SNAREs on opposing membranes physically interact to form a trans-SNARE complex, in a manner dependent on Ypt7 and HOPS (Starai et al. 2008; Kramer and Ungermann 2011; Kulkarni et al. 2012; Jiang et al. 2014; Orr et al. 2015).

Two main types of SNAREs exist, Q-SNAREs and R-SNAREs, which contain a glutamine and arginine residue in their catalytic domains, respectively (Fasshauer et al. 1998). SNAREs can also be categorized as t-SNAREs or v-SNAREs, for SNAREs located on the target membrane or vesicle membrane, respectively (Rothman 1994). The trans-SNARE complex consists of 2-3 Q-SNAREs (Vam3, Vti1, and Vam7), and a single R-SNARE (Ykt6), “intertwined”, which acts as a tether to allow both vesicle lipid bilayers to come into close proximity to one another (Antonin et al. 2002). The localization of SNAREs to vesicles is not entirely clear; however, in general, a donor vesicle (i.e. autophagosome) will contain a single R-SNARE, while the acceptor vesicle (vacuole or lysosome) contains the two or three Q-SNAREs (Ungermann and Langosch 2005). Once formed, the trans-SNARE complex generates a force on the two bilayers that allows for fusion to occur (Lu et al. 2008b; Risselada and Grumbuller 2012; Risselada et al. 2014). This initial fusion gradually expands, and the two vesicles become a single vesicle (Risselada et al. 2014). Once fused, hydrolytic enzymes can degrade the contents within the autophagosome.
In mammals, autophagosome fusion with the lysosome involves the same basic protein machinery as in yeast. The proteins involved in autophagosome-lysosome fusion include: LC3/GABARAP, UVRAG, ATG14, RAB7, the HOPS complex, the Q-SNARES, STX17 and SNAP29, and the R-SNARE, VAMP7/8. Starvation or stress, leads to the recruitment of STX17 to completed autophagosomes, in a manner dependent on the lysosomal membrane protein LAMP2 (Itakura et al. 2012; Hubert et al. 2016). STX17 then recruits SNAP29 to the autophagosome, forming the STX17-SNAP29 binary-SNARE complex, which is stabilized by ATG14L (Guo et al. 2014c; Diao et al. 2015; Hubert et al. 2016). Atg14L promotes the interaction between the STX17-SNAP29 complex on the autophagosome, with VAMP7/8 on the lysosome, forming a trans-SNARE complex (Diao et al. 2015). The STX17-SNAP29-VAMP7/8 trans-SNARE complex is then stabilized by binding to EPG5, a metazoan-specific autophagy gene that is recruited to autophagosomes by both LC3 and RAB7 (Tian et al. 2010; Wang et al. 2016b). Moreover, as discussed above, UVRAG interacts with components of the C-Vps/HOPS complex, resulting in HOPS localization to autophagosomes, and late endosomes/lysosomes (Liang et al. 2008a; Sun et al. 2010). The small GTPase, ARL8B, also facilitates the localization of the HOPS complex to the lysosome (Khattar et al. 2015). Once localized to vesicles, the HOPS complex then recruits RAB7 onto autophagosomes, late endosomes, and lysosomes (Bucci et al. 2000; Gutierrez et al. 2004; Jager et al. 2004; Stroupe et al. 2006). RAB7, in a positive feedback loop, leads to additional recruitment of the HOPS complex, and HOPS promotes autophagosome-lysosome fusion by stabilizing the STX17-SNAP29-VAMP7/8 trans-SNARE complex (Jiang et al. 2014; Hubert et al. 2016; Wijdeven et al. 2016). Furthermore, members of the GABARAP superfamily mediate autophagosome-lysosome fusion by recruiting the phosphatidylinositol 4-kinase IIα (PI4KIIα) that generates phosphatidylinositol 4-phosphate on autophagosomes, which is important for lysosomal fusion (Wang et al. 2015a).

The mechanism of autophagosome-lysosome fusion in C. elegans has been well characterized (Al Rawi et al. 2011; Djeddi et al. 2012; Manil-Segalen et al. 2014b; Wang et al. 2016b). Upon completion of autophagosome formation, LGG-1 recruits LGG-2, which in turn binds to VPS-39, a subunit of the HOPS complex (Manil-Segalen et al. 2014b). The HOPS complex is also recruited to lysosomes via interaction with ARL-8 (Nakae et al. 2010; Sasaki et al. 2013). VPS-39, found on both autophagosomes and endosomes/lysosomes, then recruits RAB-7, which allows EPG-5 to localize to autophagosomes (Wang et
al. 2016b). EPG-5 promotes the formation of the STX-17-SNAP-29-VAMP-7/8 trans-SNARE complex, which facilitates autophagosome-lysosome fusion (Djeddi et al. 2012; Manil-Segalen et al. 2014b; Wang et al. 2016b). In support of this mechanism, loss or depletion of several of the above genes results in serious defects in autophagy flux. 

Igg-2 null mutant embryos have an accumulation of P-granules and SQST-1-positive aggregates, while depletion of rab-7 and vps-39 results in the accumulation of LGG-1 and LGG-2 labeled foci in embryos and larvae (Alberti et al. 2010b; Manil-Segalen et al. 2014b). In addition, arl-8 mutants exhibit defects in endosome-lysosome fusion and phagocytosis, and arl-8 depleted animals accumulate GFP::LGG-1 puncta (Nakae et al. 2010; Sasaki et al. 2013; N. Palmisano, unpublished results). Depletion of SNAP-29 results in an accumulation of SQST-1-labeled foci in larvae, and accumulation of GFP::LGG-1 in dauers (Guo et al. 2014c; N. Palmisano, unpublished results). Lastly, epg-5 LOF embryos accumulate P-granules, along with SQST-1 and LGG-1-labeled foci (Tian et al. 2010).

1.2.4. The role of amphisomes in autophagy function

Prior to fusing with lysosomes, it is well established that autophagosomes can also fuse with endosomes, forming hybrid organelles called amphisomes (Gordon and Seglen 1988). Evidence for the existence of amphisomes came from electron micrographs of mammalian cells, such as rat liver cells or guinea pig pancreatic cells, showing vesicles containing components of both the autophagy machinery and endosomes (Gordon and Seglen 1988; Tooze et al. 1990; Punnonen et al. 1993; Liou et al. 1997; Lucocq and Walker 1997; Berg et al. 1998; Reggiori et al. 2004b; Jones et al. 2012). The mechanism of autophagosome fusion with endosomes is believed to involve the same protein machinery as lysosomal fusion (Jager et al. 2004; Liang et al. 2008a; Sun et al. 2010). The main endocytic compartment that contributes to amphisome formation is the multivesicular body (MVB), a late endocytic vesicle composed of intraluminal vesicles (Palade 1955). Interestingly, in yeast, the existence of amphisomes has not been documented, even though they contain MVBs, suggesting that amphisome formation is transient in yeast, or that it evolved later in higher eukaryotes.

One of the main functions of MVBs is to deliver endocytic cargo, such as growth factors and their associated receptors, to the lysosome for degradation (Eden et al. 2009; Hanson and Cashikar 2012).
Formation of MVBs is regulated by the GTPase, RAB11, and the Endosomal Sorting Complex Required for Transport (ESCRT) protein machinery, an evolutionarily conserved protein complex consisting of five sub-complexes (Savina et al. 2002; Savina et al. 2005; Hurley 2008; Szatmari et al. 2014). Interestingly, VPS34 and Atg9 have been shown to regulate the formation of intraluminal vesicles (ILVs) in MVBs, for mammals and flies, respectively (Futter et al. 2001; Bader et al. 2015). Therefore, both the endocytic and autophagy pathways converge to regulate cell homeostasis.

Further support for the convergence of these two pathways comes from colocalization assays in mammals, worms, and flies. In HeLa cells, vesicles co-labeled with fluorescent antibodies specific to LC3 and the ESCRT subunit, VPS27/HRS, have been observed during starvation conditions (Tamai et al. 2007). Moreover, in HEK cells treated with rapamycin, there is an increase in the number of vesicles containing GFP-LC3 and immunostained with EEA1, an early endosomal protein (Wu et al. 2016). Amphisomes have also been visualized in *C. elegans* and *D. melanogaster*. In *C. elegans*, amphisomes are immunofluorescently labeled with anti-LGG-1, and anti-VPS-27 or anti-VPS-32, two ESCRT subunits (Djeddi et al. 2012; Manil-Segalen et al. 2014b). Additionally, loss of the autophagy protein, EPG-5, results in the accumulation of amphisomes containing GFP::LGG-1 and mCherry::RAB-7 or RFP::RME-1 (marker for basolateral recycling endosomes) (Wang et al. 2016b). In *D. melanogaster*, vesicles in fat body cells are found to contain both GFP-Atg8a and Texas Red-labeled Hrs/Vps27 (Rusten et al. 2007). In addition, amino acid starvation leads to a significant increase in the number vesicles co-labeled with Atg8a and Rab11 in *D. melanogaster* fat body cells (Szatmari et al. 2014).

Highlighting the importance of amphisome formation for autophagy, defects in the endocytic machinery can adversely affect autophagy function. In mammals, loss of various subunits part of the ESCRT complex, or loss of *Rab11*, hinders amphisome formation, and reduces the autophagic degradation of cargo, such as Huntington aggregates (Filimonenko et al. 2007; Fader et al. 2008; Szatmari et al. 2014; Oshima et al. 2016). Similarly, in *Drosophila*, defects in the ESCRT machinery result in an accumulation of autophagosomes and defects in the degradation of the p62/SQST-1 ortholog, Ref(2)p (Rusten et al. 2007; Morelli et al. 2014). In *C. elegans*, loss or depletion of the ESCRT machinery increases the number of GFP::LGG-1 and GFP::SQST-1-positive foci; however, this was interpreted to be an increase in autophagy activity, since the lysosomal cleavage of GFP from these reporters was observed (Djeddi et al. 2012). One
caveat to this interpretation is that autophagosomes themselves can become acidic via lysosomal fusion, and can therefore cleave GFP (Stromhaug and Seglen 1993). Additionally, ESCRT depleted animals showed an increase in LGG-1-II and SQST-1 protein levels in western blots, commonly associated with defects in flux (Djeddi et al. 2012). In all, amphisomes are a critical component of the autophagy pathway and are necessary for the proper degradation of autophagic cargo.

1.3. Membrane Trafficking

1.3.1. Overview of membrane trafficking

Membrane trafficking is an evolutionarily conserved process that involves the sorting and transport of cargo throughout the cell, and is divided into two major pathways, the biosynthetic/secretory pathway and endocytic trafficking pathway (Mellman 1996; Urbe et al. 1997; Shaw et al. 2001; Blom et al. 2011; Sato et al. 2014a; Feyder et al. 2015). The biosynthetic/secretory pathway involves the export and trafficking of newly synthesized ER-derived cargo throughout the endomembrane system, while the endocytic pathway involves the uptake and trafficking of cargo from the plasma membrane to the endomembrane system (Fig. 1.3) (Mellman 1996; Urbe et al. 1997). The endomembrane system encompasses interconnected membrane-bound organelles such as the plasma membrane (PM), early endosomes (EE), late endosomes/multivesicular bodies (LE/MVB), recycling endosomes (RE), lysosomes, the Golgi body, and the endoplasmic reticulum (ER) (Schmid 1993; Nebenfuhr 2002).

Membrane trafficking pathways utilize membrane-bound vesicles and membrane-bound compartments derived from the endomembrane system to sort and transport cargo (Mellman 1996; Urbe et al. 1997). Intracellular cargo can include transmembrane proteins, such as protein receptors and ion channels, or membrane lipids, such as phospholipids and sphingolipids (Blom et al. 2011; Maldonado-Baez et al. 2013). Membrane trafficking pathways play vital roles in maintaining cell growth, cell signaling and cell polarity (Mellman 1996; Maxfield and McGraw 2004; Soldati and Schliwa 2006; Grant and Donaldson 2009).

Sorting and trafficking of cargo along the endomembrane system requires the coordinated efforts of various GTPases, SNAREs, protein coats, and the cytoskeletal network (Mellman 1996; Stenmark 2009; Faini et al. 2013). Intracellular vesicles can be surrounded by specific protein coats, such as clathrin, and
retromer, which function in the specificity of cargo selection via interaction with cargo adaptor proteins, and provide a “mold” for vesicle formation (Seaman et al. 1998; Faini et al. 2013). Vesicle trafficking along the endomembrane system, is regulated by various GTPases, such as RAB GTPases (Zerial and Stenmark 1993; Chavrier and Goud 1999; Zerial and McBride 2001). RAB GTPases are responsible for protein coat assembly, facilitate vesicle transport, and mediate vesicle tethering/fusion events (Zerial and Stenmark 1993; Pfeffer 1994; Chavrier and Goud 1999; Zerial and McBride 2001; Donaldson and Honda 2005). In addition, SNARE proteins operate alongside RAB GTPases to ensure vesicle fusion with target membranes (Pfeffer 1994; Mellman 1996). Lastly, microtubules and actin filaments act as a molecular roadway that vesicles can travel along to reach their destination (Qualmann et al. 2000; Qualmann and Kessels 2002; Granger et al. 2014). The coordination between GTPases, SNAREs, protein coats, and the cytoskeleton ensures the efficient transport of cargo necessary for cell homeostasis and function (Altomare and Khaled 2012).

1.3.2. Cell Polarization

Cell polarity is a fundamental property found in all eukaryotic cells. This property allows cells to become highly organized and acquire specialized functions through the asymmetric organization and specialization of the plasma membrane, organelles, and the cytoskeletal network (Glotzer and Hyman 1995; Pringle et al. 1995; Drubin and Nelson 1996; Nelson 2003). In *Saccharomyces cerevisiae*, cell polarity is established during budding and mating, where the cytoskeletal network is rearranged to allow for vesicle/membrane trafficking toward the bud neck or mating projection, resulting in distinct membrane domains at those locations (Evangelista et al. 2002; Sagot et al. 2002; Valdez-Taubas and Pelham 2003; Wedlich-Soldner et al. 2003). Both polarized and non-polarized yeast have three main endocytic compartments: the post-Golgi endosome/early endosome, the pre-vacuolar compartment/late endosome, and the vacuole/lysosome (Singer-Kruger et al. 1993; Shaw et al. 2001; Pelham 2002).

In the case of polarized epithelial cells in mammals and *C. elegans*, polarity is accomplished by the reorganization of the cellular plasma membrane into structurally and functionally distinct apical and basolateral domains, with specialized protein and lipid compositions (Almers and Stirling 1984; Nelson 2003). Moreover, polarized epithelial cells have distinct apical and basolateral early endosomes, and
specialized recycling endosomes, while non-polarized cells have a uniform plasma membrane, along with randomly distributed early endosomes, and a single large recycling endosome (Bomsel et al. 1990; Mellman et al. 1993; Mellman 1995; Drubin and Nelson 1996; Nelson 2003; Welling and Weisz 2010). In polarized cells, the apical membrane faces the external environment/lumen, while the basolateral membrane faces the extracellular matrix (ECM) or basement membrane, and both membranes regulate the directional movement of ions and solutes across the cell (Almers and Stirling 1984; Muth et al. 1997; Nelson 2003; Stoops and Caplan 2014).

1.3.3. RAB GTPase regulation and cycling

GTPases are evolutionarily conserved molecular switches that cycle between active/GTP-bound and inactive/GDP-bound conformational states (Fig. 1.2) (Hutagalung and Novick 2011). Due to their structural properties and functions, these enzymes are classified into families, such as the RAB GTPase family, ARF GTPase family, etc (Schwartz et al. 2007). Members of the RAB GTPase family (RABs) lie at the heart of membrane trafficking, with roles in vesicle budding, vesicle coating and uncoating, vesicle transport, and vesicle fusion events (Zerial and Stenmark 1993; Pfeffer 1994; Zerial and McBride 2001; Stenmark 2009; Galvez et al. 2012). In their GTP-bound form, RABs can carry out various vesicle trafficking roles by recruiting effector proteins (Grosshans et al. 2006b; Schwartz et al. 2007; Gillingham et al. 2014). For example, Ypt6/RAB6 can transport post-Golgi vesicles along microtubules or actin filaments by interacting with effector proteins, such as the molecular motor proteins, that bind to the cytoskeletal network (Tsukada and Gallwitz 1996; Echard et al. 1998; Tsukada et al. 1999; Grigoriev et al. 2007; Wanschers et al. 2008; Suda et al. 2013). To ensure that RABs respond in a timely and appropriate manner to membrane trafficking needs, they constantly undergo cycles of activation and inactivation (Hutagalung and Novick 2011).

RAB cycling is coordinated by the actions of RAB regulatory proteins, such as GDP dissociation inhibitors (GDI), GDI-displacement factors (GDF), guanine nucleotide exchange factors (GEF), and GTPase activating proteins (GAPs) (Fig. 1.2) (Hutagalung and Novick 2011; Cherfils and Zeghouf 2013). After protein translation, RABs are escorted to membranes by a RAB escort protein (REP); however, the mechanism for RAB targeting to specific membranes is not fully understood (Alexandrov et al. 1994;
Miaczynska et al. 1997). At the membrane, a GEF loads the RAB with GTP, and the activated RAB interacts with a downstream effector protein that facilitates a specific membrane trafficking event (ie. vesicle motility) (Fig. 1.2) (Burstein and Macara 1992; Lai et al. 1993; Burton and De Camilli 1994; Hutagalung and Novick 2011; Blumer et al. 2013). Inactivation of the RAB is mediated by a GAP, which binds to the RAB and accelerates GTP hydrolysis, resulting in an inactive/GDP-bound conformational state, and dissociation of effector proteins (Fig. 1.2) (Strom et al. 1993; Vollmer and Gallwitz 1995; Rak et al. 2000; Hutagalung and Novick 2011). The inactive RAB is then removed from the membrane via interaction with GDI and becomes cytosolic (Sasaki et al. 1990; Schalk et al. 1996; Pylypenko et al. 2006; Hutagalung and Novick 2011). Dissociation from GDI and reattachment to the membrane requires the activity of GDF, resulting in the reactivation of the RAB, and continuous cycling between inactive and active states (Fig. 1.2) (Dirac-Svejstrup et al. 1997; Sivars et al. 2003).

**Figure 1.2: Regulation of GTPase cycling.** RAB GTPase cycling is coordinated by the actions of several regulatory proteins, such as GDP dissociation inhibitors (GDI), GDI-displacement factors (GDF), guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Hutagalung and Novick, 2011). RAB membrane localization requires the dissociation of GDI by GDF (Dirac-Svejstrup et al., 1997). At the membrane, a GEF loads the GTPase with GTP, resulting in its activation and interaction with a downstream effector protein that facilitates a specific membrane trafficking event (Burton and De Camilli, 1994; Hutagalung and Novick, 2011). Once the membrane trafficking event is performed, a GAP then hydrolyzes GTP to GDP, resulting in the dissociation of the effector protein, and inactivation and cytosolic localization of the GTPase (Strom et al., 1993; Hutagalung and Novick, 2011). The cycle then repeats with the reactivation of the GTPase (Hutagalung and Novick, 2011).
A single RAB is usually able to interact with multiple effector proteins, either directly or indirectly, and can facilitate various vesicle trafficking events. For example, the yeast ortholog of RAB10/RAB-10, Sec4, is a GTPase involved in post-Golgi vesicle secretion (see “The role of Sec4/RAB10/RAB-10 in membrane trafficking” below) (Novick et al. 1981; Salminen and Novick 1987). Sec4 interacts with a protein complex called the exocyst, which mediates vesicle tethering and fusion at the plasma membrane (Guo et al. 1999). Sec4 also interacts with Myo2, a Myosin V ortholog important for vesicle transport along actin filaments (Jin et al. 2011; Santiago-Tirado et al. 2011). Therefore, Sec4 couples vesicle transport with vesicle tethering by recruiting and interacting with Myo2 and the exocyst, respectively. In general, this allows a RAB to perform multiple membrane trafficking functions simultaneously in a regulated fashion.

1.3.4. The Endocytic pathway

The endocytic pathway is composed of functionally distinct membrane-bound compartments and vesicles whose function is largely regulated by specific RAB GTPases (Zerial and Stenmark 1993; Pfeffer 1994; Chavrier and Goud 1999; Zerial and McBride 2001; Donaldson and Honda 2005; Schwartz et al. 2007; Stenmark 2009). The endocytic pathway itself can be categorized into two functional pathways, the recycling pathway and the degradative pathway (Fig. 1.3) (Steinman et al. 1983; Huotari and Helenius 2011). To ensure continued growth and function, cells utilize endocytic recycling to reuse specific molecules, such as the transferrin receptor (TfR) in higher eukaryotes, or chitin synthase enzymes in yeast (Yamashiro et al. 1984; Valdivia et al. 2002). On the other hand, to shut off cellular signaling pathways, or remove ubiquitinated cargo, cells will utilize the degradative properties of the late endosome/lysosome (Futter et al. 1996; Tjelle et al. 1996; Huotari and Helenius 2011).
In an evolutionarily conserved fashion, the endocytic pathway consists of four main endocytic compartments: 1. Early endosomes (EE), 2. Late endosomes/Multivesicular bodies (LE/MVB), 3. Recycling endosomes (RE), and 4. Lysosomes.

Figure 1.3: Schematic representation of membrane trafficking in *C. elegans* intestinal epithelia. Membrane trafficking ensures that cargo is correctly sorted to specific areas of the cell, allowing the cell to use those components for the synthesis of macromolecules needed for growth (Mellman, 1996). In polarized intestinal cells, basolateral and apical specific cargo are taken up into their respective early endosomes. This cargo is then sorted and delivered to either recycling endosomes for re-use (yellow arrows), or late endosomes for degradation (red arrows) (Huotari and Helenius, 2011; Steinman et al., 1983). Endosomes can be classified according to the specific proteins associated with their membranes, such as the Rab GTPases, RAB-5 and RAB-7, on early endosomes and late endosomes, respectively (Pfeffer, 1994; Zerial and Stenmark, 1993). In *C. elegans*, RAB-10 is commonly associated with basolateral early endosomes, and possibly, common recycling endosomes (Chen et al., 2006).
1.3.4.1. Early endosomes/Sorting endosomes

Early endosomes (EEs) are the first to receive internalized cargo from the plasma membrane, and are commonly referred to as sorting endosomes, since they sort and transport cargo to various locations in the cell, such as the plasma membrane, the Golgi, recycling endosomes, or lysosomes (Dunn et al. 1989; Jovic et al. 2010). The early endosome has a complex morphology, consisting of a tubulo-vesicular structure with few intraluminal vesicles (ILVs), along with distinct sub-domains, enriched with various GTPases and protein coats, which act as hubs for the sorting and trafficking of cargo to different intracellular locations (Dunn et al. 1989; Sonnichsen et al. 2000; Gruenberg 2001).

The most prominent RAB GTPase present on EEs is Ypt51/RAB5/RAB-5 (RAB5) (Fig. 1.3) (Chavrier et al. 1990a; Singer-Kruger et al. 1994; Grant and Hirsh 1999; Gerrard et al. 2000; Sato et al. 2014a). RAB5 is important for the uptake and formation of clathrin-coated vesicles and promotes endosome-to-endosome fusion (homotypic fusion) events (Gorvel et al. 1991; Bucci et al. 1992; Singer-Kruger et al. 1995; Gerrard et al. 2000).

Active RAB5 recruits VPS34 to early endosomes, which results in the production of PI3P (Christoforidis et al. 1999b; Murray et al. 2002). However, in contrast to autophagy, PI3P in the endocytic pathway leads to the recruitment of the RAB5 effector proteins, Vac1/EEA1/EEA-1 and Vac1/RABENOSYN-5/RABS-5 (Mu et al. 1995; Patki et al. 1997; Simonsen et al. 1998; Christoforidis et al. 1999a; Tall et al. 1999; Lawe et al. 2000; Nielsen et al. 2000; Sato et al. 2005). Important for the tethering/docking and homotypic fusion of EEs, EEA1 facilitates the interaction between the Q-SNAREs, SYNTAXIN6, SYNTAXIN13, and VTI1a, and the R-SNARE, VAMP4 (Simonsen et al. 1999; Brandhorst et al. 2006; Ohya et al. 2009). In yeast, early endosomes are also referred to as post-Golgi endosomes, which in addition to containing Ypt51, also contain the Q/t-SNARE, Tlg1/2 (Singer-Kruger et al. 1993; Shaw et al. 2001; Pelham 2002).

Early endosomes gradually mature into late endosomes, which further mature into multivesicular bodies (MVBs), a process mediated in part by the actions of RAB5 (discussed below) (Stoorvogel et al. 1991; Dunn and Maxfield 1992; Peplowska et al. 2007).

1.3.4.2. Late endosomes/Multivesicular bodies
Late endosomes and multivesicular bodies function in transporting cargo destined for lysosomal degradation, as well as delivering hydrolytic enzymes and membrane proteins needed for vacuole/lysosome function (Tjelle et al. 1996; Huotari and Helenius 2011). In yeast, the late endosome is also referred to as the pre-vacuolar endosome and is characterized by the presence of Pep12 (Singer-Kruger et al. 1993; Pelham 2002). The maturation of early endosomes into late endosomes is accompanied by several morphological changes. Maturing early endosomes become more acidic, develop more ILVs, due to the increased association of ESCRT components on the early endosome, and lose their tubules (Russell et al. 2006; Hurley 2008; Huotari and Helenius 2011). The ESCRT machinery is evolutionarily conserved from yeast to mammals, and consists of five sub-complexes that coordinate with one another to form ILVs, which carry sequestered ubiquitinated cargo (Katzmann et al. 2001; Urbanowski and Piper 2001; Raiborg et al. 2002; Hurley 2008). ILVs ensure that signaling receptors are not in contact with the cytosol and provides easy access for lysosomal degradation, since ILVs are not surrounded by glycoproteins that typically protect membranes from lysosomal degradation (Huotari and Helenius 2011). In addition, maturing early endosomes gradually lose RAB5 and acquire the late endocytic GTPase, Ypt7/RAB7/RAB-7 (RAB7), via the actions of the RAB7 GEF, Mon1/MON1/SAND-1 (Schimmoller and Riezman 1993; Meresse et al. 1995; Grant and Hirsh 1999; Bucci et al. 2000; Poteryaev et al. 2007) (Fig. 1.3). The recruitment of Mon1/MON1/SAND-1 to maturing early endosomes depends on PI3P levels produced by VPS-34 (Poteryaev et al. 2007; Poteryaev et al. 2010). The primary function of RAB7 is to ensure the delivery and promote the fusion of LE/MVBs with autophagosomes and lysosomes, via the recruitment of the HOPS tethering complex and molecular motor proteins (Wichmann et al. 1992; Schimmoller and Riezman 1993; Meresse et al. 1995; Bucci et al. 2000).

1.3.4.3. The Lysosome/Vacuole

The vacuole/lysosome (lysosome) is the main digestive compartment of the cell that receives cargo extracellularly, via endocytosis and phagocytosis, or intracellularly, via autophagy (De Duve et al. 1955; DeDuve 1963; DeDuve and Wattiaux 1966; Luzio et al. 2007; Li and Kane 2009). The highly acidic environment of lysosomes is due to the concentrated presence of vacuolar H⁺ ATPases on the lysosomal membrane, which pump protons into the lysosomal lumen (Ohkuma et al. 1982; Yamashiro et al. 1983;
Uchida et al. 1985; Kane et al. 1989). The acidic pH of the lysosome is optimal for the activity of various hydrolytic enzymes, such as proteases, hydrolases, and lipases, which degrade proteins, lipids, and various other macromolecules (Coffey and De Duve 1968; Ohkuma and Poole 1978). The constituent molecules produced after degradation are reused by the cell for the synthesis of new macromolecules (Maguire et al. 1983; Town et al. 1998; Russnak et al. 2001). Various glycoproteins, such as LAMP1/LMP-1 in higher eukaryotes or Vgp72 in yeast, protect the lysosomal membrane from hydrolytic degradation (Fig. 1.3) (Chen et al. 1988; Nishikawa et al. 1990). In addition, the lysosomal membrane contains various nutrient transporters, such as glucose transporters, which export various nutrients from the lysosomal lumen into the cell cytosol (Maguire et al. 1983; Russnak et al. 2001; MacDiarmid et al. 2002; Gao et al. 2005; Liu et al. 2012a; Wang et al. 2015b). Moreover, as described above, TOR is localized to the lysosomal membrane where it is able to sense and respond to differing nutrient levels, either activating or repressing cell growth and autophagy (Rohde et al. 2001; Fingar and Blenis 2004; Peli-Gulli et al. 2015; Kira et al. 2016). Lysosomes therefore play an essential role in the degradation and recycling of unneeded cellular macromolecules into usable forms.

1.3.4.4. Recycling endosomes

The endocytic recycling pathway regulates the return of various proteins and lipids back to the plasma membrane after internalization, which have vital roles in signal transduction, cell adhesion, motility, nutrient uptake, and cell polarity (Maxfield and McGraw 2004; Soldati and Schliwa 2006; Grant and Donaldson 2009). After internalization into early endosomes, cargo can be recycled back to the plasma membrane via a “fast” or “slow” recycling route (Mayor et al. 1993; Sheff et al. 1999; Hao and Maxfield 2000). Fast recycling involves the direct trafficking of cargo from the early endosome to the plasma membrane, while slow recycling involves the trafficking of cargo from EEs to recycling endosomes, prior to reaching the plasma membrane (Mayor et al. 1993; Hao and Maxfield 2000; Grant and Donaldson 2009). The recycling endosome, also known as the endocytic recycling compartment, is generally tubular-vesicular in nature, from which recycling endocytic vesicles bud from (Marsh et al. 1986; Griffiths et al. 1989).

In yeast, all cargo is recycled via the fast recycling route, while in higher eukaryotes, cargo typically will utilize both recycling routes (Wiederkehr et al. 2000; Shaw et al. 2001; Grant and Donaldson 2009).
The fast recycling route allows for the rapid return of protein receptors to the plasma membrane during high cellular demands for nutrient uptake and cell signaling, while the slow recycling route allows for receptors and other cargo to travel deeper into the cell to mediate various cellular processes, such as organelle homeostasis, nutrient utilization, etc. (Hao and Maxfield 2000; Maxfield and McGraw 2004; Grant and Donaldson 2009).

In polarized epithelial cells, cargo will travel through several specialized recycling endosomes, such as the common recycling endosome (CRE), apical recycling endosome (ARE), and basolateral recycling endosomes (BRE) (Fig. 1.3). The CRE receives and sorts cargo from both the basolateral and apical plasma membranes, and is characterized by the presence of RAB10/RAB-10 (Knight et al. 1995; Wang et al. 2000; Babbey et al. 2006). The ARE is localized near the apical membrane of epithelial cells, and is commonly associated with RAB11/RAB-11.1, ortholog of Ypt31/32 (Fig. 1.3) (Apodaca et al. 1994; Barroso and Sztul 1994; Casanova et al. 1999). The ARE functions specifically in recycling to the apical membrane and is devoid of basolateral cargo (Apodaca et al. 1994; Casanova et al. 1999; Wang et al. 2000). Basolateral recycling endosomes seem to be specific to C. elegans polarized cells and contain the Eps15 homology domain (EHD) containing protein, RME-1 (Fig. 1.3) (Grant et al. 2001; Lin et al. 2001b). Although the BREs are not formally classified in mammalian cells, RME1/EHD1 in mammals is localized to recycling endosomes and functions in general endocytic recycling (Grant et al. 2001; Caplan et al. 2002). Commonly known basolateral cargo markers include the transferrin receptor (TfR), while apical cargo markers include influenza A virus hemagglutinin (HA) (Rodriguez Boulan and Pendergast 1980; Gibson et al. 1998).

### 1.3.4.5. Endosome-to-Golgi trafficking: Roles of the Retromer complex

The retromer is an evolutionarily conserved multi-subunit protein complex that facilitates the retrograde transport of cargo from early endosomes/post-Golgi compartment to the trans-Golgi network (Seaman et al. 1998). Subunits of the retromer include: Vps35, Vps29, Vps26, Vps5, and Vps17, which form two sub-complexes. The first subcomplex, is the cargo selection complex, and consists of Vps35, Vps29, and Vps26, while the second subcomplex includes Vps5 and Vps17, which is required for vesicle formation/assembly (Seaman et al. 1998; Nothwehr et al. 1999). Retromer components in S. cerevisiae were discovered by screening for mutants defective in endosome-to-Golgi trafficking of the
carboxypeptidase Y (CPY) sorting receptor, Vps10 (Marcusson et al. 1994; Horazdovsky et al. 1997; Seaman et al. 1997; Seaman et al. 1998; Bowers and Stevens 2005). Vps10, bound to CPY, travels to early endosomes, where CPY dissociates from Vps10, and Vps10 is recycled back to the Golgi in retromer-coated vesicles (Stevens et al. 1982; Bryant and Stevens 1998). Loss of the retromer complex results in the missorting of Vps10 to the vacuole due to the eventual maturation of early endosomes to late endosomes, which then fuse with the vacuole (Horazdovsky et al. 1997; Seaman et al. 1997; Rojas et al. 2008; Liu et al. 2012b). In addition, subunits of the autophagy nucleation complex are required for retromer-mediated retrograde trafficking (Seaman et al. 1997; Kametaka et al. 1998; Burda et al. 2002). Loss of either ATG6 (Beclin1/bec-1), or VPS34 (Vps34/vps-34), results in the missorting of Vps10 to the vacuole, and CPY to the plasma membrane (Seaman et al. 1997; Kametaka et al. 1998; Burda et al. 2002).

In mammals, the retromer complex includes: SNX1/2, SNX5/6, VPS26A/B, VPS29, and VPS35, which are orthologs of Vps5, Vps17, Vps26, Vps29, and Vps35, respectively (Haft et al. 2000; Rojas et al. 2007; Wassmer et al. 2007). The best characterized cargo molecule for the retromer in mammals is the cation independent-mannose 6-phosphate receptor (CI-MPR) (Arighi et al. 2004; Seaman 2004). Failure to recycle CI-MPR results in its lysosomal degradation, inhibits cathepsin D protease maturation, and leads to the enlargement of lysosomes (Arighi et al. 2004; Seaman 2004). The mammalian retromer complex also recycles the Wnt receptor, Wntless, back to the Golgi. In mammalian cells, Wntless colocalizes with the retromer complex, and decreased levels of VPS35 reduce the secretion of WNT proteins (Belenkaya et al. 2008; Yang et al. 2008). Moreover, in microglial cells, the retromer complex functions with BECLIN1 and VPS34 in the recycling of the phagocytic receptor, CD63/LAMP3, during phagocytosis (Lucin et al. 2013).

The retromer complex in C. elegans includes: SNX-1, SNX-6, VPS-26, VPS-29, VPS-35, and RME-8 (Coudreuse et al. 2006; Shi et al. 2009; Wassmer et al. 2009). The best characterized retromer cargo in C. elegans is the Wntless ortholog, MIG-14 (Yang et al. 2008; Zhang et al. 2012). In C. elegans, Wnt signaling is important for endoderm induction, neuronal migration, and cell fate specification (Whangbo and Kenyon 1999; Hilliard and Bargmann 2006; Prasad and Clark 2006). Newly synthesized Wnts bind to MIG-14 at the Golgi and are delivered to the PM, where they are secreted out of the cell. At
the PM, MIG-14 is then endocytosed and delivered back to the Golgi in retromer-coated vesicles (Pan et al. 2008; Yang et al. 2008). MIG-14 is missorted to the lysosome in the absence of retromer, leading to a variety of developmental defects, such as polarity defects and neuronal migration defects (Pan et al. 2008; Yang et al. 2008; Shi et al. 2009). Moreover, depletion of either BEC-1 or VPS-34 results in the missorting of MIG-14 into LMP-1/LAMP1-positive lysosomal compartments (Ruck et al. 2011). BEC-1 interacts with the retromer by associating with RME-8, a J-domain protein part of the retromer complex (Shi et al. 2009; Ruck et al. 2011). Furthermore, like mammals, autophagy proteins, such as BEC-1, and the retromer complex, function in apoptotic cell corpse clearance (refer to “Phagocytosis” below) (Ruck et al. 2011; Li et al. 2012a; Huang et al. 2012; Chen et al. 2010b; Lu et al. 2011a). Overall, the retromer is an evolutionarily conserved protein complex with its basic function being mirrored in yeast, mammals, and C. elegans.

1.3.4.6. Golgi-to-Plasma Membrane trafficking: Roles of the Exocyst complex

The trans-Golgi Network (TGN) is responsible for separating cargo destined for the cell surface and endo-lysosomal compartments (Griffiths and Simons 1986). The TGN contains specialized membrane domains, which act as a platform from which distinct transport tubules and vesicles bud and form (Griffiths and Simons 1986). From yeast to mammals, cargo delivery from the TGN to the PM depends on the exocyst complex (TerBush et al. 1996; Hsu et al. 2004). The exocyst complex is a multimeric protein complex that consists of: Sec15, Sec5, Sec10, Sec6, Sec8, Sec3, Exo70, and Exo84 (TerBush et al. 1996). These subunits coordinate with one another to facilitate post-Golgi vesicle secretion at the plasma membrane (Guo et al. 1999).

In yeast, the exocyst complex can be divided into two subcomplexes, the first consisting of Sec15, Sec10, Exo84, Sec5, Sec6, and Sec8 (Sec15 complex), and the other subcomplex consisting of Sec3 and Exo70 (Exo70 complex) (Finger et al. 1998; Guo et al. 1999; Boyd et al. 2004). The Exo70 complex represents a spatial landmark for the exocyst complex, and is localized to sites of exocytosis on the plasma membrane, while the Sec15 complex assembles on secretory vesicles (Finger et al. 1998; Boyd et al. 2004). On secretory vesicles, Sec15 directly interacts with the GTPase, Sec4, and via interaction between Sec4 and the myosin motor, Myo2, secretory vesicles are transported to sites on the membrane.
PM marked by the Exo70 complex (Guo et al. 1999; Boyd et al. 2004; He et al. 2007; Jin et al. 2011; Luo et al. 2014). At the PM, all subunits of the exocyst come together, and via interaction with plasma membrane resident SNAREs, the exocyst complex mediates the tethering and fusion of secretory vesicles with the PM membrane (refer to “The role of Sec4/RAB10/RAB-10 in membrane trafficking” below) (Lehman et al. 1999; Grosshans et al. 2006a; Dubuke et al. 2015).

In mammals, the exocyst complex also functions in secretion, with additional functions in ciliogenesis and cell migration (Wu and Guo 2015). The mammalian exocyst complex consists of the same subunits as in yeast, and is also divided in to two main subcomplexes, one of which consists of SEC15, SEC10, and EXO84, while the other consists of SEC5, SEC6, SEC8, SEC3, and EXO70 (Ting et al. 1995; Hsu et al. 2004). The SEC15/EXO84 subcomplex is localized to secretory vesicles, while the SEC5 subcomplex is localized to the plasma membrane, as a result of SEC3 and EXO70 (Moskalenko et al. 2003; Liu et al. 2007). In mammals, the exocyst complex interacts with a variety of GTPases to facilitate exocytosis in a tissue-specific manner (Tracy et al. 2016). In polarized Madin-Darby Canine Kidney cells (MDCK) cells, the exocyst complex is essential for vesicle trafficking to both apical and basolateral membranes (Grindstaff et al. 1998; Yeaman et al. 2004; Oztan et al. 2007; Bryant et al. 2010). Exocyst-mediated trafficking of vesicles to the apical membrane is dependent on, RAB8 and RAB11, while the GTPase that facilitates exocyst-mediated vesicle trafficking to the basolateral membrane remains unknown (Grindstaff et al. 1998; Yeaman et al. 2004; Oztan et al. 2007; Bryant et al. 2010). In renal epithelial cells and 3T3-L1 adipocytes, RAB10 interacts with the exocyst complex to regulate post-Golgi (refer to “The role of Sec4/RAB10/RAB-10 in membrane trafficking” below) (Karunanithi et al. 2014).

The exocyst complex also plays an important role in autophagy in mammalian cells, which is dependent on the GTPase, RALB (Bielinski et al. 1993; de Leeuw et al. 1999; Moskalenko et al. 2002; Moskalenko et al. 2003; Bodemann et al. 2011). Components of the exocyst complex and RALB interact with components of the autophagy machinery, and interaction between the exocyst and RALB is required for starvation-induced autophagy (Bodemann et al. 2011). Interestingly, in regards to autophagy, two different exocyst complexes exist: one containing SEC5 and lacking EXO84 (SEC5 subcomplex), and the other containing EXO84 and lacking SEC5 (EXO84 subcomplex) (Bodemann et al. 2011). During nutrient rich conditions, the SEC5 subcomplex contains inactive RALB, ULK1 and mTORC1, where mTORC1
inhits ULK1 (Hosokawa et al. 2009; Jung et al. 2009; Bodemann et al. 2011). During starvation, RALB activation occurs, and SEC5 is replaced with EXO84, leading to mTORC1 dissociation, ULK1 activation, and recruitment of the PI3KC3 complex (Bodemann et al. 2011). Therefore, in coordination with RALB, the exocyst acts as a scaffold for autophagy complex assembly (Farre and Subramani 2011).

In C. elegans, the exocyst functions in endocytic trafficking events in cooperation with the GTPases, RAB-10 and RAL-1. SEC-10 functions downstream of RAB-10, in the regulation of basolateral endocytic recycling (refer to “The role of Sec4/RAB10/RAB-10 in membrane trafficking” below) (Chen et al. 2014). In addition, the exocyst functions with the GTPase, RAL-1 (RALA/B ortholog) in intracellular lumenogenesis (Frische et al. 2007; Armenti et al. 2014). In excretory cell lumen formation, RAL-1 recruits the exocyst complex to sites on the apical PM that contain PAR proteins (Polarity proteins). At these sites, the exocyst complex binds to RAL-1-positive exocytic vesicles that carry proteins important for lumenogenesis, and promotes their fusion (Armenti et al. 2014).

In summary, the exocyst is an evolutionarily conserved multimeric protein complex that coordinates with GTPases to mediate functions in vesicle secretion, as well as other processes, such as autophagy.

1.4. Phagocytosis

Phagocytosis is an evolutionarily conserved process found in higher eukaryotes that involves the uptake of large particles, such as pathogens or apoptotic/necrotic cell corpses, from the extracellular environment of an engulfing cell (Metchnikoff 1921). Phagocytic cells can be classified as professional phagocytes (i.e. macrophages) or non-professional phagocytes (i.e. epithelial cells), with non-professional phagocytes having a limited range in particle recognition compared to professional phagocytes (Rabinovitch 1995). Phagocytosis operates under the same principles as basic endocytosis and is divided up into two main parts: first, the recognition and uptake of cargo, and second, the maturation and degradation of cargo (Aderem and Underhill 1999).

The mechanism of apoptotic/necrotic cell corpse engulfment and degradation is well characterized in C. elegans. During development, specific somatic cells and several hundred germ cells undergo programmed cell death (Sulston and Horvitz 1977; Sulston et al. 1983; Gumienny et al. 1999). These
apoptotic cells are rapidly engulfed and degraded by neighboring cells (Gumienny et al. 1999; Gumienny and Hengartner 2001). *C. elegans* have two functionally redundant cell engulfment pathways which comprise specific *ced* (cell death abnormality) genes. One pathway consists of *ced-1, ced-6,* and *ced-7,* while the other consists of *ced-2, ced-5, ced-12* (Hedgecock et al. 1983; Ellis et al. 1991; Kinchen et al. 2005).

In the *ced-1* pathway, CED-1 is a transmembrane receptor that recognizes and binds to phosphatidylserine (PS) located on the surface of apoptotic cells (Fadok et al. 1992; Zhou et al. 2001b; Yu et al. 2008; Li et al. 2015). CED-1 is recruited to the PM of engulfing cells by CED-7, an ABC transporter (Wu and Horvitz 1998a; Mapes et al. 2012). CED-6, an adaptor protein, then interacts with CED-1 and recruits downstream proteins required for phagosome degradation (Liu and Hengartner 1998; Su et al. 2002). CED-1 is potentially recycled by the retromer subunits, SNX-1 and SNX-6, allowing for its continued reuse by the cell (Chen et al. 2010b; Wang and Yang 2016). In the *ced-2* pathway, PSR-1 acts as the transmembrane receptor that binds to PS on apoptotic/necrotic cell corpses (Yang et al. 2015). CED-2 relays engulfment signals downstream of PSR-1, and activates the CED-5/CED-12 complex (Wu and Horvitz 1998b; Reddien and Horvitz 2000; Gumienny et al. 2001; Zhou et al. 2001a). The CED-5/CED-12 complex acts as a GEF for the Rac GTPase, CED-10, which integrates signals from both the CED-1 and CED-2 pathways to reorganize the cytoskeletal network for cell corpse engulfment and phagosome formation (Wu and Horvitz 1998b; Gumienny et al. 2001; Zhou et al. 2001a; Kinchen et al. 2005; Kinchen et al. 2008).

Components downstream of CED-10 that regulate phagosome maturation include the same components that regulate endosome maturation (Wang and Yang 2016). In phagosome maturation, RAB-5 is recruited to phagosomes, in a manner dependent on the GTPase, DYN-1/DYNAMIN1 (Kinchen et al. 2008). On phagosomes, RAB-5 recruits VPS-34, which along with the class II PI3K, PIKI-1, produces PI3P (Kinchen et al. 2008; Lu et al. 2012). PI3P then results in the recruitment of the RAB-7 GEF, the SAND-1/CCZ-1 complex, which recruits RAB-7 and the HOPS complex to phagosomes (Kinchen et al. 2008; Yu et al. 2008; Xiao et al. 2009; Kinchen and Ravichandran 2010; Nieto et al. 2010). The HOPS complex and RAB-7 also recruit ARL-8, and both ARL-8 and RAB-7 promote phagosome fusion with the lysosome (Kinchen et al. 2008; Kinchen and Ravichandran 2010; Sasaki et al. 2013).
Components of the autophagy machinery have been shown to play an important role in cell corpse clearance. Loss of *unc-51*, *bec-1*, *vps-34*, *atg-7*, *atg-18*, and *epg-5* results in a persistent cell corpse phenotype in the germline and/or embryos (Takacs-Vellai et al. 2005; Ruck et al. 2011; Huang et al. 2012; Li et al. 2012a). Interestingly, loss of *epg-5* and/or *atg-18* delays the recruitment of RAB-5 and RAB-7 to phagosomes, suggesting that EPG-5 and ATG-18 function in RAB-5 recruitment to phagosomes (Huang et al. 2012). LGG-1 is also recruited to phagosomes, which is interesting, since it is also observed in mammals in a process termed LC3-associated phagocytosis (LAP), and therefore may be evolutionarily conserved (Huang et al. 2012; Lai and Devenish 2012).

1.5. The role of Sec4/RAB10/RAB-10 in membrane trafficking

Sec4 was the first RAS-like GTPase discovered to play a role in membrane trafficking, specifically in the secretion of post-Golgi vesicles to the plasma membrane, and shares high sequence similarity to mammalian RAB10 and RAB8 (Salminen and Novick 1987; Chavrier et al. 1990b; Chen et al. 1993). Sec4 has a GTP-binding domain with high sequence similarity to the GTP-binding domains of the mammalian RAS GTPase, p21, and yeast RAS-like GTPase, Ypt1/RAB1/RAB-1. The membrane surface of secretory vesicles is positive for Sec4, where it cycles between its active and inactive states to mediate vesicle fusion with the plasma membrane (Goud et al. 1988; Walworth et al. 1989). In line with this, *sec4* temperature sensitive mutants, and Sec4 mutants defective in GTP hydrolysis, have a block in general protein secretion and accumulate secretory vesicles (Novick et al. 1980; Salminen and Novick 1987; Walworth et al. 1989).

Sec4 mediates post-Golgi vesicle fusion with the plasma membrane by interacting with the exocyst complex and components of the exocytic SNARE complex (Brennwald et al. 1994; Guo et al. 1999; Grosshans et al. 2006a). On secretory vesicles, Sec4 becomes GTP-bound by its GEF Sec2, and directly interacts with Sec15, recruiting the exocyst complex to secretory vesicles (Salminen and Novick 1987; Walch-Solimena et al. 1997; Guo et al. 1999; Luo et al. 2014). Via interaction between Sec4 and the myosin motor, Myo2, the secretory vesicles then travel to the plasma membrane where they interact with the exocytic SNARE complex (Novick et al. 2006; Jin et al. 2011). The exocytic SNARE complex consists of the secretory vesicle SNARE, Snc1/2, and the plasma membrane SNAREs, Sso1/2 and Sec9.
Via interaction between Sec4, the exocytic SNARE complex, and the exocyst complex, secretory vesicles can tether and fuse to the plasma membrane (Lehman et al. 1999; Grosshans et al. 2006a; Dubuke et al. 2015). In addition to post-Golgi vesicle secretion, Sec2 and Sec4 promote autophagy activity during starvation (Geng et al. 2010). Similar to atg1 deletion mutants, sec2 and sec4 temperature sensitive mutants have a decrease in Pho8 phosphatase activity (assay for measuring autophagy activity), when grown at the non-permissive temperature during starvation conditions. Moreover, as measured by Pho8 activity, yeast constitutively expressing a GDP-locked form of Sec4 display a dominant negative phenotype for autophagy activity during starvation conditions, suggesting that the GTPase activity of Sec4 is required for functional autophagy during starvation (Geng et al. 2010). In wild type yeast, Atg-9::GFP puncta are dispersed throughout the cytosol, irrespective of nutrient status (Reggiori et al. 2005). Recycling of Atg9 from the PAS and back to the periphery depends on the Atg1 complex, and in atg1 deletion mutants, Atg9::GFP is sequestered to a single punctum, representing the PAS (Shintani and Klionsky 2004; Reggiori et al. 2005). During starvation, atg1 deletion mutants carrying temperature sensitive mutations in either sec2 or sec4, have restored Atg9::GFP localization to multiple puncta at the restrictive temperature (Geng et al. 2010). Therefore, Sec4 and Sec2 promote starvation-induced autophagy by mediating the anterograde/forward trafficking of Atg9 to the PAS. Interestingly, as discussed above, trafficking of Atg9 to the PAS also requires the SNARE proteins Sso1/2 and Sec9 (Nair et al. 2011). Although a direct connection between Sec9 and Sec4 was made for the secretion of secretory vesicles, a direct connection between Sec4 and Sec9 in autophagy regulation has not yet been shown (Nair et al. 2011). In all, both Sec2 and Sec4 regulate post-Golgi secretion of secretory vesicles and the anterograde trafficking of Atg9 to the PAS.

In mammals, RAB10 regulates basolateral endocytic recycling and vesicle secretion (Babbey et al. 2006; Schuck et al. 2007; English and Voeltz 2013). In mice, RAB10 is essential for viability, since Rab10−/− homozygous mice are embryonic lethal at E7.5, and contain a large number of enlarged vacuoles in cells, along with ER hyperplasia (Lv et al. 2015). In polarized cells, RAB10 is mainly localized to common recycling endosomes (CRE) (Fig. 1.3) (Chen et al. 1993; Babbey et al. 2006; Schuck et al. 2007; English and Voeltz 2013). RAB10 GTPase cycling is required for its ability to localize to CREs,
since RAB10 in either a constitutively inactive/GDP-locked form, or constitutively active/GTP-locked form, accumulates at the Golgi or apical recycling endosomes (ARE), respectively (Babbey et al. 2006). Furthermore, RAB10 in its constitutively inactive or active form increases the recycling rates of basolaterally derived cargo from basolateral early endosomes (BEE) to the PM; due to the inability of cargo to enter the CRE, and properly sort throughout the cell (Babbey et al. 2006). Conversely, the recycling rates of apically derived cargo remain normal in the presence of active or inactive RAB10, confirming specificity of RAB10 in the basolateral endocytic pathway, specifically in the transport of cargo from the BEE to the CRE (Babbey et al. 2006).

During the early stages of cell polarization, RAB10 is mainly localized to the Golgi to regulate post-Golgi secretion; however, as cells become more polarized, RAB10 changes its localization pattern and resides at the CRE (Chen et al. 1993; Babbey et al. 2006; Schuck et al. 2007). Hence, in the early stages of polarization, RAB10 functions in vesicle secretion to the PM. RAB10 in its constitutively active form delays the secretion of the basolateral specific cargo molecules, such as vesicular stomatitis virus glycoprotein (VSV-G), from the Golgi to the basolateral membrane, which eventually results in their missorting to the apical plasma membrane (Schuck et al. 2007). The inactive form of RAB10 could not be evaluated for secretion due to its instability (Schuck et al. 2007). Further supporting a role for RAB10 in secretion, constitutively active RAB10 results in an accumulation of VSV-G at the TGN, the main site for cargo secretion (Griffiths and Simons 1986; Schuck et al. 2007).

In mammalian adipocytes, RAB10, along with its GEF, DENND4C, and its GAP, AS160, are important for the insulin stimulated secretion/translocation of GLUT4 storage vesicles (GSV’s) to the plasma membrane (Eguez et al. 2005; Larance et al. 2005; Miine et al. 2005; Sano et al. 2007; Yoshimura et al. 2010; Sano et al. 2011; Chen et al. 2012; Karunanithi et al. 2014). GLUT4 is the mammalian glucose transporter/receptor, which is delivered to the PM and binds to glucose in response to insulin signaling (Suzuki and Kono 1980; Karnieli et al. 1986; Martin et al. 2000; Bryant et al. 2002; Watson et al. 2004; Foley et al. 2011). In the absence of insulin stimulation, GLUT4 is localized to the TGN, where GSVs form (Slot et al. 1991).

The mechanism of GLUT4 translocation by RAB10 is well elucidated. In the absence of insulin stimulation, RAB10, AS160, and DENND4C are localized to GLUT4 vesicles, where RAB10 activity is
inhibited by AS160 (Miinea et al. 2005; Yoshimura et al. 2010; Chen et al. 2012). In response to insulin stimulation, the AKT/PKB kinase inhibits AS160, which allows for RAB10 activation by DENND4C (Sano et al. 2003; Larance et al. 2005; Sano et al. 2011; Chen et al. 2012; Sadacca et al. 2013). Active RAB10 then activates the GTPase, RALA, via the recruitment of the RALA GEF, RLF/RGL2 (Karunanithi et al. 2014). RALA recruits and assembles the exocyst complex onto GSVs, which also binds to RAB10, via interaction between RAB10 and SEC15 (Moskalenko et al. 2003; Chen et al. 2007; Chen et al. 2011; Karunanithi et al. 2014; Sano et al. 2015). RAB10 then binds to the myosin-Va motor protein, which attaches to actin to deliver GLUT4 vesicles to the PM (Chen et al. 2012). In support of the above mechanism, siRNA-mediated depletion of RAB10 in insulin stimulated cells reduces GLUT4 translocation to the PM, which is suppressed by overexpression of RLF/RGL2 (Sano et al. 2007; Karunanithi et al. 2014). Moreover, constitutively active and inactive RAB10 results in an increase and decrease of RALA activity, respectively, while expression of GTP-locked RAB10, overexpression of DENND4C, or AS160 depletion, increase the translocation of GLUT4 vesicles to the PM (Sano et al. 2007; Sano et al. 2011; Chen et al. 2012). Lastly, reduced SEC5 or SEC15 activity decreases GLUT4 translocation to the PM (Sano et al. 2011; Sano et al. 2015).

Further support for RAB10 function in vesicle secretion, comes from studies in axon growth. RAB10 functions in axon growth by providing proteins and lipids needed for axon membrane expansion during neuronal polarization (Wang et al. 2011; Liu et al. 2013b; Deng et al. 2014; Xu et al. 2014). Membrane components are provided to growing axons by post-Golgi derived plasmalemmal precursor vesicles (PPVs) (Hughes 1953; Dotti et al. 1988; Bradke and Dotti 1997; Pfenninger et al. 2003). In hippocampal and cortical neurons, RAB10 associates with post-Golgi derived PPVs, and delivers them to the axon PM (Wang et al. 2011; Deng et al. 2014). In neurons, RAB10 activation requires LGL1, the mammalian ortholog of lethal giant larvae in D. melanogaster, which displaces GDI from RAB10, and allows for RAB10 to localize to the TGN and PPVs (Wang et al. 2011; Deng et al. 2014). At the TGN, active RAB10 then interacts with the myosin-Vb (MYO5B) motor protein, which promotes the budding and fission of PPVs from the TGN (Liu et al. 2013b). PPVs then associate with the c-Jun N-terminal kinase-
interacting protein 1 (JIP1), via interaction with RAB10, where JIP1 binds to the kinesin-1 light chain protein (KLC1), and promotes the anterograde transport of PPVs to the PM (Dajas-Bailador et al. 2008; Deng et al. 2014). Once at the PM, RAB10 promotes the docking and fusion of PPVs (Stumpo et al. 1989; Xu et al. 2014).

In hippocampal and cortical neurons, expression of GDP-locked RAB10, or siRNA-mediated depletion of RAB10, or LGL1, significantly reduces axon length (Wang et al. 2011; Xu et al. 2014). Likewise, siRNA-mediated depletion of MYO5B disrupts axon growth, and reduces the number of RAB10-positive PPVs at the TGN (Liu et al. 2013b). Similarly, JIP1 depletion disrupts the interaction between RAB10 and KLC1, and reduces the number of motile RAB10 PPVs (Deng et al. 2014). In all, the role of RAB10 in post-Golgi secretion, and the general mechanism by which it does so, is conserved in multiple cell types.

Roles for RAB10 in processes other than endocytosis/secretion are also evident. In mouse macrophages, RAB10 functions in phagosome maturation (Cardoso et al. 2010). Specifically, RAB10 localizes to phagocytic cups, and functions in the recruitment of the lysosome membrane glycoprotein, LAMP2, to the surface of phagosomes (Cardoso et al. 2010). LAMP2, together with its paralog, LAMP1, functions in recruiting RAB7 to the phagosome for lysosomal fusion (Huynh et al. 2007). RNAi-mediated depletion of RAB10 or constitutively inactive RAB10 delays the recruitment of LAMP2 to phagosomes, and delays phagosome maturation (Cardoso et al. 2010). Additionally, RAB10, along with RAB3A, function in lysosomal exocytosis and plasma membrane repair (PMR) (Encarnacao et al. 2016). However, RAB3A contributes to the process of PMR more so than RAB10, since RAB3A shRNA-depletion had a more drastic effect on PMR than RAB10 depletion (Encarnacao et al. 2016). Lastly, RAB10 plays a role in lipophagy (Li et al. 2016). During starvation conditions, GTP-bound RAB10 associates with autophagosomes labeled with ATG16L1 and LC3, and these RAB10-positive pre-autophagosomes localize to lipid droplets (LDs), and participate in LD lysosomal degradation (Li et al. 2016). RAB10-mediated degradation of LDs occurs via the recruitment of the effector proteins, EHBP1 and EHD2, both of which bind to one another and link endocytic vesicles to actin (Guilherme et al. 2004; Li et al. 2016). The RAB10-EHBP1-EHD2 complex then delivers LD-engulfed autophagosomes to LAMP1-positive lysosomes (Li et al. 2016). siRNA-mediated depletion or knockout of RAB10, EHBP1, or EHD2, results in
an accumulation of LDs in MEF cells, and reduces the amount of LC3 and ATG16L1 associated with autophagosomes (Li et al. 2016). Overall, RAB10 has maintained its role in endocytic trafficking and autophagy from yeast, yet its role in these processes has become more specialized.

The GTPase RAB8 seems to function in a partially redundant manner with RAB10, since RAB8 is localized to basolateral vesicles derived from the TGN, and functions in the trafficking of newly synthesized basolateral cargo to the basolateral membrane (Huber et al. 1993; Henry and Sheff 2008). However, the role of RAB8 in endocytic trafficking still remains elusive, since in mice intestinal cells, RAB8 functions in protein transport and localization to the apical membrane, with no apparent role in protein localization to the basolateral membrane (Sato et al. 2007; Sato et al. 2014b). Therefore, RAB8 may function in redundant and non-redundant manner with RAB10 in endocytic trafficking.

RAB-10 is ubiquitously expressed in *C. elegans*, but its role in endocytic trafficking has come from studies done in intestinal cells (Chen et al. 2006; Shi et al. 2012). In the intestine, RAB-10 localizes to multiple endocytic compartments, such as basolateral early endosomes (RAB-5), the TGN (MANS), late endosomes (RAB-7), and apical recycling endosomes (RAB-11). Loss of function (LOF) or null mutations in *rab-10* result in the formation of enlarged vacuoles in intestinal cells, which represent abnormal early endosomes defective in maturation to late endosomes (Chen et al. 2006; Liu and Grant 2015). Indeed, RAB-10 interacts with AMPH-1, a pinchase orthologous to mammalian Amphiphysin/BIN1, to recruit the RAB-5 GAP, TBC-2, to basolateral early endosomes (Chotard et al. 2010; Liu and Grant 2015). TBC-2 functions with RAB-7 to inhibit RAB-5 during endosome maturation (Chotard et al. 2010; Liu and Grant 2015).

Supporting a role for RAB-10 in basolateral recycling, basolaterally derived endocytic tracer dyes that normally recycle back to BPM, accumulate inside the enlarged vacuoles of *rab-10* mutants. Conversely, apically derived endocytic tracer dyes do not accumulate in *rab-10* mutant vacuoles, suggesting that apical trafficking is normal (Chen et al. 2006). Moreover, the enlarged vacuoles of *rab-10* mutants accumulate basolateral specific transmembrane proteins, such as human interleukin alpha chain (hTAC), LMP-1, and to a minor extent, human transferrin receptor (hTfR) (Chen et al. 2006; Shi et al. 2012). Enlarged vacuoles are also found in *me-1* LOF and null mutants, which accumulate basolaterally derived cargo, but these vacuoles appear to represent abnormal basolateral recycling endosomes since
they are devoid of RAB-5 (Grant et al. 2001; Chen et al. 2006; Chen et al. 2014). Lastly, compared to wild-type animals, *rab-10* mutants have a reduced number of RME-1-positive basolateral recycling endosomes (BREs), an increased number of RAB-5 and RAB-7-positive endosomes, and no change in the number of RAB-11-positive AREs. In summary, the accumulation of basolaterally derived cargo in the enlarged vacuoles and disruption of BREs in *rab-10* mutants, suggests that endocytic uptake from the basolateral PM (BPM) is normal, but recycling back to the BPM from BEEs is blocked (Chen et al. 2006).

RAB-10 facilitates basolateral recycling in part by recruiting the ARF6/ARF-6 GAP, CNT-1 (ACAP1/2 in mammals) (Shi et al. 2012). ARF-6 is a GTPase that converts PI(4)P into PI(4,5)P2 (PIP2) (Radhakrishna and Donaldson 1997; Brown et al. 2001). PIP2 is mainly localized to the PM and REs, and functions in the recruitment of membrane bending and fission proteins, such as RME-1, SDPN-1, and AMPH-1, and also functions in the recycling of cargo, such as hTAC. Changes in PIP2 levels lead to the aberrant localization of membrane bending proteins, and retention of basolateral cargo in endocytic vesicles (Shi et al. 2012; Gleason et al. 2016; Wang et al. 2016a). RAB-10 in its active form recruits CNT-1 to basolateral endosomes, which negatively regulates ARF-6 activity to ensure a proper balance between the various lipid species on basolateral endosomes (Shi et al. 2012). Loss of *rab-10* or *cnt-1* results in an accumulation of PIP2 on basolateral endocytic vesicles, due to an accumulation of ARF-6, which disrupts the localization of SDPN-1 and RME-1, disrupting vesicle formation and cargo recycling (Shi et al. 2012; Wang et al. 2016a). Moreover, to regulate basolateral recycling, RAB-10 associates with the calponin homology domain (CH) containing protein, EHBP-1 (mammalian ortholog of EHBP1 that binds actin) (Guilherme et al. 2004; Shi et al. 2010; Li et al. 2016; Wang et al. 2016a). EHBP-1 binds to PIP2, and directly interacts with and recruits GTP-bound RAB-10 to endosomes (Shi et al. 2010; Wang et al. 2016a). GTP-bound RAB-10 in turn promotes EHBP-1 binding to actin filaments, which is necessary for endosomal tubulation and vesicle formation (Wang et al. 2016a). EHBP-1 colocalizes extensively with RAB-10-positive endosomes and PIP2-positive endocytic tubules. Additionally, the phenotypes of *ehbp-1* LOF mutants strongly resemble the phenotypes of *rab-10* mutants, such as the formation of enlarged vacuoles, accumulation of basolateral cargo, and disruption of RME-1-positive BREs (Shi et al. 2010; Wang et al. 2016a).
As discussed above, the exocyst subunit, SEC-10, functions in basolateral endocytic recycling in intestinal cells (Chen et al. 2014). sec-5 and sec-10 deletion mutants have defects in the basolateral recycling of FM4-64, a fluid-phase endocytic marker. Moreover, similar to what was found for a loss of rab-10, a loss of sec-10 disrupts the tubular nature of RME-1-positive basolateral recycling endosomes, and increased the number of RAB-5 and RAB-7, respectively (Grant et al. 2001; Chen et al. 2014).

Deletion of sec-10 suppresses the enlarged vacuole phenotype of rme-1 mutants, but not that of rab-10 mutants, suggesting that SEC-10 acts upstream of RME-1, but downstream of RAB-10 in the regulation of basolateral recycling (Chen et al. 2014). In the intestine, the endocytic marker, hTAC, is found mainly in basolateral recycling endosomes/tubules (Chen et al. 2006; Chen et al. 2014). Both RAB-10 and SEC-10 are required for the formation of hTAC-positive tubules. RAB-10 functions in the growth and extension of hTAC tubules, while SEC-10 functions in the tethering and fusion of hTAC tubules with other hTAC tubules (Chen et al. 2014).

Like in mammals, C. elegans RAB-10 is paralogous to RAB-8, and RAB-10 extensively colocalizes with RAB-8 on endocytic vesicles; however, RAB-10 and RAB-8 appear to have distinct functions in polarized cells (Shi et al. 2010). EHBP-1 is an effector for both RAB-8 and RAB-10 and can bind to both in their GTP-loaded forms; yet as discussed above, in intestinal cells and interneurons, the phenotypes of ehbp-1 LOF mutants strongly resemble the phenotypes of rab-10 mutants, but not rab-8 mutants (Shi et al. 2010). In addition, loss of ehbp-1 diminishes the formation of basolaterally localized RAB-10 endosomes, while RAB-8-positive endosomes accumulate at the apical membrane (Shi et al. 2010). Further supporting a distinct role for RAB-8 in endocytic trafficking, rab-8 deletion mutants accumulate the apically localized glycoprotein, PGP-1, and PIP2 in apical vesicles, as opposed to basolateral vesicles (Sato et al. 2007; Shi et al. 2012). Moreover, loss of rab-10 and ehbp-1 disrupt DCV secretion, while loss of rab-8 had no effect on DCV secretion (see below) (Sasidharan et al. 2012). Interestingly, RAB-8 and RAB-10 do appear to function redundantly in non-polarized cells, such as the germline. rab-8 and rab-10 single deletion mutants each have a reduced brood size, but are not sterile (Shi et al. 2010). Conversely, rab-8(RNAi); rab-10(−) double mutants are sterile, similar to ehbp-1 LOF mutants. Moreover, as is found for ehbp-1 LOF mutants, animals co-depleted of rab-8 and rab-10 accumulate puncta labeled with the SNARE protein, SNB-1, in germ cells (Shi et al. 2010). These results
suggest that RAB-8 and RAB-10 function in a redundant and non-redundant manner, in non-polarized and polarized cells, respectively (Shi et al. 2010).

The role of RAB-10 in endocytic trafficking is not limited to intestinal cells. RAB-10 also functions in neurons to regulate neuropeptide secretion and recycling (Glodowski et al. 2007; Sasidharan et al. 2012). In DA/DB cholinergic motor neurons, RAB-10 and RAB-5 function in the secretion of dense core vesicles (DCVs), a type of secretory vesicle, derived from the Golgi, that contains neuropeptides (Matteoli et al. 1988; Thureson-Klein and Klein 1990; Sasidharan et al. 2012). GTPase activity is required for neuropeptide secretion, since GTP-locked RAB-10 and RAB-5 results in reduced DCV secretion. Moreover, deletions in the RAB-5 and RAB-10 GAPs, tbc-2 and tbc-4, respectively, as well as depletion of the RAB-5 and RAB-10 effectors, RABN-5 and EHBP-1, respectively, result in DCV secretion defects similar to rab-5 and rab-10 LOF mutants (Sasidharan et al. 2012). Lastly, RAB-10 functions cell-autonomously, since expression of mCherry::RAB-10 specifically in DA/DB cholinergic motor neurons, rescues the DCV secretion defect in rab-10 null mutants (Sasidharan et al. 2012).

In interneurons, RAB-10 functions in the recycling of the AMPA-type glutamate receptor subunit, GLR-1, to post-synaptic membranes from intracellular early endosomal compartments (Glodowski et al. 2007). AMPA-type glutamate receptors play a role in synapse signal strength, and animals lacking GLR-1 have defects in backwards movement (Bredt and Nicoll 2003; Malinow 2003; Gerges et al. 2005; Glodowski et al. 2007). RAB-10 functions in parallel to the LIN-10 pathway in GLR-1 recycling (Rongo et al. 1998; Glodowski et al. 2005; Glodowski et al. 2007). Loss of rab-10 results in the formation of GLR-1 accretions/aggregations in neurite endosomal compartments. Moreover, RAB-10 cDNA driven by the GLR-1 promoter is able to rescue the GLR-1 accretion phenotype in rab-10 mutants, suggesting that RAB-10 regulates GLR-1 recycling cell-autonomously in interneurons. Lastly, rab-10 mutants display reduced backward movement in response to nose-touch, a phenotype observed in the absence of GLR-1 (Glodowski et al. 2007).

RAB-10 also facilitates proximal arborization/branching of the PVD neuron (Taylor et al. 2015; Zou et al. 2015). rab-10 null mutants have reduced dendritic branching in the proximal region, but not the distal region, of the PVD neuron, suggesting that distal arborization precedes via a RAB-10-independent mechanism (Taylor et al. 2015; Zou et al. 2015). Similar to all other neurons investigated, RAB-10 functions
cell-autonomously in the PVD neuron to regulate dendritic branching, and requires GTP-binding activity to do so, since GTP-locked RAB-10, but not GDP-locked RAB-10, rescues the dendritic branching defect in rab-10 null mutants. Moreover, PVD proximal arborization requires the actions of the exocyst complex subunits, EXOC-8, SEC-8, and SEC-5. Loss of function mutations in exoc-8, sec-5, or sec-8, all result in arborization defects similar to rab-10 null mutations. As evidence for RAB-10 mediating dendrite arborization through vesicle trafficking, RAB-10 is strongly localized to the Golgi and early endosomes, and vesicles labeled with EXOC-8. Moreover, RAB-10 is localized to vesicles containing DMA-1 and HPO-30, which are transmembrane proteins that facilitate dendrite branching due to their localization at the PM (Liu and Shen 2011; Dong et al. 2013; Taylor et al. 2015; Zou et al. 2015). Loss of sec-8 or exoc-8 results in the accumulation of intracellular vesicles containing RAB-10, and either DMA-1 or HPO-30. Furthermore, loss of either rab-10 or exoc-8 reduces DMA-1 and HPO-30 localization to the PM. These results suggest that RAB-10 and the exocyst complex regulate the delivery of DMA-1 and HPO-30 to the PM, which facilitates dendrite arborization (Taylor et al. 2015; Zou et al. 2015).

Lastly, RAB-10 is also implicated regulating dietary restricted induced autophagy. As discussed above, animals with a LOF mutation in the nicotinic acetylcholine receptor, eat-2, are dietary restricted and exhibit elevated levels of autophagy (Avery 1993; Lakowski and Hekimi 1998; Hansen et al. 2008a). The elevated levels of autophagy in eat-2 LOF mutants are at least partly a result of reduced LET-363 activity (Hansen et al. 2007; Hansen et al. 2008a). Importantly, the autophagic response elicited by eat-2 is also mediated by the FOXA transcription factor, PHA-4, and RAB-10 (Hansen et al. 2008a). rab-10 null mutants display DR-like phenotypes that include: reduced fecundity, increased lifespan, DR-age related pigments, and elevated levels of GFP::LGG-1-positive autophagosomes in seam cells (Hansen et al. 2008a). rab-10 mRNA levels fall in response to DR, which was thought to be due to the transcriptional regulation of rab-10 by PHA-4. However, pha-4 depletion reduces autophagy levels in rab-10 mutants, suggesting that pha-4 is required for the elevated levels of autophagy in rab-10 mutants, and acts either in parallel or downstream of rab-10. Importantly, whether the elevated levels of autophagy found in rab-10 mutants was due to autophagy induction or autophagy inhibition, was not investigated (Hansen et al. 2008a).

1.6. Thesis rationale
Autophagy and membrane/endocytic trafficking are two quality control mechanisms employed by the cell to maintain homeostasis. Endocytic trafficking ensures that proteins and other molecules, involved in processes such as cell signaling and growth, are properly transported and utilized by the cell. Autophagy, on the other hand, is responsible for the removal of dysfunctional proteins and organelles, which contribute to cell toxicity and intracellular damage.

In line with this, defects in various endocytic and autophagy genes have been implicated in neurodegenerative diseases and cancer. For example, mutations in \textit{ALS2}, a GEF for \textit{RAB5}, contribute to Amyotrophic lateral sclerosis (ALS) (Yamanaka et al. 2003). Furthermore, deletion of \textit{TSG101}, part of the ESCRT complex, can lead to sporadic forms of breast and prostate cancers (Li et al. 1997; Sun et al. 1997). The autophagy gene \textit{BECLIN1} has been documented to be monoallelically deleted in several types of cancers, such as ovarian, breast, and prostate cancers (Liang et al. 1999). Additionally, genetic reduction of \textit{BECLIN1} increases the accumulation of amyloid-\(\beta\) proteins, a common cause of Alzheimer’s disease (Pickford et al. 2008). Lastly, the small GTPase Rab25, a protein important for apical recycling endocytosis and transcytosis, is found to be upregulated in both ovarian and breast cancers (Cheng et al. 2004). Thus, further understanding of the relationship between autophagy and endocytosis can provide insights as to how defects in these processes contribute to similar types of diseases.

Although it is clear that crosstalk exists between autophagy and endocytosis, the extent to which endocytic proteins and/or compartments function in autophagy, and how these endocytic components contribute to autophagosome biogenesis, are still unknown (Jing and Tang 1999b; Hirota et al. 2013; Lamb et al. 2013). For example, multiple endocytic compartments contribute membrane to initiate autophagosome biogenesis via the actions of Atg9/ATG9/ATG-9, but the exact signaling mechanisms that decide which compartment is used to form an autophagosome are not well understood (Noda et al. 2000; Young et al. 2006; Mari et al. 2010b; Kim et al. 2011; Russell et al. 2013; Papinski et al. 2014). In addition, although it is known that \textit{RAB} GTPases associate with autophagosomes, whether \textit{RAB} conversion cascades (i.e. \textit{RAB5} exchange with \textit{RAB7}) take place on autophagosomes has been unexplored. Moreover, and most importantly, there are at least five main GTPase families, which include: \textit{RAS}, \textit{RAB}, \textit{RAN}, \textit{ARF}, and \textit{RHO} GTPase families (Wennerberg et al. 2005). Each family consists of multiple GTPases, with multiple GEFs, GAPs, and effector proteins. Many of these molecules have
already been identified to function in autophagy, but for those molecules not yet tested for a role in autophagy, how they function in and regulate autophagy is a question largely unanswered.

To further investigate the relationship between autophagy and endocytosis, we conducted an RNAi screen for endocytic genes that altered GFP::LGG-1 expression and blocked tissue remodeling in daf-2/IIR mutants. daf-2/IIR temperature sensitive mutants display a constitutive dauer phenotype at the non-permissive temperature, associated with elevated foci labeled with the autophagosome reporter, GFP::LGG-1. From our screen, we identified, RAB-10, as a regulator of autophagy. Consistent with a previous report, we find that rab-10 mutants have an increase in the number of GFP::LGG-1 positive foci in wild type seam cells (Hansen et al. 2008a). We show that a loss of rab-10 also results in an increase in GFP::LGG-1 foci in daf-2 mutant non-dauer/L3 animals at the permissive temperature. In contrast, loss of rab-10 in daf-2 dauers display a decrease in the number of GFP::LGG-1 positive foci and SQST-1/p62::GFP positive foci. These observations suggest a role for rab-10 in autophagic flux, and indeed, we find that a block of autophagic flux with chloroquine does not further increase the number of GFP::LGG-1 foci in rab-10 mutants. Moreover, colocalization of the lysosomal marker LMP-1::tagRFP and GFP::LGG-1 significantly decreases in daf-2; rab-10 dauers, further supporting a defect in autophagic flux. To investigate the possible mechanism by which rab-10 functions in autophagy, we evaluate the localization of intestinally localized GFP::ATG-9, a marker for the autophagosome membrane. We find that loss of rab-10 increases or decreases the size of GFP::ATG-9 positive structures in daf-2 dauer or non-dauer animals, respectively. Additionally, transgenic rescue experiments suggest that RAB-10 GTPase cycling activity is required to control the size of GFP::ATG-9 foci. In conclusion, these data support a role for rab-10 in autophagosome formation and in autophagosome maturation.
Chapter 2

2. The recycling endosome protein RAB-10 promotes autophagic flux and localization of the transmembrane protein ATG-9

N. J. Palmisano¹,², Rosario, N., Wysocki, M., Hong, M., Grant, B.³ and A. Meléndez¹,² *

¹Biology Department, Queens College, CUNY, Flushing, NY; ²The Graduate Center of the City University of New York, New York; ³Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ.

*, Correspondence should be addressed to A.M, Alicia.Melendez@qc.cuny.edu

Running Title: The role of RAB-10 in autophagy

Acknowledgement: This manuscript has been accepted for publication in the journal, “Autophagy”, on January, 26th 2017, by Taylor and Francis publishing, an informa company.

Keywords: autophagy, daf-2, dauer, endocytosis, GFP::ATG-9, GFP::LGG-1, rab-10
2.1. Abstract:

Autophagy is a degradative process that involves the formation of an autophagosome, a double-membrane vesicle, which delivers sequestered cytoplasmic cargo to late endosomes and lysosomes for degradation. Closely related to autophagy, endocytic trafficking mediates the sorting and transport of cargo throughout the cell, via the formation of single-membrane endocytic vesicles. Endocytic trafficking and autophagy are important for cellular homeostasis, however, the extent to which endocytic proteins function in autophagy, and how they contribute to this process, is not fully understood. To further investigate the connection between autophagy and endocytosis, we conducted an RNAi screen for genes that altered GFP::LGG-1 expression and blocked tissue remodeling in daf-2/IIR mutants. Insulin-like receptor daf-2/IIR mutants display a constitutive dauer phenotype at the non-permissive temperature and have an increase in foci labeled with the autophagosome reporter, GFP::LGG-1. From our screen, we identified the small GTPase, RAB-10, a regulator of basolateral endocytic trafficking in C. elegans and mammals. Consistent with a previous report, we found that rab-10 mutants have an increase in GFP::LGG-1 positive foci in wild type animals. Similarly, we found that loss of rab-10 also resulted in an increase of GFP::LGG-1 foci in daf-2 mutant non-dauer animals at the permissive temperature. In contrast, loss of rab-10 in daf-2 dauers displayed a decrease in the number of GFP::LGG-1 positive foci and SQST-1/p62::GFP positive foci, an autophagic cargo marker. These observations suggested a role for rab-10 in autophagic flux. Consistent with this interpretation, we found that a block of autophagic flux with chloroquine did not further increase the number of GFP::LGG-1 foci. Moreover, colocalization of the lysosomal marker LMP-1::tagRFP and GFP::LGG-1 was significantly decreased in daf-2; rab-10 dauers. To investigate the possible mechanism of rab-10 function in autophagy, we used GFP::ATG-9, a marker for the autophagosome membrane. We found that loss of rab-10 increased or decreased the size of GFP::ATG-9 positive structures in daf-2 dauer or non-dauer animals, respectively. Moreover, transgenic rescue experiments suggest that RAB-10 GTPase cycling activity is required to control the size of GFP::ATG-9 structures. In conclusion, our data support a role for rab-10 in autophagosome formation and in autophagosome maturation.
2.2. Introduction:

Autophagy is an evolutionarily conserved stress response pathway in eukaryotic cells that leads to the bulk degradation and recycling of cellular components through a lysosomal pathway (Deter et al. 1967; Levine and Klionsky 2004; Mizushima 2007; Melendez and Neufeld 2008). This process occurs at basal levels to maintain cellular homeostasis, but is significantly upregulated in response to stress, such as nutrient deprivation, hypoxia, damaged organelles, and the accumulation of defective/long lived proteins (Melendez and Neufeld 2008). Autophagy is unique when compared to other membrane trafficking processes in that it involves the formation of a double-membrane vesicle, called the autophagosome (Clark 1957; Deter et al. 1967; Levine and Klionsky 2004).

In autophagy, vesicle formation begins with the activation of the serine/threonine kinase, UNC-51/ULK1/2, and recruitment of the multispanning transmembrane protein ATG-9/mAtg9, which is important for providing membrane to the developing pre-autophagosome (Noda et al. 2000; Young et al. 2006; Kim et al. 2011; Russell et al. 2013; Papinski et al. 2014). The class III PI3K complex, comprising the class III PI3-kinase VPS-34/VPS34, and its binding partner BEC-1/BECN1, is then responsible for the nucleation of the pre-autophagosome and recruitment of additional autophagy proteins (Kihara et al. 2001; Suzuki et al. 2007; Russell et al. 2013). These additional proteins include two conjugation complexes, LGG-1/LC3 conjugated to phosphatidylethanolamine, and LGG-3/ATG12 conjugated to ATG-5/ATG5 and ATG-16.1/2/ATG16L1. Both complexes are required for elongation and completion of the pre-autophagosome to form a mature autophagosome (Kabeya et al. 2000; Kuma et al. 2002; Mizushima et al. 2003; Xie et al. 2008). In the process of autophagosome elongation, LGG-1 binds to the adaptor protein, SQST-1/p62, which recognizes and binds to polyubiquitinated protein aggregates and organelles destined for degradation (Vadlamudi et al. 1996; Pankiv et al. 2007). Lastly, the autophagosome fuses with the lysosome leading to the degradation and recycling of cellular constituents, which is dependent on the late endocytic protein, RAB-7, and the autophagy proteins, EPG-5 and LGG-2 (Tian et al. 2010; Manil-Segalen et al. 2014b).

Related to autophagy, endocytic trafficking is a cellular process that involves the formation of single-membrane intracellular vesicles termed endosomes/exosomes. These vesicles are responsible for the uptake, sorting, and transport of cargo inside the cell (Mellman 1996). Trafficking of specific cargo,
such as ligand-receptor complexes, occurs through the endomembrane system, which includes the endoplasmic reticulum (ER), Golgi complex, endosomes, and lysosomes (Mellman 1996; Nebenfuhr 2002). In general, endocytic membrane compartments are found associated with unique sets of proteins (Hutagalung and Novick 2011). These proteins include protein coats, such as Clathrin, important for vesicle budding and cargo recognition, Arf GTPases, important for protein coat assembly, Rab GTPases, which recruit effector proteins needed for vesicle transport and membrane fusion, and SNARE proteins, such as syntaxins, important for vesicle fusion to target membranes (Grosshans et al. 2006b; Stenmark 2009; Huotari and Helenius 2011). All these proteins function to tightly regulate cargo sorting and transport, which similar to autophagy, is important for cellular homeostasis and function.

Evidence for crosstalk between autophagy and endocytosis has been demonstrated (Seglen and Bohley 1992; Jing and Tang 1999b; Hirota et al. 2013; Lamb et al. 2013). For example, in mammalian cells, Atg14L/Barkor, a Beclin 1 binding partner important for isolation membrane nucleation, interacts with the protein Snapin, a SNARE-associated protein important for endocytic trafficking (Ilardi et al. 1999; Buxton et al. 2003; Matsunaga et al. 2009). Together, both Atg14L and Snapin regulate the endocytic degradation of the epidermal growth factor receptor (Kim et al. 2012). Additionally, in C. elegans, yeast, and mammals, the small GTPase RAB-7/ Ypt7/RAB7, an important regulator of the late endocytic pathway, is important for fusion of autophagosomes with lysosomes to form autolysosomes (Wichmann et al. 1992; Vitelli et al. 1997; Kirisako et al. 1999; Jager et al. 2004; Poteryaev et al. 2010; Djeddi et al. 2012; Manil-Segalen et al. 2014b). Although it is clear that both autophagy and membrane trafficking are two interconnected processes, the extent to which membrane trafficking proteins function in autophagy, and how these proteins contribute to autophagosome biogenesis, are still not well understood.

Here, we show that the small GTPase, RAB-10, is important for autophagosome biogenesis and autophagic flux. In C. elegans and mammals, RAB-10/RAB10 mediates the efficient recycling of basolaterally derived cargo in polarized epithelial cells, and facilitates the transport of cargo important for various aspects of neuronal development, such as dendritic arborization and axonal growth (Babbey et al. 2006; Chen et al. 2006; Schuck et al. 2007; Shi et al. 2010; Shi et al. 2012; Liu et al. 2013b; Deng et al. 2014; Liu and Grant 2015; Taylor et al. 2015; Zou et al. 2015). We find that a rab-10 loss of function mutation, or depletion by RNAi, alters the localization of foci labeled with the autophagosome reporter,
GFP::LGG-1. RAB-10 is also required for the degradation of SQST-1/p62::GFP, as an increase in the size of SQST-1/p62::GFP positive structures was observed in *rab-10* loss of function mutants. Interestingly, we find that RAB-10 is required for normal levels of autophagic flux, which is defined as the transit from autophagosome formation to degradation (Loos et al. 2014). In all, our results demonstrate that RAB-10 promotes autophagy in *C. elegans*.

### 2.3. Results:

#### 2.3.1. Identifying the small GTPase, *rab-10*, as a mediator of autophagy function

To investigate the role of endocytic genes in autophagy, we carried out an RNAi screen for endocytic genes that when depleted, resulted in dauer defective phenotypes and/or the mislocalization of the autophagy reporter, GFP::LGG-1, in *daf-2(e1370)* temperature sensitive mutants (Swanson and Riddle 1981; Gottlieb and Ruvkun 1994; Meléndez et al. 2003). *daf-2* encodes the insulin-like/IGF-1 receptor (IIR) in *C. elegans*, which is an evolutionarily conserved upstream regulator of autophagy (Mortimore and Mondon 1970; Pfeifer 1977; Pfeifer 1978; Petiot et al. 2000). *daf-2(e1370)* mutants display a dauer constitutive (Daf-c) phenotype at the restrictive temperature, which was previously shown to require autophagy (Meléndez et al. 2003). When grown at the permissive temperature, *daf-2(e1370)* mutants do not display a Daf-c phenotype, can reach adulthood, and have a long lifespan that is also dependent on autophagy activity (Swanson and Riddle 1981; Kenyon et al. 1993; Gottlieb and Ruvkun 1994; Kimura et al. 1997; Riddle et al. 1997; Meléndez et al. 2003). Compared to wild-type animals, which have low/basal levels of autophagy, *daf-2(e1370)* mutants have elevated levels of autophagy at the permissive temperature, with an even greater increase of autophagy at the restrictive temperature (Meléndez et al. 2003). In *C. elegans*, autophagy can be visualized with the autophagy reporter, GFP::LGG-1, in hypodermal seam cells, a cell type that has been shown to undergo remodeling during dauer formation (Singh and Sulston 1978; Meléndez et al. 2003).

RNAi-mediated depletion or loss of function mutations of autophagy genes in *daf-2/IIR* dauers, results in the formation of enlarged GFP::LGG-1-positive punctate structures/foci in seam cells, a phenotype indicative of defective autophagosomes (Fig. 2.1) (Meléndez et al. 2003; Kuma et al. 2007; Shvets and Elazar 2008). From a collection of 74 RNAi clones that were previously shown to be required
FIG. 2.1: Endocytic genes isolated in candidate gene RNAi screen. A) Representative epifluorescent images of GFP::LGG-1 in the hypodermal seam cells of daf-2(e1370) dauers treated with dsRNA to target genes, as part of the RNAi screen for genes that altered GFP::LGG-1 localization. B) Genes identified in the RNAi screen that alter GFP::LGG-1 localization were part of the ESCRT (Endosomal Sorting Complex Required for Transport) complex, the HOPS (Homotypic fusion and Protein vacuole Sorting) complex, Ras and arl-like GTPase superfamily, and syntaxins. *RNAi resulted in embryonic lethality. ‡Representative images not shown. Magnification 630X. scale bar: 2 µm
for endocytosis, inactivation of several genes resulted in the formation of enlarged GFP::LGG-1-positive foci in *daf-2* dauers (Fig. 2.1) (Balklava et al. 2007). One of the genes found in our screen encoded for the small GTPase RAB-10 (Fig. 2.1A-B). Interestingly, the GFP::LGG-1 positive foci in the *daf-2; rab-10*(RNAi) animals appeared comparable in size to that of *daf-2; atg-7*(RNAi) or *daf-2; atg-18*(RNAi) animals, and slightly smaller than those observed in *daf-2; bec-1*(RNAi) or *daf-2; unc-51*(RNAi) animals. Since UNC-51 (in mammals ULK1/2) acts at the inductive step, and BEC-1 (in mammals BECN1/Beclin1) acts at the nucleation step, we hypothesized that RAB-10 may function at a later step in the autophagy process. From our screen, we also identified several genes previously shown to be required for autophagy, such as genes part of the ESCRT (Endosomal Sorting Complex Required for Transport)-related machinery, confirming the validity of our screen (Fig. 2.1A-B) (Jager et al. 2004; Filimonenko et al. 2007; Rusten et al. 2007; Djeddi et al. 2012; Manil-Segalen et al. 2014b).

### 2.3.2. RAB-10 is required for proper localization of GFP::LGG-1 in hypodermal seam cells

To evaluate how RAB-10 is required for autophagy, we investigated the phenotype of a null allele for *rab-10, ok1494*, in *daf-2* mutants, and assessed changes in the number and size of GFP::LGG-1 positive foci, at the permissive temperature (L3, non-dauer larvae) where autophagy levels are mildly elevated, and in animals grown at the restrictive temperature (dauer larvae), where autophagy levels are high.

In *daf-2* dauers, otherwise wild-type for *rab-10*, GFP::LGG-1 foci are localized in close proximity to one another (Fig. 2.2A). We assayed the number of GFP::LGG-1 positive foci in *daf-2; rab-10*, and in *daf-2; atg-7*, or *daf-2; atg-9* double mutant dauers, as autophagy deficient controls (Fig. 2.2A-C). *daf-2; rab-10* double mutant dauers displayed a reduction in the number of GFP::LGG-1 positive foci in seam cells, yet larger, when compared to those in *daf-2* single mutant dauers (Fig. 2.2A-C). Similar results were obtained in *daf-2(e1370)* dauers that carry the *atg-7* or *atg-9* null mutations (Fig. 2.2A-C). These results suggest that *rab-10* is required for autophagosome biogenesis.

*daf-2/IIR* animals, otherwise wild-type for *rab-10*, when grown at the permissive temperature until the L3 larvae stage, display low levels of GFP::LGG-1 positive foci that are not in very close proximity (Fig. 2.2D). *daf-2; rab-10* double mutant L3 larvae had a significant increase in the number, but no
FIG. 2.2: RAB-10 loss alters the localization of GFP::LGG-1 foci in daf-2(e1370) dauers and non-dauer seam cells. A and D) Representative deconvolved epifluorescent images of seam cells (white outlines) in daf-2(e1370), daf-2(e1370); rab-10(ok1494), daf-2(e1370); atg-7(bp411), and daf-2(e1370); atg-9(bp564) dauers (A) and L3 larvae (D) expressing GFP::LGG-1. B-C) Quantification of the average number (B) and size (C) of GFP::LGG-1-positive foci per seam cell in dauer animals; n ≥ 25 per strain, # of seam cells/strain ≥ 77. E-F) Quantification of the average number (E) and size (F) of GFP::LGG-1-positive foci per seam cell in L3 larvae; n ≥ 15 per strain, # of seam cells/strain ≥ 48. Data shown represents an average of two independent trials. Statistical analysis was done using an unpaired, two tailed t-test. p-value *≤0.05, **≤0.01 and *** ≤0.001. Error bars = ± S.E. Arrow heads point to GFP::LGG-1 foci; arrows point to enlarged GFP::LGG-1 foci. Magnification 630X. scale bar: 2 μm.
change in the size, of GFP::LGG-1 positive foci, when compared to control daf-2/IIR single mutants (Fig. 2.2D-F). In contrast, we found that daf-2; atg-7 or daf-2; atg-9 double mutants display a decrease in the number, and an increase in the size of GFP::LGG-1 positive foci, compared to control daf-2/IIR single mutants (Fig. 2.2D-F). Overall, these results suggest that RAB-10 plays a role in autophagosome formation, however, we note that RAB-10 may function in a step of the autophagy pathway distinct from core autophagy proteins. In summary, RAB-10 is required for the proper number and morphology of GFP::LGG-1 positive foci, under both mild and moderate levels of autophagy activity.

2.3.3. RAB-10 is required to maintain basal levels of autophagy

Basal levels of autophagy ensure cellular homeostasis and prevent the accumulation of protein aggregates and damaged mitochondria or organelles (Kuma et al. 2004; Komatsu et al. 2006; Ryter et al. 2013). To investigate if RAB-10 was required for basal levels of autophagy, we measured the number and size of GFP::LGG-1 positive foci in rab-10 single mutants, otherwise wild-type for daf-2 (Brenner 1974; Kang et al. 2007; Djeddi et al. 2012). As previously reported, rab-10 mutants had an increase in the number of GFP::LGG-1 positive foci in seam cells (Fig. 2.3A-C) (Hansen et al. 2008a). However, we found that rab-10 mutants have smaller GFP::LGG-1 positive foci, when compared to wild-type animals (Fig. 2.3C). The number of GFP::LGG-1 positive foci in atg-7 or atg-9 single mutants did not change, when compared to wild-type animals; but the size of the GFP::LGG-1 foci was significantly increased (Fig. 2.3A-C). The elevated number of GFP::LGG-1 positive foci in rab-10 single mutants, and in daf-2; rab-10 non-dauers, together with the enlarged GFP::LGG-1 positive foci in daf-2; rab-10 dauers, suggests that RAB-10 may be required for autophagic flux, and that different levels of autophagy activity may influence the defects associated with the loss of rab-10 activity. A reduction in the number of functional autophagosomes may be evidenced by the accumulation of GFP::LGG-1 positive foci, or the increase in size of GFP::LGG-1 punctate structures.

2.3.4. RAB-10 is required for autophagy flux in C. elegans

To investigate if RAB-10 is important for autophagic flux, we treated animals with chloroquine, a lysosomotropic agent that neutralizes the acidic pH of the lysosome and inhibits lysosomal activity,
FIG. 2.3: Loss of rab-10 increases the number of GFP::LGG-1-positive foci in hypodermal seam cells of wild type animals. A) Representative deconvolved epifluorescent images of GFP::LGG-1 expressed in hypodermal seam cells (white outlines) of wild type, rab-10(ok1494), atg-7(bp411), and atg-9(bp564) single mutants. B&C) Quantification of the average number (B) and average size ($\mu$m$^2$) (C) of GFP::LGG-1-positive foci per seam cell. n ≥ 19 per strain, # of seam cells/strain ≥ 54. Data shown represents an average of two independent trials. Statistical analysis was done using an unpaired, two tailed t-test. p-value *≤0.05 and *** ≤0.001. Error bars = ± S.E. Arrow heads point to GFP::LGG-1 foci; Arrows point to enlarged GFP::LGG-1 foci. Magnification 630X. scale bar: 2 $\mu$m.
decreasing autophagic flux (Ohkuma and Poole 1978). In cells with normal autophagic flux, chloroquine treatment results in an increase in the number of GFP::LGG-1 foci, due to the failed lysosomal degradation of GFP::LGG-1-positive autophagosomes. In cells with a disruption in autophagic flux, treatment with chloroquine has no further increase in the number of GFP::LGG-1 foci.

Chloroquine treatment of wild-type animals increased the number and size of GFP::LGG-1 positive foci in seam cells of wild-type animals, compared to non-treated animals (Fig. 2.4A-C), as expected for a block in autophagic flux. Importantly, chloroquine treatment of rab-10 mutants resulted in no further increase in the number or size of GFP::LGG-1 foci, when compared to the non-treated controls (Fig. 2.4A-C), suggesting that the loss of rab-10 mutants blocks autophagic flux. To validate our methodology, we treated atg-7 single mutants with chloroquine and found that similar to rab-10 mutants, both treated and non-treated atg-7 mutants had a similar number and size of GFP::LGG-1 positive foci (Fig. 2.4A-C). Our results suggest that autophagic flux is defective in rab-10 mutants and that the increase in GFP::LGG-1 positive punctate structures in rab-10 mutants results from an accumulation of isolation membranes or pre-autophagosomes.

We next investigated the role of RAB-10 in promoting autophagy flux in daf-2/IIR dauers (Fig. 2.4D and 2.4E). We utilized daf-2/IIR mutants that co-express GFP::LGG-1 and LMP-1::tagRFP, a lysosomal membrane protein, and treated them throughout development with dsRNA specific to rab-10, atg-7, or the arf-like GTPase arl-8, a mediator of lysosomal biogenesis (Nakae et al. 2010). We reasoned that under normal conditions, the colocalization of GFP::LGG-1 and LMP-1::tagRFP positive foci would represent the fusion of autophagosomes with lysosomes. Conversely, under autophagy flux-disrupted conditions, we would expect to see a decrease in the colocalization of GFP::LGG-1 and LMP-1::tagRFP, or a complete lack of colocalization of the autophagy and lysosome reporters. In daf-2/IIR dauers treated with control dsRNA, colocalization of LMP-1::tagRFP and GFP::LGG-1-positive vesicles was observed (Fig. 2.4D and 2.4E). However, daf-2 mutants treated with rab-10 RNAi displayed a reduction in the colocalization of GFP::LGG-1 and LMP-1::tagRFP, when compared to daf-2/IIR mutants treated with control RNAi (Fig. 2.4D and 2.4E), confirming a role for RAB-10 in autophagic flux. Similarly, the colocalization of LMP-1::tagRFP and GFP::LGG-1 was strongly reduced in atg-7 and arl-8 RNAi depleted animals, confirming defects in autophagic flux in these animals (Fig. 2.4D and 2.4E). Our results indicate
FIG. 2.4: RAB-10 is required for proper autophagic flux. A) Representative deconvolved epifluorescent images of GFP::LGG-1 expression in seam cells of wild type, rab-10(ok1494), and atg-7(bp411) mutants treated with control (dH2O) or 30 mM chloroquine. B-C) Quantification of the average number (B) and size (C) of GFP::LGG-1-positive foci per seam cell in animals treated with control (dH2O) or 30 mM chloroquine. D) Representative deconvolved epifluorescent images of seam cells in daf-2(e1370) mutants that co-express GFP::LGG-1 and LMP-1::tagRFP reporters, treated with RNAi against L4440 empty vector control, atg-7, rab-10 or arl-8. E) Quantification of the Pearson's correlation coefficient for colocalization between GFP::LGG-1 and LMP-1::tagRFP. A-C) Animals were analyzed at the L3 stage at 20°C. n ≥ 17 animals/strain; seam cell # ≥ 33/strain. D&E) Animals were analyzed at the dauer stage at 25°C. n ≥ 14 animals/RNAi clone; seam cell # ≥ 46/RNAi clone. Data shown is an average of two independent trials. Statistical analysis was done using an unpaired, two tailed t-test. *≤0.05 **≤0.01 and ***≤0.001. Error bars = ± S.E. Arrow heads point to representative GFP::LGG-1 positive foci in (A) or GFP::LGG-1 and LMP-1::tagRFP colocalized foci in (D), and arrows point to GFP::LGG-1 and LMP-1::tagRFP non-colocalizing foci (D). Magnification 630X. scale bar: 2 μm.
that RAB-10 is required to promote autophagic flux in *C. elegans*.

### 2.3.5. RAB-10 loss results in an enlargement of SQST-1::GFP foci

SQST-1 is the *C. elegans* ortholog of the autophagy cargo adaptor protein, p62/SQSTM1, which mediates the autophagic degradation of poly-ubiquitinated cargo, by binding to LC3/LGG-1 (Vadlamudi et al. 1996; Pankiv et al. 2007). In both non-dauer and dauer animals, SQST-1::GFP is expressed in the pharynx/head, vulva, and tail (data not shown). RNAi-mediated depletion or loss of function mutations in autophagy genes, including genes important for autophagic flux, results in the formation of enlarged SQST-1::GFP positive punctate structures throughout the worm (Pankiv et al. 2007; Korolchuk et al. 2009; Guo et al. 2014a; Guo et al. 2014c).

To determine if RAB-10 is required for SQST-1/p62::GFP degradation by autophagy, we examined the expression levels of the SQST-1/p62::GFP reporter in *daf-2; rab-10* double mutants, grown at the permissive and restrictive temperatures ([Fig. 2.5](#)). We found that *daf-2; rab-10* dauer and non-dauer animals contained larger SQST-1/p62::GFP positive punctate structures, when compared to *daf-2* single mutants otherwise wild-type for *rab-10* ([Fig. 2.5A and 2.5B](#)), suggesting that RAB-10 is required for the normal degradation of SQST-1/p62::GFP. We observed a slight increase, although not significant, in the average number of SQST-1/p62::GFP structures in *daf-2; rab-10* non-dauer double mutants, when compared to *daf-2/IIR* non-dauer single mutants. Interestingly, *daf-2/IIR* dauers (raised at the restrictive temperature) displayed a decrease in the number, but an increase in the size of SQST-1/p62::GFP punctate structures, when compared to the *daf-2/IIR* single mutant control animals ([Fig. 2.5B](#)). Altogether, our results suggest that *rab-10* is required for the proper degradation of SQST-1/p62::GFP during autophagy inducing conditions, and these results further support our observations that RAB-10 is required for autophagic flux.

### 2.3.6. RAB-10 is required for trafficking of GFP::ATG-9

In yeast and mammals, autophagosome formation requires the constant cycling of the sole transmembrane protein, Atg9/ATG9, from membrane-bound organelles to the preautophagosomal structure (PAS), and vice versa (Noda et al. 2000; Young et al. 2006; Suzuki et al. 2007; Yen and
FIG. 2.5: RAB-10 is required to maintain the number and size of SQST-1/p62::GFP positive foci in daf-2(e1370) mutants. A) Representative deconvolved epifluorescent images of SQST-1/p62::GFP expression in the body of daf-2(e1370) and daf-2(e1370); rab-10(ok1494) L3 larvae (nondauer) and dauer animals. B-C) Quantification of the average number (B) and size (C) of SQST-1/p62::GFP-positive foci in the body of daf-2(e1370) and daf-2(e1370); rab-10(ok1494) L3 larvae and dauer larvae. Data shown represents an average of two independent trials. Error bars ± S.E. of the mean. Arrows point to representative SQST-1::GFP punctate structures/foci in (A). Statistical analysis was done using an unpaired, two tailed t-test, p-value *≤0.05 **≤0.01 and ***≤0.001. Magnification 400X. scale bar: 2 μm.
In yeast, Atg9 was found to localize to mitochondria, Golgi, recycling endosomes, and unique vesicles referred to as “Atg9 reservoirs”, while in mammals, ATG9 was found on Golgi, early endosomes, late endosomes, recycling endosomes, and similarly on ATG9-specific vesicles that could also be referred to as “ATG9 reservoirs” (Young et al. 2006; Mari and Reggiori 2010; Longatti et al. 2012; Orsi et al. 2012; Popovic and Dikic 2014). The localization of Atg9/ATG9 to pre-autophagosomes, is a transient process, where once Atg9/ATG9 is localized to the PAS, it is quickly recycled back to the periphery (Mari and Reggiori 2010; Orsi et al. 2012). In yeast, the anterograde trafficking of Atg9 to the site of autophagosome formation was dependent on Sec4, the yeast ortholog of RAB-10 (Geng et al. 2010). In mammals, ATG9 translocates to autophagosomes in an ULK1 and PI3K dependent manner (Young et al. 2006; Longatti et al. 2012; Orsi et al. 2012; Popovic and Dikic 2014).

To test whether RAB-10 plays a role in ATG-9 trafficking in C. elegans, we examined GFP::ATG-9 localization in the intestine of *rab-10* loss of function mutants. GFP::ATG-9 positive punctate structures were visualized in the apical and basolateral regions of intestinal cells (Fig. 2.6, 2.8). GFP::ATG-9 positive punctate structures may represent endocytic compartments, since its mammalian ortholog associates with similar compartments in mammals (Young et al. 2006; Longatti et al. 2012; Orsi et al. 2012). We evaluated GFP::ATG-9 localization in the intestinal cells of *daf-2; rab-10* mutants compared to the *daf-2* single mutant controls (Fig. 2.6A-C), and found that loss of *rab-10* had no effect on the number of GFP::ATG-9 positive punctate structures in *daf-2/IIR* dauer or non-dauer animals (Fig. 2.6A and 2.6B). However, loss of *rab-10* resulted in a significant change in the size of the intestinal GFP::ATG-9 positive structures in *daf-2/IIR* dauer and non-dauer animals, when compared to controls. GFP::ATG-9 positive structures were significantly larger in dauer animals (Fig. 2.6A and 2.6C), where autophagy is significantly elevated. These data show that RAB-10 may be required to provide GFP::ATG-9 membrane to the phagophores/pre-autophagosomes, or be involved in the retrieval of ATG-9. Another possibility is that RAB-10 involvement in recycling may indirectly alter the trafficking of GFP::ATG-9 to the sites of autophagosome formation.

To verify that RAB-10 is required for autophagy in the intestine, similarly to what was observed in seam cells, we examined an intestinal mCherry::LGG-1 reporter in *daf-2/IIR* dauer and non-dauer animals.
FIG. 2.6: RAB-10 controls the size of GFP::ATG-9-positive foci in daf-2 intestinal cells. A) Representative confocal images of GFP::ATG-9 expression in intestinal cells of daf-2(e1370), and daf-2(e1370); rab-10(ok1494) mutants. B-C) Quantification of the average number (B) and size (C) of intestinal GFP::ATG-9 positive foci in daf-2(e1370) and daf-2(e1370); rab-10(ok1494) non-dauer (L3) and dauer animals. Data shown represents an average of two independent trials. n= 25 animals/strain. Statistical analysis was done using an unpaired, two tailed t-test. p-value *≤0.05, ***≤0.001. Error bars = ± S.E. Arrows point to representative GFP::ATG-9 intestinal punctate structures in (A). DAPI filter was used to identify autofluorescent vesicles. Magnification 630X. scale bar: 2 µm.
Non-dauer daf-2/IIR mutant animals displayed mCherry::LGG-1 localized to the apical plasma membrane and its expression was mostly diffuse with occasional punctate structures (Fig. 2.7A). In daf-2/IIR dauers, mCherry::LGG-1 expression was more punctate in nature (Fig. 2.7A and 2.7D).

Interestingly, the daf-2; rab-10 non-dauers (raised at the permissive temperature) displayed an increase in the number of intestinal mCherry::LGG-1 positive foci, but no change in size, when compared to daf-2 non-dauer controls (Fig. 2.7B-C). daf-2; rab-10 dauers (raised at the restrictive temperature) had an increase in the size of GFP::LGG-1 foci, although no further accumulation in the number of mCherry::LGG-1 positive foci was observed, when compared to daf-2 dauers, otherwise wild-type for rab-10.

Furthermore, consistent with the results in seam cells, rab-10 single mutants had an increase in the number of mCherry::LGG-1 foci in the intestine, but no change in the size of GFP::LGG-1 foci, when compared to wild-type control animals (Fig. 2.7D-F). These data show that RAB-10 is also required for autophagy in intestinal cells, and the loss of rab-10 may significantly change the number or the size of autophagy reporters depending on the level of autophagy induction.

### 2.3.7. GTPase cycling of RAB-10 is required for GFP::ATG-9 traffic in intestinal cells

Essential to the function of RAB GTPases is their ability to cycle between GTP-bound (active) and GDP-bound (inactive) states (Stenmark 2009; Hutagalung and Novick 2011). In the GTP-bound state, RAB GTPases associate with membranes and bind to effector proteins important for cellular processes, such as vesicle transport and/or membrane fusion events. In the GDP-bound state, they dissociate from their effector proteins and become cytosolic (Stenmark 2009; Hutagalung and Novick 2011). It is well established that GTPases in the active form are important for membrane trafficking events, and thus, we hypothesized that this would also be true for RAB-10 and autophagy (Grosshans et al. 2006b; Stenmark 2009; Hutagalung and Novick 2011).

To determine if the GTP-bound form of RAB-10 is required for autophagy, we investigated the rescue potential for tagRFP::RAB-10(wt) or tagRFP::RAB-10(Q68L); Q68L is an amino acid change in the GTP hydrolysis domain that results in constitutive binding to GTP (Fig. 2.8) (Wiegandt et al. 2015). rab-10 deletion mutants displayed a decrease in the number and size of intestinal GFP::ATG-9 foci (Fig. 2.8A-
FIG. 2.7: RAB-10 is required for the proper localization of mCherry::LGG-1 in intestinal cells.  
A) Representative deconvolved epifluorescent images of mCherry::LGG-1 in intestinal cells of daf-2(e1370), daf-2(e1370); rab-10(ok1494) L3 larvae and dauer larvae.  
B&C) Quantification of the average number (B) and size (C) of mCherry::LGG-1 foci in daf-2(e1370) and daf-2(e1370); rab-10(ok1494) L3 and dauer animals.  
D) Representative deconvolved epifluorescent images of mCherry::LGG-1 in intestinal cells of wild type and rab-10(ok1494) L3 larvae.  
E&F) Quantification of the average number (E) and size (F) of mCherry::LGG-1 foci in wild type and rab-10(ok1494) L3 larvae.  
Data shown represents an average of two independent trials, except for data found in D-F, which consists of three trials. n ≥ 26/strain (A-C) and n ≥ 40/strain (D-F).  
Statistical analysis was done using an unpaired, two tailed t-test. p-value *≤0.05, ***≤0.001. Error bars = ± S.E. Arrows point to representative intestinal mCherry::LGG-1 foci. Magnification 400X. scale bar: 2 μm.
FIG. 2.8: Proper GTP hydrolysis of RAB-10 is required for the recruitment of ATG-9 to vesicles in intestinal cells. **A)** Representative confocal images of GFP::ATG-9 expression in intestinal cells of wild type and *rab-10(ok1494)* mutants that contain the wild-type or GTP-locked version of RAB-10. **B)** Quantification of the average number of intestinal GFP::ATG-9 positive foci per unit area. **C)** Quantification of the average size of intestinal GFP::ATG-9 positive foci per unit area. Data shown represents an average of two independent trials. *n* ≥ 20/strain. Statistical analysis was done using an unpaired, two tailed t-test. *p*-value *≤* 0.05, *p** ≤ 0.01, and *** ≤ 0.001. Error bars = ± S.E. Arrows point to representative GFP::ATG-9 positive foci. DAPI filter was used to identify autofluorescent vesicles. Magnification 630X. scale bar: 2 μm.
suggesting that RAB-10 is responsible for the recruitment of GFP::ATG-9 to vesicular structures, or potentially in the retrieval step from autophagosomes. Expression of the RFP::RAB-10(wt) transgene mostly rescued the reduced number and the decreased size of GFP::ATG-9 positive foci in rab-10 deletion mutants (Fig. 2.8A-C). Interestingly, we found that expression of tagRFP::RAB-10(Q68L) only partially rescued the reduced size of intestinal GFP::ATG-9 positive foci and failed to rescue the decrease in the number of GFP::ATG-9 positive foci of rab-10(ok1494) mutants (Fig. 2.8A-C). These results show that the constitutively active form of RAB-10 is not sufficient to rescue the altered size and number of GFP::ATG-9 foci in rab-10 mutants, and indicate that the cycling between the GDP and GTP bound forms of RAB-10 may be required for the recruitment of GFP::ATG-9 to punctate structures, or that alternatively, RAB-10 may be required for the retrieval of ATG-9.

In an effort to better understand the function of RAB-10 in the trafficking of ATG-9, we investigated the colocalization between RAB-10 and ATG-9 and failed to observe significant colocalization between RFP::ATG-9 and GFP::RAB-10 in daf-2 mutant L3 non-dauer animals or dauers (Fig. 2.9). We note that dauers had rare colocalization of the two markers but the Pearson’s Correlation Coefficient remained negative. Thus, the role of RAB-10 in ATG-9 localization may not be direct. Similarly, we investigated the colocalization of ATG-9 and LGG-1 in rab-10 mutants. A CERULEAN::VENUS::LGG-1 reporter, referred to as dFP::LGG-1 was used (Chapin et al. 2015), because it is only expressed in the intestine and contained less background fluorescence, to measure the colocalization between LGG-1 and ATG-9. We found that in rab-10 loss of function mutants, there was no change in the level of colocalization between tagRFP::ATG and dFP::LGG-1 (Fig. 2.10).

2.4. Discussion

In conclusion, the small GTPase RAB-10 is required for autophagy flux in C. elegans. Compared to daf-2 single mutant dauers, we observed that daf-2; rab-10 double mutant dauers have enlarged GFP::LGG-1 positive foci in the seam cells, as well as enlarged mCherry::LGG-1 positive foci in the intestine. The number of GFP::LGG-1 positive foci decreased in daf-2; rab-10 double mutant dauers, when compared to daf-2 dauers, but did not show a significant change with the mCherry::LGG-1 reporter in the intestine. When comparing the accumulation of mCherry::LGG-1 foci in the intestine and the
FIG. 2.9: ATG-9 does not colocalize with RAB-10-positive endosomes. A) Representative deconvolved epifluorescent images of daf-2(e1370) L3 and dauer animals that coexpress tagRFP::ATG-9 and GFP::RAB-10 in intestinal cells. B) Quantification of the Pearson’s correlation coefficient for colocalization between tagRFP::ATG-9 and GFP::RAB-10. A-B) Animals were analyzed at the L3 or dauer larval stages at 15°C and 25°C. n# ≥ 10 animals/strain; # of units per area analyzed/animal = 2. Statistical analysis was done using an unpaired, two tailed t-test. Error bars = ± S.E. Arrow heads point to representative GFP::RAB-10-positive vesicles, arrows point to representative tagRFP::ATG-9-positive vesicles, and notched arrow points to colocalized puncta Magnification 630X.
FIG. 2.10: Loss of rab-10 does not alter the localization of ATG-9 to LGG-1-positive autophagosomes. A) Representative deconvolved epifluorescent images of wild type and rab-10(ok1494) animals that coexpress tagRFP::ATG-9 and CERULEAN::VENUS::LGG-1 in intestinal cells. B) Quantification of the Pearson’s correlation coefficient for colocalization between tagRFP::ATG-9 and CERULEAN::VENUS::LGG-1. A-B) Animals were analyzed at the L3 larval stage at 15°C and 25°C. n# ≥ 6 animals/strain; # of units per area analyzed/animal = 2. Statistical analysis was done using an unpaired, two tailed t-test. Error bars = ± S.E. Magnification 630X.
accumulation of GFP::LGG-1 foci in hypodermal seam cells, it is important to note that mCherry is insensitive to acid quenching and degradation in lysosomes, and thus slight differences in the accumulation of the two reporters can be expected. We observed consistent results between the number of GFP::LGG-1 and mCherry::LGG-1 positive foci in both the intestine and seam cells, as we found an increase in the number of either reporter in *daf-2; rab-10* mutants when compared to the *daf-2* non dauer L3 larvae. Clearly, when compared to *daf-2* single mutants, the change in number and size of LGG-1-labeled foci in the intestine, and hypodermal seam cells, of *daf-2; rab-10* double mutants, suggest a defect in autophagosome biogenesis.

SQST-1/p62::GFP positive punctate structures were also increased in the *daf-2; rab-10* double mutant dauers. Our results indicate a disruption in autophagic flux in *rab-10* mutants. To determine this, we found that treatment of *rab-10* mutants with chloroquine to inhibit lysosomal activity, did not increase the number of GFP::LGG-1 positive foci in seam cells, when compared to control mock-treated *rab-10* mutants. We also found that the colocalization of GFP::LGG-1 and LMP-1::tagRFP was significantly reduced in *daf-2; rab-10* dauers. We conclude from these experiments that RAB-10 is required for autophagosome degradation or autophagic flux. However, we cannot exclude the possibility that RAB-10 has an earlier role in the autophagy pathway, such as in autophagosome induction. We also, have not excluded that the RAB-10 recycling function is important at this step.

In yeast, the anterograde transport of Atg9 is dependent on Sec4, and the Sec4-dependent GDP/GTP exchange factor (GEF), Sec2, as well as the SNARE proteins, Tlg2, Sec22, and Sso1 (Geng et al. 2010; Nair et al. 2011). Defects in Atg9 cycling in yeast results in defects in autophagy dynamics and flux (Young et al. 2006; Suzuki et al. 2007; Yen and Klionsky 2007; Suzuki et al. 2015). Our results show that the localization of GFP::ATG-9 to punctate structures depends on RAB-10 function. *rab-10* mutants have a reduction in the number and size of GFP::ATG-9 positive punctate structures in epithelial intestinal cells, which was rescued only by the expression of the tagRFP::RAB-10(wt) transgene, and not by the GTP-locked RAB-10 mutant, suggesting RAB-10 GTPase cycling is required for proper ATG-9 localization. In *daf-2/IIR* mutants, the loss of *rab-10* altered the size of GFP::ATG-9 foci, since non-dauer L3 larvae had smaller sized GFP::ATG-9 punctate structures and dauer animals had larger GFP::ATG-9 punctate structures. Thus, RAB-10 may be important for recruiting ATG-9 to vesicular/punctate structures
and/or regulating ATG-9 trafficking dynamics. Finally, transgenic rescue experiments demonstrated that
the GTP locked RAB-10 GTPase transgene failed to rescue the changes in size or number of GFP::ATG-
9 positive structures in *rab-10* mutants completely, suggesting that the GTPase cycling activity is
required.

Overall, our data show that the small GTPase RAB-10, a major regulator of basolateral recycling
in polarized cells, also positively regulates autophagic flux in *C. elegans*, possibly by facilitating the proper
localization of GFP::ATG-9 to endocytic compartments, which in turn is required for the initial steps of
autophagosome formation. However, in experiments where we evaluated colocalization between RAB-10
and ATG-9, using tagRFP::ATG-9 and GFP::RAB-10, colocalization was rarely observed in L3 larvae or
dauer animals. This may suggests that RAB-10 is not found on the same vesicles as ATG-9, or that both
proteins transiently interact beyond detection levels. In addition, we also evaluated the colocalization
between LGG-1 and ATG-9 in *rab-10* loss of function mutants, using CERULEAN::VENUS::LGG-1
(referred to as dFP::LGG-1) and tagRFP::ATG-9 reporters. Interestingly, we found that the level of
colocalization between dFP::LGG-1 and tagRFP::ATG-9 was unchanged in *rab-10* mutants. This
suggests that ATG-9 can localize to autophagosomes in the absence of RAB-10. Our findings suggest
that the accumulation of autophagosomes is due to defective autophagy flux, and that RAB-10 may act
on the fusion of autophagosome with recycling endosomes, or on the retrieval of ATG-9 from
autophagosomes/ autolysosomes.

### 2.5. Materials and Methods

#### 2.5.1. *C. elegans* strains: All strains were maintained on standard OP50 *Escherichia coli* diet.(Brenner
1974) *daf-2(e1370)* strains were analyzed at the permissive temperature (15°C) for L3 larvae or the
restrictive temperature (25°C) for dauer larvae. A full list of strains is found below.

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA2123</td>
<td>adls2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]</td>
</tr>
<tr>
<td>MAH14</td>
<td>daf-2(e1370); adls2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]</td>
</tr>
<tr>
<td>VK1241</td>
<td>vkEx1241[Pnhx-1::mCherry::LGG-1 + Pmyo-2::GFP]</td>
</tr>
<tr>
<td>RT533</td>
<td>unc-119(ed3); pwl214[PRAB-10::GFP::RAB-10, Cb unc-119(+)]</td>
</tr>
<tr>
<td>QU140</td>
<td>daf-2(e1370); bpIs151[PSQST-1::SQST-1::GFP, unc-76]</td>
</tr>
</tbody>
</table>
2.5.2. Construction of ATG-9 transgenic strains: Construction of the GFP and tagRFP::ATG-9 transgenes was performed using the Gateway Technology cloning system (ThermoFisher Scientific). To
specifically express the GFP::ATG-9 and tagRFP::ATG-9 transgenes in the intestine, a vha-6 promoter-driven-vector, modified with a Gateway cassette, was inserted at the EcoRI or NaeI site, downstream of the GFP or RFP coding region. The atg-9 cDNA was amplified by PCR using the Herculase II high-fidelity DNA polymerase kit (Agilent technologies) and primers with modified attB sites (Untergasser 2006) from an initial clone kindly gifted by Dr. Daniel Colón Ramos. The resulting PCR product was cloned into the Gateway pDONR221 entry vector via the BP-clonase II reaction (ThermoFisher Scientific). The pDONR221 vectors containing the atg-9 cDNA were then subcloned into the modified vha-6 promoter-driven-vector via the LR-clonase II reaction (ThermoFisher Scientific). Integrated transgenic lines were isolated by microparticle bombardment.(Praitis et al. 2001)

2.5.3. Microscopy and Image analysis: Live worms were mounted on 3% agarose pads with 50mM sodium azide (sigma) in M9 buffer. Fluorescence images were obtained using an Axioimager M2 microscope equipped with a CCD camera (Carl Zeiss microscopy), captured using the Axiovision release 4.8 software (Carl Zeiss microscopy), and deconvolved using the Axiovision 3D deconvolution module (Carl Zeiss microscopy). To obtain confocal images, we used a Leica TCS-SP5 laser scanning confocal microscope (Leica microsystems), equipped with a PMT (photomultiplier) detector, and captured images with the Leica Application Suite AF software (Leica microsystems). All images were acquired as z-stacks at a thickness of 0.5 microns. Quantification of images was performed using the open source software FIJI (FIJI is just image J, National Institute of Health).

For quantification of fluorescently labeled punctate structures, in seam cells and/or intestinal cells, z-stacks were converted to maximum intensity projections using either the GFP or RFP/mCherry channel, and adjusted using the subtract background option in FIJI. Foci/punctate structures were then isolated by thresholding-based segmentation. GFP::LGG-1, mCherry::LGG-1, and GFP::ATG-9 foci ≥ 0.01µm², and SQST-1/p62::GFP foci ≥ 0.069µm², were quantified. For quantification of intestinal foci, a consistent region of interest was selected for the anterior and posterior intestine. To analyze the mCherry::LGG-1 expression in the intestine, we measured the number and size of mCherry::LGG-1 positive foci in the posterior region of the intestine only, since this region consistently expressed the mCherry::LGG-1 transgenic reporter. In the seam cells of dauer animals, the size of GFP::LGG-1 foci was quantified by
scanning through z-slices, and adjusting the threshold to highlight the entire area of individual foci found. The add to manager tool in FIJI was used to ensure a single foci was not counted more than once. For colocalization analysis, the colocalization threshold plugin in FIJI was used to calculate Pearson’s correlation coefficient (R). For colocalization analysis in seam cells, a single z-slice corresponding to the mid-region of each seam cell was used to determine Pearson’s correlation coefficient between GFP::LGG-1 and LMP-1::RFP. For colocalization analysis in intestinal cells, single z-slices with the greatest amount of GFP and RFP-positive foci were chosen. Autofluorescent vesicles labeled with the DAPI filter were removed from green and red channels using the image calculator feature, and both channels were adjusted using the subtract background option.

2.5.4. RNA interference: RNAi experiments for the candidate gene screen were performed as previously described, with slight modifications (Timmons and Fire 1998). In short, daf-2(e1370) L4 larvae were placed overnight at 15°C on NGM plates supplemented with 2mM IPTG, 50mg/ml carbenicillin, and HT115 bacteria containing dsRNA expression vectors (RNAi plates). F1 progeny from mothers were synchronized as L1 larvae and transferred to 25°C on RNAi plates, and were allowed to grow at this temperature until reaching the dauer larval stage. Image acquisition and analysis was carried out as described above. For RNAi clones resulting in embryonic lethality, synchronized progeny were fed dsRNA at the start of L1 and analyzed when they reached the dauer stage. For the RNAi screen, dauer animals were evaluated for changes in the localization of GFP::LGG-1 in seam cells, and RNAi clones that altered GFP::LGG-1 localization were considered as positives. RNAi clones were obtained from the Ahringer (Kamath and Ahringer 2003) and Vidal (Rual et al. 2004) genomic RNAi libraries and were sequence verified using the M13-forward primer. For all RNAi experiments, the empty vector, L4440, was used as a control.

2.5.5. Chloroquine treatment: Standard NGM plates seeded with OP50 were UV-treated at 500J/cm² for 10 minutes using a UV-crosslinker (Fisher Scientific). UV-treated plates were then supplemented with either 30 mM chloroquine (Sigma) or dH2O as a control. Animals were raised on live OP50 NGM plates at 20°C and synchronized as L1 larvae. L1 larvae were then transferred onto UV treated plates with either
chloroquine or control, and allowed to grow at 20°C until they reached the L3 larval stage.

2.6. Acknowledgements

This work was supported by a grant from the NIH (R15 GM102846) to A. M. and (GM067237) to B. G. We thank Drs. Daniel Colón Ramos, and Hong Zhang, as well as the C. elegans Knockout Consortium, and Caenorhabditis Genetics Center (GCG), which is funded by the NIH (P40 OD010440), for strains and reagents. We also thank Drs. Anne Norris, and Nathalia Holtzman, for assistance with microscopy, as well as the Core Facility for Imaging, Cellular and Molecular Biology at Queens College. We are grateful to Drs. Cathy Savage-Dunn, and Hannes Buelow for critical reading of the manuscript and the Savage-Dunn, and Meléndez labs for advice and helpful discussions. A.M. was an Ellison Medical Foundation New Scholar in Aging (AG-NS-0521-08).
Chapter 3

3. The Atg6/Vps30/Beclin 1 ortholog BEC-1 mediates endocytic retrograde transport in addition to autophagy in C. elegans (See Appendix B for attached manuscript)


Acknowledgement: This is an accepted manuscript of an article published in Autophagy on April 1st, 2011; available online: http://www.tandfonline.com/doi/abs/10.4161/auto.7.4.14391

Abstract

Autophagy and endocytosis are dynamic and tightly regulated processes that contribute to many fundamental aspects of biology including survival, longevity, and development. However, the molecular links between autophagy and endocytosis are not well understood. Here, we report that BEC-1, the C. elegans ortholog of Atg6/Vps30/Beclin1, previously described as a key regulator of the autophagic machinery, also contributes to endosome function. In particular we identify a defect in retrograde transport from endosomes to the Golgi in bec-1 mutants. MIG-14/Wntless is normally recycled from endosomes to the Golgi through the action of the retromer complex and its associated factor RME-8. Lack of retromer or RME-8 activity results in the aberrant transport of the cargo protein MIG-14/Wntless to the lysosome where it is degraded. Similarly, we find that lack of bec-1 also results in mislocalization and degradation of MIG-14-GFP, reduced levels of RME-8 on endosomal membranes, and the accumulation of morphologically abnormal endosomes. We further identify a requirement for BEC-1 in the clearance of apoptotic corpses, suggesting a role for BEC-1 in phagosome maturation, a process recently reported to depend upon retrograde transport

Contribution to this paper:

In this manuscript, I analyzed the role of bec-1 in cell corpse engulfment and degradation. In the adult hermaphrodite, the gonad consists of two U-shaped tubes, with each tube consisting of a distal and
proximal arm (Hirsh et al. 1976; Sulston et al. 1983; Gumienny et al. 1999; Hubbard and Greenstein 2000). These tubes are surrounded by a single layer of cells, called sheath cells, and are filled with a large number of germ cells (Hirsh et al. 1976; Kimble and Hirsh 1979; Gumienny et al. 1999; Hubbard and Greenstein 2000). Germ cells near the distal end of the gonad undergo mitosis, however, as they progress towards the proximal end, they undergo meiosis, and eventually form oocytes (Austin and Kimble 1987; Hubbard and Greenstein 2000). As germ cells progress towards the bend of the gonad, many undergo apoptosis and appear as "refractile" bodies/buttons by differential interference contrast (DIC) microscopy, and are quickly recognized and engulfed by sheath cells (Gumienny et al. 1999; Hubbard and Greenstein 2000). *bec-1(ok691)* null mutants exhibit a large number of apoptotic cell corpses in the germline, which could be due to increased apoptosis or decreased engulfment/degradation (Takacs-Vellai et al. 2005). Germ cell corpses in wild type animals are rapidly degraded within one hour of engulfment, while corpses in various CED mutants (i.e. *ced-1(e1735)*) are not engulfed and persist for hours (Sulston and Horvitz 1977; Gumienny et al. 1999; Gumienny et al. 2001). We found that corpses in *bec-1(ok691)* mutants were engulfed by sheath cells, suggesting defects in degradation and not engulfment (*Figure B5A in attached manuscript*). To determine if *bec-1(ok691)* mutants did indeed have defects in corpse degradation, I conducted a time lapse experiment to determine the duration of time that cell corpses persisted. As expected, I found that cell corpses persisted for over one hour in *bec-1(ok691)* null mutants, compared to wild type animals (*Figure B5B in attached manuscript*). From this result, and the results showing that *bec-1(ok691)* corpses are indeed engulfed by sheath cells, I concluded that *bec-1(ok691)* null mutants have a defect in corpse degradation, but not engulfment. Similar results were found for the depletion of additional autophagy genes, such as *vps-34* (*Figure B5C in attached manuscript*). Taken together, these results showed for the first time, a role of autophagy genes in apoptotic germ cell corpse clearance in *C. elegans.*
4. Discussion and future directions

To improve our understanding of the connections that exist between autophagy and endocytosis, we conducted an RNAi screen for endocytic genes that altered GFP::LGG-1 expression and blocked tissue remodeling in *daf-2/IIR* mutants. The goal of this screen was to identify new or previously characterized endocytic genes that function in autophagy, further elucidate the mechanism by which these genes control autophagy, and enhance our understanding as to how distantly related processes are interconnected.

We identified the small GTPase, RAB-10, as a mediator of autophagic flux in *C. elegans*. A connection between RAB-10 and autophagy was first made when *rab-10* depletion failed to further enhance the longevity phenotype of *eat-2* DR LOF mutants, which have increased levels of autophagy (Hansen et al. 2007). In addition, *rab-10* mRNA levels were significantly reduced in *eat-2* LOF mutants (Hansen et al. 2007). *rab-10* null mutants have an increase in the number of GFP::LGG-1 foci, when compared to wild type animals, which was interpreted as an increase in autophagy activity (Hansen et al. 2008a). However, this report did not investigate if the loss of *rab-10* increased autophagy activity or decreased autophagy flux (Hansen et al. 2008a). The results presented in this thesis show that RAB-10 is a positive regulator of autophagy flux.

4.1. RAB-10 promotes autophagy flux in *C. elegans*

We were interested in investigating the potential crosstalk between endocytic genes in the autophagy pathway. Based on our studies, RAB-10 promotes autophagy activity in *C. elegans*. We found that *daf-2; rab-10* double mutant dauers have enlarged LGG-1-labeled foci in both seam cells and intestinal cells, compared to *daf-2* dauer controls (Fig. 2.2C and Fig. 2.7C). A similar phenotype was observed in *daf-2; atg-7* or *daf-2; atg-9* double mutants (Fig. 2.2C). Interestingly, *daf-2; rab-10* dauers, had no change in the number of mCherry::LGG-1 intestinal foci, when compared to *daf-2/IIR* single mutants. In contrast, a significant decrease in the number of GFP::LGG-1 foci in seam cells was observed in the *daf-2; rab-10* dauers (Fig. 2.2A,B and 2.7A,B). When comparing mCherry::LGG-1 in the intestine, to GFP::LGG-1 foci in seam cells, it is important to note that mCherry is insensitive to the acidic environment of the lysosome,
and thus slight differences in the expression of the two reporters can be expected, especially in dauer as with elevated levels of autophagy (Patterson et al. 1997a; Campbell and Choy 2001; Ward 2006).

Compared to controls, the consistent increase in the number of LGG-1-positive foci in intestinal cells (Fig. 2.7A-F), and seam cells (Fig. 2.2D-F and 2.3A-C), found in daf-2; rab-10 double and rab-10 single mutant non-dauers (L3s), suggests that defective autophagic bodies accumulate as a result of rab-10 loss. Likewise, we observed an increase in the size of SQST-1::GFP foci in daf-2; rab-10 double mutant non-dauers and dauers, compared to controls, which indicates an accumulation of autophagy cargo (Fig. 2.5A-C). These results, combined with our results showing that lysosomal inhibition does not further increase the number of GFP::LGG-1 foci in rab-10 mutants (Fig. 2.4A-C), and that GFP::LGG-1 and LMP-1::tagRFP colocalization is significantly reduced in daf-2; rab-10 dauers (Fig. 2.4D and E), supports a role for rab-10 in promoting autophagy flux.

4.2. RAB-10 GTPase cycling is required for autophagy activity

Our data show that RAB-10 GTPase cycling, between its GTP-bound and GDP-bound states, is required for autophagy function, since GTP-locked RAB-10 was unable to restore the number of GFP::ATG-9 foci to wild type levels in rab-10 LOF mutants (Fig. 2.8A-C). RAB-10 in either its GTP-bound or GDP-bound state, may differentially regulate autophagy activity, and therefore act as a switch regulatory element. This is commonly seen in RAB GTPase cascades, where a GTPase (A), in its GTP-bound form, recruits a GEF for a downstream GTPase (B), and GTPase B recruits the GAP for GTPase A and GEF for another downstream GTPase (Hutagalung and Novick 2011). Having a GTPase constitutively locked in its active or inactive state, can severely affect the activity of upstream and downstream GTPases, which can hamper a specific cellular process (Grosshans et al. 2006b; Geng et al. 2010; Hutagalung and Novick 2011).

Interestingly, in mammals, ATG9 cycling depends on a RAB cascade between RAB1 and RAB11 (Lamb et al. 2016). RAB11 binds to the GAP, TBC1D14, which in turn binds to the TRAPP complex, a GEF for RAB1, and activated RAB1 facilitates the trafficking of ATG9 to ATG9-specific compartments in an ULK1 dependent manner (Lamb et al. 2016).

To determine if autophagy requires a RAB-10 dependent cascade, one approach would be to identify candidate RAB GTPases that when mutated or overexpressed, result in a similar defect in...
autophagy as *rab-10* mutants. Once identified, the next step would be to identify potential GAPs and GEFs associated with that GTPase, possibly by conducting protein-pulldown assays. Potential GAPs and GEFs that arise from these pulldown assays can then be tested for their effects on autophagy when deleted or overexpressed. It would be predicted that if the particular GTPase functions downstream of RAB-10, it would bind to a GAP specific to RAB-10, which if overexpressed, would disrupt autophagy in a similar manner as a GDP-locked *rab-10* mutant. Conversely, when dealing with a potential GEF, its overexpression should disrupt autophagy in a similar manner to a GTP-locked *rab-10* mutant.

4.3. RAB-10 is required for the localization of ATG-9 to punctate structures

We proposed that RAB-10 functions in the trafficking of ATG-9 to the site of autophagosome biogenesis. The RAB-10 ortholog in yeast, Sec4, was shown to perform this function (Geng et al. 2010). Indeed, *rab-10* mutants have a decrease in the number and size of intestinal GFP::ATG-9-positive foci (Fig. 2.8A-C), suggesting that in the absence of *rab-10*, ATG-9 fails to localize to endocytic vesicles, possibly corresponding to endocytic vesicles or ATG-9-reservoirs (Young et al. 2006; Mari and Reggiori 2010; Longatti et al. 2012; Orsi et al. 2012; Popovic and Dikic 2014). Under conditions of high autophagy activity, we predicted that RAB-10 would localize to ATG-9-positive vesicles to deliver ATG-9 to the site of autophagosome formation. However, colocalization experiments between GFP::RAB-10 and tagRFP::ATG-9, in *daf-2* non-dauer, and dauer animals, failed to show significant colocalization, although there were some instances of colocalization in dauers (Fig. 2.9A and 2.9B). As discussed in chapter 2, the transient nature of ATG-9 trafficking may render the detection of any changes in colocalization between tagRFP::ATG-9 and GFP::RAB-10, difficult. An alternative approach for examining an interaction between ATG-9 and RAB-10 would be to conduct a yeast two hybrid (Y2H) experiment. If a direct interaction between RAB-10 and ATG-9 is found through a Y2H, we could further determine the domains of each protein necessary for the interaction. Alternatively, RAB-10 may interact with ATG-9 indirectly through an effector protein. Therefore, a GST-pulldown assay, or a co-immunoprecipitation experiment, could evaluate if RAB-10 indirectly interacts with ATG-9, and at the same time, identify potential candidates that bridge that interaction. Such results could help elucidate the mechanism by which RAB-10 regulates autophagy flux.
In experiments to evaluate colocalization between CERULEAN::VENUS::LGG-1 and tagRFP::ATG-9, we found that the colocalization between the two reporters did not significantly change in a rab-10 mutant background, compared to wild-type controls (Fig. 2.10). This suggests that ATG-9 is able to localize to autophagosomes in the absence of rab-10, and that RAB-10 does not control ATG-9 trafficking; however, careful interpretation of these results is required. As discussed in chapter 1, the ULK1 and PI3KC3 complexes are important for ATG9 cycling (Young et al. 2006; He et al. 2013; Takahashi et al. 2014). In MEF and HeLa cells lacking the PI3KC3 subunits, BIF1, UVRAG, and BECLIN1, ATG9 localization to punctate structures is disrupted (He et al. 2013; Takahashi et al. 2014). This is similar to what we observed for GFP::ATG-9 foci in rab-10 mutants (Fig. 2.8 and A5). ATG9 cycling in mammals and yeast also depends on the TRAPPIII complex (Kakuta et al. 2012; Shirahama-Noda et al. 2013; Lamb et al. 2016), however, a block in TRAPPIII activity does not alter the ability of Atg9/ATG9 to localize to autophagosomes (Kakuta et al. 2012; Lamb et al. 2016) (Fig. 2.10). Likewise, compared to controls, siRNA-mediated depletion of ULK1 reduced the number of ATG9-positive foci, but ATG9 is still able to localize to autophagosomes (Young et al. 2006; Orsi et al. 2012). Overall, we find that the loss of RAB-10, has a similar phenotype to the loss of BIF1, or TRAPPIII activity, on ATG9 trafficking, and therefore we cannot exclude the possibility that RAB-10 participates in ATG-9 cycling. An additional mechanism for how RAB-10 might regulate autophagy is proposed below.

In all, our results show that RAB-10 is an evolutionarily conserved mediator of autophagy function, and expand our knowledge on the role of GTPases in autophagy.

4.4. Elucidating the mechanism by which RAB-10, and other endocytic genes, regulate autophagy activity in C. elegans (Discussion on work described in Appendix A)

4.4.1 RAB-10 functions in a distinct step of the autophagy pathway

Our results provide evidence of rab-10 function in a step of the autophagy pathway distinct from atg-7 and atg-9, since we found that atg-7 and unc-51 are epistatic to rab-10 (Fig. A3). It would be interesting to determine if atg-7 and unc-51 are also epistatic to rab-10 in daf-2; rab-10 dauers, where the levels of autophagy are increased. Additionally, given that the levels of autophagy activity influence the phenotypes associated with a loss of rab-10, atg-7, and atg-9, it would also be important to investigate the epistatic relationship between rab-10 and various autophagy genes in non-dauer animals, such as wild type
or daf-2 L3 larvae. Our GTPase cycling data suggest that \textit{rab-10} may act upstream of \textit{unc-51} to promote autophagy flux (Fig. 2.8A-C, A4 and A5). Therefore, genes upstream of \textit{unc-51} should be tested for their effect on GFP::LGG-1 localization in daf-2; \textit{rab-10} dauers. One possible candidate would be \textit{let-363}/TOR, since the \textit{let-363}/TOR kinase is a major regulator of autophagy (Kamada et al. 2000)(Hosokawa et al. 2009; Jung et al. 2009). This experiment would also determine whether \textit{rab-10} is required to inhibit TOR function.

As described in chapter 1, in mammalian adipocytes, activated RAB10 recruits the RALA GEF, RLF/RGL2 (Karunanithi et al. 2014), however, RGL2 also acts as a GEF for RALB (Wolthuis et al. 1997). RALA and RALB, are two paralog GTPases that function with the exocyst in vesicle exocytosis. RALB specifically works with the exocyst complex to induce autophagy during times of stress (Bielinski et al. 1993; de Leeuw et al. 1999; Moskalenko et al. 2002; Moskalenko et al. 2003). Active RALB leads to the dissociation of mTORC1 from the exocyst scaffold complex, inducing autophagy (Bodemann et al. 2011; Martin et al. 2014). In \textit{C. elegans}, the RALA/B ortholog, RAL-1, functions with the exocyst complex in excretory canal lumenogenesis, while RAB-10 interacts with the exocyst in basolateral recycling (Frische et al. 2007; Armenti et al. 2014; Chen et al. 2014). Therefore, another mechanism by which RAB-10 could promote autophagy activity is by utilizing the exocyst complex to promote RAL-1 activation and LET-363/TOR inhibition. Currently, a connection between RAB-10, LET-363/TOR, and RAL-1 in \textit{C. elegans} has not been determined (Martin et al. 2014). One approach for investigating such a connection would be to evaluate if the loss of \textit{ral-1} results in a similar defect in autophagy as the loss of \textit{rab-10}. Moreover, colocalization experiments between fluorescent reporters labeling RAL-1, RAB-10, and the RGL2 ortholog, RGL-1, can determine if these proteins potentially interact, which can be further validated with a Y2H or GST-pull down experiment. Since a fluorescent reporter does not exist for LET-363, we could evaluate if an interaction exists between RAB-10 and RHEB-1, a positive regulator of Tor/mTOR/LET-363 (Long et al. 2005).

mTORC1 directly regulates the activity of the transcription factor, TFEB, which regulates the transcription of lysosomal genes and autophagy genes (Settembre et al. 2011; Martina et al. 2012; Rocziak-Ferguson et al. 2012). One of the autophagy genes under the control of TFEB activity is \textit{Atg9} (Rocziak-Ferguson et al. 2012). In \textit{glp-1}/germline-less mutants, the TFEB ortholog, HLH-30, regulates the transcriptional activity of \textit{atg-9}, with a loss of \textit{hlh-30} reducing \textit{atg-9} expression (Lapierre et al. 2013a).
Therefore, to further connect RAB-10 and RAL-1 to LET-363/TOR, we could evaluate how a loss of RAB-10 and RAL-1 effects the nuclear localization of HLH-30, and then evaluate the mRNA levels of \textit{atg-9} using qRT-PCR or an \textit{atg-9} transcriptional reporter. Overall, a loss of \textit{rab-10} could hinder RAL-1 activation, which could promote LET-363/TOR activity, reduce HLH-30 activity, and decrease \textit{atg-9} transcription and translation. If found true, this could explain the decrease in the number and size of GFP::ATG-9 foci in \textit{rab-10} LOF mutants, and the disruption of autophagy flux.

Future experiments will investigate if RAB-10 promotes autophagy flux by mediating ATG-9 trafficking or LET-363/TOR regulation, or both. As discussed in appendix A1, we attempted to investigate whether RAB-10 promotes ATG-9 trafficking from early and late endocytic compartments, and/or ATG-9-positive reservoirs (\textbf{Fig. A10, A11, and A12}). However, we failed to see any significant change in the colocalization between GFP::ATG-9, and the early or late endosomal reporters, RFP::RAB-5, or RFP::RAB-7, in \textit{rab-10} and \textit{daf-2} single mutants, compared to controls, which we attributed to the transient nature of ATG-9 trafficking (\textbf{Fig. A10, A11, and A12}). Future experiments should focus on determining if any interaction exists between ATG-9 and RAB-10, and investigate the potential interaction between RAB-10 and LET-363/TOR.

\textbf{4.4.2 Identification of additional endocytic genes that regulate autophagy activity}

As discussed in chapter 2 and appendix A1, we found that RNAi-mediated depletion of several endocytic genes disrupted the localization of GFP::LGG-1 and SQST-1::GFP (\textbf{Fig. 2.1, Fig. A1, and Table A1}). The formation of enlarged GFP::LGG-1 foci in \textit{daf-2/IIR} mutants (grown at the restrictive temperature to induce dauer formation), is a phenotype indicative of defective autophagy, since a similar phenotype is found upon the depletion or loss of autophagy genes (\textbf{Fig. 2.1 and Fig. 2.2}). Confirming the validity of our screen, we found that the depletion of genes previously found to impact autophagy, also disrupted the localization of GFP::LGG-1 and SQST-1::GFP in \textit{daf-2/IIR} dauers (\textbf{Fig. 2.1, Fig. A1, and Table A1}) (Jager et al. 2004; Filimonenko et al. 2007; Rusten et al. 2007; Djeddi et al. 2012; Manil-Segalen et al. 2014b; Szatmari et al. 2014). Some examples include subunits of the ESCRT machinery, the GTPase \textit{rab-11.1}, and the SNARE protein, \textit{syn-1} (Jager et al. 2004; Filimonenko et al. 2007; Rusten et al. 2007; Nair et al. 2011; Djeddi et al. 2012; Szatmari et al. 2014).
In our RNAi screen, depletion of the ESCRT machinery severely disrupted the localization of GFP::LGG-1 and SQST-1::GFP in *daf-2* dauers (Fig. 2.1, Fig. A1, and Table A1). In mammals and *D. melanogaster*, loss of the ESCRT complex disrupts amphisome formation and blocks autophagy (Jager et al. 2004; Filimonenko et al. 2007; Rusten et al. 2007). Interestingly, in *C. elegans*, the loss or depletion of certain ESCRT components, such as *vps-37*, has been reported to increase autophagy activity (Djeddi et al. 2012; Guo et al. 2014b). The reasons for these discrepancies have been attributed to differences in autophagosome maturation events when comparing *C. elegans* and other species. However, thus far, autophagosome maturation events between worms, flies, and mammals appear to be evolutionarily conserved (Djeddi et al. 2012; Manil-Segalen et al. 2014b). Thus, further studies should investigate the basis of these differences.

In yeast, mammals, and *D. melanogaster*, Ypt31/32, RAB11, and Rab11, respectively, have been shown to positively regulate autophagy (Fader et al. 2008; Geng et al. 2010; Szatmari et al. 2014). In yeast, similar to *sec4* mutants, *ypt31/32* mutants lack GFP-Atg8 foci and autophagic bodies near the vacuole (Geng et al. 2010). In mammals, RAB11 facilitates autophagosome formation by delivering recycling endosomes to growing autophagosomes, and is a critical component of amphisome formation (Fader et al. 2008; Longatti et al. 2012). Similarly, in flies, Rab11 promotes autophagy by translocating from recycling endosomes to autophagosomes, and in the process, sequesters the protein, HOOK, which negatively regulates endosome/autophagosome maturation (Szatmari et al. 2014). In our candidate RNAi screen, depletion of *rab-11.1* resulted in the formation of enlarged SQST-1::GFP foci (Fig. A1). It would be interesting to further elucidate the function of RAB-11.1 in autophagy, and to determine if it also promotes autophagic flux in *C. elegans*. In mammals, similar to worms, RAB11 is an important regulator of endocytic recycling, and interestingly, ATG9 localizes to RAB-11-positive recycling endosomes (Puri et al. 2013). One way to determine if this also occurs in *C. elegans* would be to investigate if ATG-9 localizes to RAB-11.1-positive recycling endosomes. In addition, to determine if RAB-11.1 promotes autophagy, we could investigate whether the number of GFP::LGG-1 foci is increased, or remains constant, in *rab-11.1* mutants treated with chloroquine, a lysosomal inhibitor. Results from these experiments would support an evolutionarily conserved role for RAB-11.1 involvement in autophagy.
SYN-1 is another protein of interest, since depletion of syn-1 results in the formation of enlarged GFP::LGG-1 foci similar to that found in rab-10 RNAi treated animals (Fig. 2.1 and Table A1). SYN-1 is the ortholog of Sso1/2 in yeast, and interacts with the Sec9-Sro7 complex, which in turn interacts with Sec4 to mediate the anterograde/forward trafficking of ATG9 to the site of autophagosome formation (PAS) (Lehman et al. 1999; Grosshans et al. 2006a; Nair et al. 2011; Dubuke et al. 2015). Whether a similar interaction between RAB-10 and these SNAREs exists in C. elegans is unknown. However, it would be exciting to find that RAB-10 interacts with SYN-1, either directly or indirectly, to regulate autophagy. A yeast two-hybrid approach could determine if RAB-10 and SYN-1 interact, and if RAB-10 interacts with additional SNAREs found in our screen, such as the Sec9 ortholog, AEX-4 (Mahoney et al. 2008; Nair et al. 2011). aex-4 depletion only resulted in the formation of enlarged SQST-1::GFP foci and did not significantly change the number or size of GFP::LGG-1 positive foci (Table A1). It is possible that the degree of gene knockdown via RNAi was insufficient to produce a result for GFP::LGG-1 after RNAi depletion of aex-4, and therefore a LOF genetic mutation should be tested (Mahoney et al. 2008). In addition, we could investigate how syn-1 or aex-4 LOF mutations disrupt the localization of GFP::ATG-9 in intestinal cells and compare that to other autophagy mutants.

Interestingly, another gene identified in our RNAi screen that when depleted significantly affected the localization of SQST-1::GFP positive foci, but not that of GFP::LGG-1 positive foci, was rab-35 (Table A1). RAB-35 functions together with RME-4 to facilitate the endocytic recycling of the yolk receptor, RME-2, and functions parallel to RAB-11.1 and RME-1 (Sato et al. 2008b). In addition, RAB-35 interacts with the RAB-10 effector, CNT-1 (Shi et al. 2012). Whether a genetic loss of function mutation in rab-35 results in the formation of enlarged GFP::LGG-1 foci in daf-2 mutants and/or wild-type animals, remains unknown. Similarly, it is not known if rab-35 LOF disrupts the localization of GFP::ATG-9. Such results would further expand the network of endocytic GTPases that regulate autophagy.

To identify potential RAB-10 effectors involved in autophagy, we carried out an RNAi screen that tested all potential effectors. We investigated how RNAi-mediated depletion of RAB-10 effectors altered the localization of GFP::LGG-1 in daf-2/IIR dauers. We found that TBC-2 may regulate autophagy activity through RAB-10, and may therefore be a RAB-10 effector protein for autophagy, as a loss of function mutation resulted in the abberant mislocalization of GFP::LGG-1 to the membranes of abnormal enlarged
vesicles (Fig. A7). This should be further investigated by evaluating the expression of various autophagy reporters, such as GFP::ATG-9 and SQST-1::GFP, in the tbc-2 null mutant. In addition, if TBC-2 has a role in autophagy, we would expect it to colocalize with the autophagosome marker GFP::LGG-1. A direct interaction between TBC-2 and LGG-1 or SQST-1 can also be investigated by yeast two-hybrid analysis or GST-pulldown experiments.

Another effector that may participate with RAB-10 in the regulation of autophagy is the exocyst complex. In endocytosis, the exocyst complex functions with RAB-10 in facilitating the formation of hTAC tubules in intestinal cells (Chen et al. 2014). We found that depletion of sec-15 significantly disrupted seam cell morphology and GFP::LGG-1 localization (Fig. A7A); however, it is difficult to determine if this is due to a direct role of sec-15 in autophagy regulation or its general role in plasma membrane trafficking. Therefore, the relationship between the exocyst complex and RAB-10, in the regulation of autophagy, should be investigated further.

We found that RNAi depletion of several candidate genes disrupted the localization of the autophagy reporters, but did not disrupt dauer morphogenesis (Fig. 2.1, Fig. A1B, and Table A1). A simple explanation may be that RNAi knockdown is not very efficient, and that a complete loss of function mutation would abolish dauer morphogenesis. Another explanation is that the block on dauer formation requires RNAi depletion of the gene activity in neurons, and RNAi is not as efficient in neurons. Finally, it is still possible that there are genes that function solely in autophagy and have no role in dauer remodeling, or are redundant at this step. Future work will be needed to investigate these possibilities.
Chapter 5

5. Materials and Methods

5.1. *C. elegans* strains

All strains were maintained on standard OP50 *Escherichia coli* diet (Brenner, 1974). *daf-2(e1370)* strains were analyzed at the permissive temperature (15 °C) for L3 larvae or the restrictive temperature (25 °C) for dauer larvae. A full list of strains is found below:

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA2123</td>
<td>adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]</td>
</tr>
<tr>
<td>HZ946</td>
<td>rpl-43(bp399); bpl151[PSQST-1::SQST-1::GFP, unc-76]</td>
</tr>
<tr>
<td>MAH114</td>
<td>daf-2[e1370]; adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]</td>
</tr>
<tr>
<td>RT533</td>
<td>unc-119(ed3); pws214[PRAB-10::GFP::RAB-10, Cb unc-119(+)]</td>
</tr>
<tr>
<td>RT2618</td>
<td>pws957[Pvha-6::tagRFP::RAB-10(wt)]</td>
</tr>
<tr>
<td>VK1241</td>
<td>vEx2124[Pnhx-1::mCherry::LGG-1 + Pmyo-2::GFP]</td>
</tr>
<tr>
<td>QU140</td>
<td>daf-2[e1370]; bpl151[PSQST-1::SQST-1::GFP, unc-76]</td>
</tr>
<tr>
<td>QU141</td>
<td>daf-2[e1370]; adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]; cdlg194[PLMP-1::LMP-1::tagRFP(S158T); unc-119(+)-ttx-3::GFP]</td>
</tr>
<tr>
<td>QU168</td>
<td>rab-10(ok1494); adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]</td>
</tr>
<tr>
<td>QU177</td>
<td>daf-2[e1370]; gck-2(tm2537); adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]</td>
</tr>
<tr>
<td>QU184</td>
<td>daf-2[e1370]; rab-10(ok1494); adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]</td>
</tr>
<tr>
<td>QU187</td>
<td>daf-2[e1370]; pws206[Pvha-6::GFP::RAB-10(wt) + Cb unc-119(+)]</td>
</tr>
<tr>
<td>QU191</td>
<td>daf-2[e1370]; tac-2(tm2241); adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]</td>
</tr>
<tr>
<td>QU192</td>
<td>atg-9(bp564); adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]</td>
</tr>
<tr>
<td>QU193</td>
<td>daf-2[e1370]; atg-9(bp564); adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]</td>
</tr>
<tr>
<td>QU201</td>
<td>atg-7(bp411); adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]</td>
</tr>
<tr>
<td>QU202</td>
<td>daf-2[e1370]; atg-7(bp411); adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]</td>
</tr>
<tr>
<td>QU218</td>
<td>daf-2[e1370]; vEx2124[Pnhx-1::mCherry::LGG-1 + Pmyo-2::GFP]</td>
</tr>
<tr>
<td>QU219</td>
<td>daf-2[e1370]; rab-10(ok1494); vEx2124[Pnhx-1::mCherry::LGG-1 + Pmyo-2::GFP]</td>
</tr>
<tr>
<td>QU223</td>
<td>daf-2[e1370]; rab-10(ok1494); bpl151[PSQST-1::SQST-1::GFP, unc-76]</td>
</tr>
<tr>
<td>QU227</td>
<td>daf-2[e1370]; rab-10(ok1494); pws206[Pvha-6::GFP::RAB-10(wt); vEx2124[Pnhx-1::mCherry::LGG-1 + Pmyo-2::GFP]</td>
</tr>
<tr>
<td>QU233</td>
<td>daf-2[e1370]; izls91[Pvha-6::tagRFP::ATG-9 + Cb unc-119(+)]; pws206[Pvha-6::GFP::RAB-10(wt) + Cb unc-119(+)]</td>
</tr>
<tr>
<td>QU257</td>
<td>daf-2[e1370]; rab-10(ok1494); pws210[Pvha-6::GFP::RAB-10(Q68L); vEx2124[Pnhx-2::mCherry::LGG-1 + Pmyo-2::GFP]</td>
</tr>
<tr>
<td>QU258</td>
<td>izls91[Pvha-6::GFP::ATG-9 + Cb unc-119(+)]; pws846[Pvha-6::TagRFP::RAB-5]</td>
</tr>
<tr>
<td>QU259</td>
<td>daf-2[e1370]; rab-10(ok1494); pws211[Pvha-6::GFP::RAB-10(T23N)]; vEx2124[Pnhx-2::mCherry::LGG-1 + Pmyo-2::GFP]</td>
</tr>
<tr>
<td>QU264</td>
<td>rab-10(ok1494); adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]; cdlg194[PLMP-1::LMP-1::tagRFP(S158T); unc-119(+)-ttx-3::GFP]</td>
</tr>
<tr>
<td>QU265</td>
<td>adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]; cdlg194[PLMP-1::LMP-1::tagRFP(S158T); unc-119(+)-ttx-3::GFP]</td>
</tr>
<tr>
<td>QU266</td>
<td>atg-7(bp411); adIs2122[PLGG-1::GFP::LGG-1 + rol-6(su1006)]; cdlg194[PLMP-1::LMP-1::tagRFP(S158T); unc-119(+)-ttx-3::GFP]</td>
</tr>
<tr>
<td>QU269</td>
<td>izIs90[Pvha-6::GFP::ATG-9]; pwIs849[Pvha-6::TagRFP::RAB-7]</td>
</tr>
<tr>
<td>QU270</td>
<td>rab-10(ok1494); izIs90[Pvha-6::GFP::ATG-9]; pwIs849[Pvha-6::TagRFP::RAB-7]</td>
</tr>
<tr>
<td>QU276</td>
<td>rab-10(ok1494); izIs90[Pvha-6::GFP::ATG-9 + unc-119(+)]; pwIs957[Pvha-6::tagRFP::RAB-10(wt)]</td>
</tr>
<tr>
<td>QU277</td>
<td>rab-10(ok1494); izIs90[Pvha-6::GFP::ATG-9 + unc-119(+)]; Is[Pvha-6::tagRFP::RAB-10(Q68L)]</td>
</tr>
<tr>
<td>QU287</td>
<td>izIs90[Pvha-6::GFP::ATG-9 + unc-119(+)]</td>
</tr>
<tr>
<td>QU288</td>
<td>rab-10(ok1494); izIs90[Pvha-6::GFP::ATG-9 + unc-119(+)]</td>
</tr>
<tr>
<td>QU289</td>
<td>daf-2[e1370]; izIs90[Pvha-6::GFP::ATG-9 + unc-119(+)]</td>
</tr>
<tr>
<td>QU290</td>
<td>daf-2[e1370]; rab-10(ok1494); izIs90[Pvha-6::GFP::ATG-9 + unc-119(+)]</td>
</tr>
<tr>
<td>QU301</td>
<td>daf-2[e1370]; izIs91[Pvha-6::tagRFP::ATG-9 + Cb unc-119(+)]; uwaEx2[Pvha-6::Cerulean-Venus::LGG-1]</td>
</tr>
<tr>
<td>QU302</td>
<td>izIs91[Pvha-6::tagRFP::ATG-9 + Cb unc-119(+)]; uwaEx2[Pvha-6::Cerulean-Venus::LGG-1]</td>
</tr>
<tr>
<td>QU310</td>
<td>daf-2[e1370]; izIs90[Pvha-6::GFP::ATG-9]; pwIs849[Pvha-6::TagRFP::RAB-7]</td>
</tr>
<tr>
<td>QU314</td>
<td>daf-2[e1370]; izIs90[Pvha-6::GFP::ATG-9 + unc-119(+)]; txuEx12[Pvha-6::tagRFP::RAB-10(T23N) + rol-6(su1006)]</td>
</tr>
<tr>
<td>QU315</td>
<td>rab-10(ok1494); izIs91[Pvha-6::tagRFP::ATG-9 + unc-119(+)]; uwaEx2[Pvha-6::Cerulean-Venus::LGG-1]</td>
</tr>
<tr>
<td>QU323</td>
<td>rab-10(ok1494); izIs90[Pvha-6::GFP::ATG-9 + Cb unc-119(+)]; pwIs846[Pvha-6::tagRFP::RAB-5]</td>
</tr>
<tr>
<td>QU324</td>
<td>daf-2[e1370]; izIs90[Pvha-6::GFP::ATG-9 + Cb unc-119(+)]; pwIs846[Pvha-6::tagRFP::RAB-5]</td>
</tr>
</tbody>
</table>

### 5.2. Construction of ATG-9 transgenic strains

Construction of the GFP and tagRFP::ATG-9 transgenes was performed using the Gateway Technology cloning system (ThermoFisher Scientific). To specifically express the GFP::ATG-9 and tagRFP::ATG-9 transgenes in the intestine, a vha-6 promoter-driven-vector, modified with a Gateway cassette, was inserted at the EcoRI or NaeI site, downstream of the GFP or RFP coding region. The *atg-9* cDNA was amplified by PCR using the Herculase II high-fidelity DNA polymerase kit (Agilent technologies) and primers with modified attB sites (Untergasser, 2006) from an initial clone kindly gifted by Dr. Daniel Colón Ramos. Primers used to amplify *atg-9* cDNA were:

**Forward Primer:** GGGGCAACTTTGTACAAAAAAGTTGTGTCAACTCAGTCAAACCGGGG

**Reverse Primer:** GGGGCAACTTTGTACAAAAAAGTTGTGTCAACTCAGTCAAACCGGGG

The resulting PCR product was cloned into the Gateway pDONR221 entry vector via the BP-clonase II reaction (ThermoFisher Scientific). The pDONR221 vectors containing the *atg-9* cDNA were then subcloned into the modified vha-6 promoter-driven-vector via the LR-clonase II reaction (ThermoFisher Scientific). Integrated transgenic lines were isolated by microparticle bombardment (Praitis et al., 2001).
5.3. Microscopy and Image analysis

Live worms were mounted on 3% agarose pads with 50mM sodium azide (sigma) in M9 buffer. Fluorescence images were obtained using an Axioimager M2 microscope equipped with a CCD camera (Carl Zeiss microscopy), captured using the Axiovision release 4.8 software (Carl Zeiss microscopy), and deconvolved using the Axiovision 3D deconvolution module (Carl Zeiss microscopy). Confocal images were obtained using a Leica TCS-SP5 laser scanning confocal microscope (Leica microsystems), equipped with a PMT (photomultiplier) detector, with images captured through the Leica Application Suite AF software (Leica microsystems). All images were acquired as z-stacks at a thickness of 0.5 microns. Quantification of images was performed using the open source software FIJI (FIJI is just image J, National Institute of Health).

For quantification of fluorescently labeled punctate structures, in seam cells and/or intestinal cells, z-stacks were converted to 8-bit maximum intensity projections using either the GFP or RFP/mCherry channel, and adjusted using the subtract background option in FIJI. Foci/punctate structures were then isolated by thresholding-based segmentation. GFP::LGG-1, mCherry::LGG-1, and GFP::ATG-9 foci ≥ 0.01µm², and SQST-1/p62::GFP foci ≥ 0.069µm², were quantified. To quantify intestinal foci, a consistent region of interest was selected for the anterior and posterior intestine. For quantification of mCherry::LGG-1 foci in intestinal cells, we measured the number and size of mCherry::LGG-1 positive foci in the posterior region of the intestine only, since this region consistently expressed the mCherry::LGG-1 transgenic reporter. In the seam cells of dauer animals, the size of GFP::LGG-1 foci was quantified by scanning through z-slices, and adjusting the threshold to highlight the entire area of individual foci found. The add to manager tool in FIJI was used to ensure a single foci was not counted more than once.

Colocalization analysis was performed using the colocalization threshold plugin in FIJI, and the Pearson’s correlation coefficient (R) was calculated. For colocalization analysis in seam cells, a single z-slice corresponding to the mid-region of each cell was used to determine the R value between GFP::LGG-1 and LMP-1::RFP. For colocalization analysis in intestinal cells, single z-slices with the greatest amount of GFP and RFP-positive foci were chosen. Autofluorescent vesicles labeled with the DAPI filter were removed from green and red channels using the image calculator feature, and both the green and red channels were adjusted using the subtract background option.
To quantify GFP::RAB-10 intestinal foci (Fig. A2C), for each animal/image, a single z-slice of similar focal plane, with an adequate number of foci, was chosen. Images were then adjusted using the subtract background option in FIJI. A region of interest was selected, and foci were tallied using the cell counter plugin.

For quantification of fluorescence intensity, camera exposure time was kept constant, and mid-focal plane images were acquired at the anterior intestine, mid intestine, and posterior intestine. For each region, the corrected total cell fluorescence (CTCF) was calculated in FIJI using the formula:

\[
\text{CTCF} = \text{Integrated Density} - (\text{Area of selected cell} \times \text{Mean fluorescence of background})
\]

The CTCF for each region was the summed and averaged per animal (Ansari et al. 2013).

5.4. RNA interference

RNAi experiments for the candidate gene screen were performed as previously described (Timmons and Fire, 1998), with slight modifications. In short, daf-2(e1370) L4 larvae were placed overnight at 15°C on NGM plates supplemented with 2mM IPTG, 50mg/ml carbenicillin, and HT115 bacteria containing dsRNA expression vectors (RNAi plates). F1 progeny from mothers were synchronized as L1 larvae and transferred to 25°C on RNAi plates, and were allowed to grow at this temperature until reaching the dauer larval stage. Image acquisition and analysis was carried out as described above. For RNAi clones resulting in embryonic lethality, synchronized progeny were fed dsRNA at the start of L1 and analyzed when they reached the dauer stage. For the RNAi screen, dauer animals were evaluated for changes in the localization of GFP::LGG-1 in seam cells, and RNAi clones that altered GFP::LGG-1 localization were considered as positives. RNAi clones were obtained from the Ahringer (Kamath et al. 2003) and Vidal genomic (Rual et al. 2004) RNAi libraries and were sequence verified using the M13-forward primer. For all RNAi experiments, the empty vector, L4440, was used as a control.

5.5. Chloroquine treatment

Standard NGM plates seeded with OP50 were UV-treated at 500J/cm² for 10 minutes using a UV-crosslinker (Fisher Scientific). UV-treated plates were then supplemented with either 30 mM chloroquine (Sigma) or dH₂O as a control. Animals were raised on live OP50 NGM plates at 20°C and synchronized
as L1 larvae. L1 larvae were then transferred onto UV treated plates with either chloroquine or control, and allowed to grow at 20°C until they reached the L3 larval stage
Appendix A

A. Additional support for RAB-10 promoting autophagy in *C. elegans*

A1. RNAi screen to identify endocytic genes with a role in autophagy

As described in chapter 2, we conducted an RNAi screen against a panel of endocytic genes, previously identified in the Grant lab to be important regulators of endocytic trafficking, such as RAB GTPases, ARF GTPases, syntaxins/SNAREs, ESCRT-related genes, retromer-related genes (Table A1) (Balklava et al. 2007). Using RNAi, we screened for genes that disrupted dauer morphogenesis and lead to the formation of enlarged GFP::LGG-1 foci/puncta in *daf-2(lir)* dauer seam cells. From 86 endocytic genes screened, the inactivation of 14 (*syn-1, rab-10, rab-11.2, rab-7, vps-24, vps-16, vps-22, vps-36, arl-8, rab-1, rab-5, dyn-1, snap-29, and vps-37*) resulted changes in GFP::LGG-1 expression relative to controls (Table A1 and Fig. 2.1). RNAi against several genes, such as *syn-1, rab-10, rab-1,* or *dyn-1* resulted in the formation of GFP::LGG-1 positive aggregates, similar to that observed in animals treated with dsRNA specific for autophagy genes controls (Table A1 and Fig. 2.1). In addition, RNAi against genes such as *rab-11.2, vps-24, rab-7, vps-22, vps-36, dyn-1,* and *snap-29,* disrupted alae production, a ridge in the cuticle that appears in dauer animals (Table A1, Fig. A1B, and Fig. 2.1). We found that alae formation was not disrupted in *rab-10* RNAi treated animals or mutants, and therefore, we did not evaluate *rab-10* loss for effects on dauer morphogenesis any further (Fig. A1B and data not shown). As described in chapter 1 and chapter 2, several of these genes, such as *vps-24* and *snap-29,* have been implicated to function in autophagy in *C. elegans,* mammals and/or yeast (Jager et al. 2004; Filimonenko et al. 2007; Rusten et al. 2007; Djeddi et al. 2012; Guo et al. 2014c; Manil-Segalen et al. 2014b).

A second screen for endocytic genes that when inactivated by RNAi, resulted in significant changes in the pattern of SQST-1::GFP expression in *daf-2(e1370)* mutant dauers. This work was carried out by David Jimenez, a former undergraduate in the lab. RNAi treatment against autophagy genes results in the aggregation of SQST-1::GFP in the body, noticeably in the hypodermis (Fig. A1A). Since depletion of autophagy genes resulted in a significant change of SQST-1::GFP in the hypodermis, compared to *L4440* control treated animals, we relied mostly on this phenotype to identify endocytic genes required for autophagy (Fig. A1A). From 59 endocytic genes screened, RNAi against 24 resulted in a change in the expression of SQST-1::GFP, when compared to the control animals (Table A1 and Fig. A1A). 11 out of 24
Figure A1: Changes in SQST-1::GFP localization and alae formation in *daf-2(e1370)* mutants treated with endocytic RNAi screen candidates. A) Representative epifluorescent images of SQST-1::GFP in the body of *daf-2(e1370)* RNAi treated animals at 25°C. Magnification 630x. B) Representative DIC images of dauer alae in *daf-2(e1370); Is[Plgg-1::GFP::LGG-1 + rol-6(su1006)]* animals treated with RNAi specific to a subset of endocytic gene candidates. Magnification 630X.
total identified genes were found to affect both GFP::LGG-1 and SQST-1::GFP expression, while RNAi against 13 genes were found to uniquely affect SQST-1::GFP expression (Table A1).

### Table A1: RNAi clones identified to alter GFP::LGG-1 and SQST-1::GFP localization in *daf-2(e1370)* dauers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Enlarged GFP::LGG-1 foci</th>
<th>Enlarged SQST-1::GFP foci</th>
<th>Defective alae</th>
<th>Brief description</th>
</tr>
</thead>
<tbody>
<tr>
<td>snap-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>SNAP29 ortholog(Guo et al. 2014c)</td>
</tr>
<tr>
<td>syn-4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Q/t-SNARE(Jantsch-Plunger and Glotzer 1999; Sato et al. 2011)</td>
</tr>
<tr>
<td>syn-1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Sso1/2 ortholog(Jantsch-Plunger and Glotzer 1999)</td>
</tr>
<tr>
<td>vti-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Vti1/Vti1a ortholog(Luo et al. 2011)</td>
</tr>
<tr>
<td>syx-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Syntaxin/SNARE protein(Jantsch-Plunger and Glotzer 1999)</td>
</tr>
<tr>
<td>syx-7</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Pep12 ortholog(Jantsch-Plunger and Glotzer 1999)</td>
</tr>
<tr>
<td>syx-17</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>SYNTAXIN17/STX17 ortholog(Sato et al. 2011)</td>
</tr>
<tr>
<td>syx-6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>SYNTAXIN6/STX6 ortholog(Luo et al. 2011)</td>
</tr>
<tr>
<td>syn-16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>SYNTAXIN16/STX16 ortholog(Gengyo-Ando et al. 2007; Luo et al. 2010)</td>
</tr>
<tr>
<td>aex-4</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>SNAP25 family member/SNARE protein(Mahoney et al. 2008)</td>
</tr>
<tr>
<td>syx-5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>t-SNARE(Hyenne et al. 2015)</td>
</tr>
<tr>
<td>unc-64</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>SNARE protein(Saifee et al. 1998; Wu et al. 2010)</td>
</tr>
<tr>
<td>ric-4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Ortholog of Sec9(Wu et al. 2010)</td>
</tr>
<tr>
<td>arl-1</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>Arf-like GTPase(Li et al. 2004b)</td>
</tr>
<tr>
<td>arl-8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ARL8 ortholog(Nakae et al. 2010; Sasaki et al. 2013)</td>
</tr>
<tr>
<td>arl-5</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Arf-like GTPase/ARF-2 paralog(Antoshechkin and Han 2002)</td>
</tr>
<tr>
<td>arf-2</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>Arf-like GTPase involved in mitosis(Antoshechkin and Han 2002)</td>
</tr>
<tr>
<td>arf-3</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>Arf-like GTPase(Skorobogata et al. 2014)</td>
</tr>
<tr>
<td>arl-3</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>Neuronal Arf-like GTPase (Li et al. 2004b)</td>
</tr>
<tr>
<td>arl-6</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>Neuronal Arf-like GTPase (Wiens et al. 2010)</td>
</tr>
<tr>
<td>arf-6</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>Arf-like GTPase (Shi et al. 2012)</td>
</tr>
<tr>
<td>arf-1.1</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>Arf-like GTPase(Todd et al. 2016)</td>
</tr>
<tr>
<td>rab-1</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>Ypt1 ortholog(Nollen et al. 2004)</td>
</tr>
<tr>
<td>rab-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Ras-like GTPase/phagocytosis(Lu et al. 2008a)</td>
</tr>
<tr>
<td>rab-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Neuronal Ras-like GTPase(Nonet et al. 1997)</td>
</tr>
<tr>
<td>rab-5</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>Ypt51/RAB5 ortholog(Grant and Hirsh 1999)</td>
</tr>
<tr>
<td>rab-6.1</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>Ras-like GTPase(Zhang et al. 2016)</td>
</tr>
<tr>
<td>rab-6.2</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>Ras-like GTPase(Zhang et al. 2016)</td>
</tr>
<tr>
<td>rab-7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Ypt7/RAB7 ortholog(Grant and Hirsh 1999)</td>
</tr>
<tr>
<td>rab-8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Ras-like GTPase/paralogue of RAB-10(Chen et al. 2006; Sato et al. 2011)</td>
</tr>
<tr>
<td>rab-10</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Ras-like GTPase/basolateral recycling(Chen et al. 2006)</td>
</tr>
<tr>
<td>rab</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Ypt31/RAB11 ortholog(Chen et al. 2006; Winter et al. 2012)</td>
</tr>
<tr>
<td>rab</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>RAB-11.1 paralog(Szumowski et al. 2014)</td>
</tr>
<tr>
<td>Gene</td>
<td>Expression</td>
<td>Protein Function</td>
<td>References</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
<td>-----------------------------------------------</td>
<td>-------------------------------------</td>
<td></td>
</tr>
<tr>
<td>rab-14</td>
<td>-</td>
<td>-</td>
<td>Ras-like GTPase/phagocytosis(Guo et al.)</td>
<td></td>
</tr>
<tr>
<td>rab-18</td>
<td>-</td>
<td>-</td>
<td>Ras-like GTPase(Zorio et al. 1997)</td>
<td></td>
</tr>
<tr>
<td>rab-19</td>
<td>-</td>
<td>-</td>
<td>Ras-like GTPase(Shaye and Greenwald 2011)</td>
<td></td>
</tr>
<tr>
<td>rab-21</td>
<td>+</td>
<td>-</td>
<td>Ras-like GTPase(Shaye and Greenwald 2011)</td>
<td></td>
</tr>
<tr>
<td>rab-27</td>
<td>-</td>
<td>-</td>
<td>Ras-like GTPase(Feng et al. 2012)</td>
<td></td>
</tr>
<tr>
<td>rab-28</td>
<td>-</td>
<td>-</td>
<td>Ras-like GTPase(Jensen et al. 2016)</td>
<td></td>
</tr>
<tr>
<td>rab-30</td>
<td>+</td>
<td>-</td>
<td>Ras-like GTPase(Shaye and Greenwald 2011)</td>
<td></td>
</tr>
<tr>
<td>rab-33</td>
<td>+</td>
<td>-</td>
<td>Ras-like GTPase(Shaye and Greenwald 2011)</td>
<td></td>
</tr>
<tr>
<td>rab-35</td>
<td>+</td>
<td>-</td>
<td>Ras-like GTPase(Sato et al. 2008b)</td>
<td></td>
</tr>
<tr>
<td>rab-37</td>
<td>-</td>
<td>-</td>
<td>Ras-like GTPase(Shaye and Greenwald 2011)</td>
<td></td>
</tr>
<tr>
<td>rab-38</td>
<td>-</td>
<td>-</td>
<td>Ras-like GTPase(Shaye and Greenwald 2011)</td>
<td></td>
</tr>
<tr>
<td>rab-39</td>
<td>-</td>
<td>-</td>
<td>Ras-like GTPase(Takenaka et al. 2013)</td>
<td></td>
</tr>
<tr>
<td>sar-1</td>
<td>ND</td>
<td>+</td>
<td>Sar1 GTPase(Hanna et al. 2016)</td>
<td></td>
</tr>
<tr>
<td>dyn-1</td>
<td>+</td>
<td>+</td>
<td>dynamin GTPase(Clark et al. 1997)</td>
<td></td>
</tr>
<tr>
<td>chc-1</td>
<td>ND</td>
<td>ND</td>
<td>Clathrin heavy chain(Kaplan et al. 2010)</td>
<td></td>
</tr>
<tr>
<td>rme-2</td>
<td>-</td>
<td>-</td>
<td>yolk receptor(Grant and Hirsh 1999)</td>
<td></td>
</tr>
<tr>
<td>rme-1</td>
<td>-</td>
<td>ND</td>
<td>dyn-1-like GTPase(Grant et al. 2001)</td>
<td></td>
</tr>
<tr>
<td>let-60</td>
<td>-</td>
<td>-</td>
<td>RAS-related gene(Han and Sternberg 1991)</td>
<td></td>
</tr>
<tr>
<td>rppk-1</td>
<td>ND</td>
<td>ND</td>
<td>Mitogen-activated protein kinase(Lackner et al. 1994)</td>
<td></td>
</tr>
<tr>
<td>let-23</td>
<td>+</td>
<td>-</td>
<td>EGF receptor ortholog(Aroian and Sternberg 1991)</td>
<td></td>
</tr>
<tr>
<td>src-1</td>
<td>ND</td>
<td>ND</td>
<td>tyrosine kinase(Hirosue et al. 2003)</td>
<td></td>
</tr>
<tr>
<td>emb-27</td>
<td>ND</td>
<td>ND</td>
<td>Anaphase promoting complex subunit(Cassada et al. 1981)</td>
<td></td>
</tr>
<tr>
<td>mbk-2</td>
<td>ND</td>
<td>-</td>
<td>Yak1-related kinase(Pang et al. 2004)</td>
<td></td>
</tr>
<tr>
<td>ncr-1</td>
<td>-</td>
<td>ND</td>
<td>Transmembrane glycoprotein(Li et al. 2004a)</td>
<td></td>
</tr>
<tr>
<td>ncr-2</td>
<td>-</td>
<td>ND</td>
<td>Transmembrane glycoprotein(Li et al. 2004a)</td>
<td></td>
</tr>
<tr>
<td>vps-16</td>
<td>+</td>
<td>+</td>
<td>HOPS subunit(Solinger and Spang 2014)</td>
<td></td>
</tr>
<tr>
<td>vps-28</td>
<td>-</td>
<td>ND</td>
<td>HOPS subunit(Solinger and Spang 2014)</td>
<td></td>
</tr>
<tr>
<td>vps-36</td>
<td>+</td>
<td>+</td>
<td>ESCRT-II subunit(Djeddi et al. 2012)</td>
<td></td>
</tr>
<tr>
<td>vps-37</td>
<td>+</td>
<td>ND</td>
<td>ESCRT-I subunit(Djeddi et al. 2012)</td>
<td></td>
</tr>
<tr>
<td>vps-22</td>
<td>+</td>
<td>ND</td>
<td>ESCRT-II subunit(Djeddi et al. 2012)</td>
<td></td>
</tr>
<tr>
<td>vps-24</td>
<td>+</td>
<td>+</td>
<td>ESCRT-III subunit(Djeddi et al. 2012)</td>
<td></td>
</tr>
<tr>
<td>cup-5</td>
<td>ND</td>
<td>+</td>
<td>Mucolipin 1 ortholog(Fares and Greenwald 2001a)</td>
<td></td>
</tr>
<tr>
<td>xrep-1</td>
<td>-</td>
<td>-</td>
<td>Ubiquitin ligase complex subunit(Choe et al. 2009)</td>
<td></td>
</tr>
<tr>
<td>kel-8</td>
<td>-</td>
<td>-</td>
<td>Kelch-like family member(Schaefer and Rongo 2006)</td>
<td></td>
</tr>
<tr>
<td>dnf-1</td>
<td>ND</td>
<td>-</td>
<td>DNA J domain-containing protein(Pan et al. 2008; Shaye and</td>
<td></td>
</tr>
<tr>
<td>vps-35</td>
<td>-</td>
<td>ND</td>
<td>retromer subunit(Pan et al. 2008; Shi et al. 2009)</td>
<td></td>
</tr>
<tr>
<td>snx-1</td>
<td>ND</td>
<td>-</td>
<td>Sorting nexin/retromer subunit(Shi et al. 2009; Chen et al. 2010b)</td>
<td></td>
</tr>
<tr>
<td>raby1</td>
<td>-</td>
<td>-</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>raby2</td>
<td>-</td>
<td>-</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>raby3</td>
<td>-</td>
<td>+</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>raby4</td>
<td>-</td>
<td>-</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>raby5</td>
<td>-</td>
<td>-</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>raby6</td>
<td>-</td>
<td>-</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>av-1N</td>
<td>ND</td>
<td>-</td>
<td>unknown</td>
<td></td>
</tr>
</tbody>
</table>
A2. GFP::RAB-10 expression levels decrease during autophagy induced conditions

We reasoned that endocytic genes required for autophagy, would change in expression or localization during autophagy induced conditions, such as during dauer formation. To determine if rab-10 expression is changed during autophagy induced conditions, we evaluated the expression of GFP::RAB-10 (under an endogenous rab-10 promoter) in both wild-type and daf-2(e1370) mutants, at both 15°C and 25°C (Fig. A2A and A2B). We found that both wild type and daf-2(e1370) mutants grown at the permissive temperature displayed a similar intensity level for GFP::RAB-10 (Fig. A2A and A2B). Interestingly, in both wild type and daf-2(e1370) dauers grown at the restrictive temperature, GFP::RAB-10 intensity levels were significantly lower than that observed in L3 larvae grown at the permissive temperature (Fig. A2A and A2B). Similarly, we found that wild type and daf-2(e1370) L3 animals grown at the permissive temperature had an equal number of GFP::RAB-10 intestinal puncta/foci (Fig. A2C), and the number of GFP::RAB-10 intestinal foci was significantly decreased in daf-2(e1370) dauers (Fig. A2C).

To determine if the change in GFP::RAB-10 expression had resulted from defects in autophagy, we RNAi depleted bec-1, atg-7, or atg-9, in daf-2(e1370) mutants, expressing GFP::RAB-10 in intestinal cells, and used rab-10 RNAi as a positive control (Fig. A2D and A2E). Interestingly, compared to controls, we found that RNAi treatment against atg-7 or atg-9 resulted in a significant increase in the expression levels of GFP::RAB-10 (Fig. A2D and A2E). Surprisingly, bec-1 RNAi resulted in a significant decrease in GFP::RAB-10 expression compared to control RNAi (Fig. A2D and A2E). Confirming the efficiency of our RNAi method, we found that the expression levels of GFP::RAB-10 were significantly decreased in animals treated with RNAi against rab-10 (Fig. A2D and A2E). A decrease in GFP::RAB-10 expression in bec-1 RNAi treated animals could be due to a role for bec-1 in retromer function. Further studies will be needed to investigate this possibility. In conclusion, our results show that atg-7 or atg-9 RNAi depletion results in
Figure A2: GFP::RAB-10 expression is reduced during dauer formation and increased during reduced autophagy activity (See legend on next page).
Figure A2: GFP::RAB-10 expression is reduced during dauer formation and increased during reduced autophagy activity. A) Representative epifluorescent images of the pharyngeal region of wild type and daf-2(e1370) animals expressing the transgene Is[Prab-10::GFP::RAB-10 + Cbr-unc-119(+)], at 15°C and 25°C. Magnification 630x. B) Quantification of the mean GFP::RAB-10 fluorescent intensity, from three regions of interest, in wild type and daf-2(e1370) animals raised at 15°C and 25°C. Data is an average of 3 independent trials (except for 25°C wild-type dauers, which had only one trial). C) Quantification of the average number of GFP::RAB-10 intestinal foci, in wild type and daf-2(e1370) animals raised at 15°C and 25°C. Data is an average of a single trial. Magnification 400x. D) Representative epifluorescent images of anterior intestinal cells in daf-2(e1370); Is[Pvha-6::GFP::RAB-10 + Cbr-unc-119(+)] treated with RNAi. Magnification 630x. E) Quantification of the average GFP::RAB-10 fluorescent intensity in daf-2(e1370) mutants treated with RNAi. Data is an average of 3 independent trials. B,C,E) Statistical analysis done using an unpaired, two tailed t-test, p-value ***.001. n ≥ 20 animals/strain. Error bars ±S.E. of the mean.
an increase in GFP::RAB-10, and possibly alters the localization of GFP::RAB-10, although the morphology of GFP::RAB-10 puncta was not investigated further.

A3. unc-51 and atg-7 are epistatic to rab-10 in the autophagy pathway

In the RNAi screen, we noticed that the enlarged GFP::LGG-1 foci found in daf-2(e1370); rab-10(RNAi) animals, compared to controls, appeared somewhat similar to foci found in daf-2(e1370); atg-7(RNAi) animals. However, GFP::LGG-1 foci found in daf-2(e1370); atg-7(bp564) animals were larger than those found in daf-2(e1370); rab-10(ok1494) double mutants (Fig. 2.2 and data not shown). To determine the genetic relationship between rab-10 and atg-7, we compared daf-2(e1370) single mutants, daf-2(e1370); rab-10(ok1494) double mutants, and daf-2(e1370); atg-7(bp564) double mutants, and analyzed GFP::LGG-1 foci formation when RNAi treated against rab-10, atg-7, or L4440 control (Fig. A3). We included unc-51 RNAi in the analysis, since unc-51 is a critical component of the autophagy induction complex, which initiates autophagosome formation during stress (Matsuura et al. 1997; Kamada et al. 2000; Hosokawa et al. 2009; Mercer et al. 2009; Kamada et al. 2010). The size and number of GFP::LGG-1 foci in daf-2(e1370); atg-7(bp564) double mutants treated with rab-10 RNAi was similar to that of daf-2(e1370); atg-7(bp564) control animals (Fig. A3A, A3B, and A3D). Conversely, we found that size and number of GFP::LGG-1 foci in daf-2(e1370); rab-10(ok1494) double mutants treated with atg-7 RNAi were similar to that of daf-2 mutants treated with RNAi against atg-7 or daf-2(e1370); atg-7(bp564) control animals (Fig. A3A, A3B, and A3C). Depletion of unc-51 in daf-2(e1370); rab-10(ok1494) or daf-2(e1370); atg-7(bp564) mutants resulted in the formation of GFP::LGG-1 foci similar to daf-2(e1370) controls fed unc-51 RNAi (Fig. A3A-D). These results show that both unc-51 and atg-7 are epistatic to rab-10. Whether rab-10 acts upstream or downstream of unc-51 and atg-7 requires further analysis to determine if rab-10 acts as part of a switch regulatory pathway or acts in a substrate dependent pathway. Additionally, to fully determine the epistatic relationship between rab-10 and various autophagy genes, daf-2(e1370); rab-10(ok1494) double mutants with null mutations in atg-7, unc-51, and other autophagy genes, should be constructed to overcome some of the pitfalls associated with RNAi, such as gene knockdown variability.
Figure A3: unc-51 and atg-7 are epistatic to rab-10 in the autophagy pathway. A) Representative deconvolved epifluorescent images of seam cells in daf-2(e1370), daf-2(e1370); atg-7(bp411), and daf-2(e1370); rab-10(ok1494) mutants expressing Is[Plgg-1::GFP::LGG-1 + rol-6(su1006)] and treated with dsRNA against L4440 empty vector control, rab-10, atg-7, or unc-51. Magnification 630X. B) Quantification of the average number of GFP::LGG-1 puncta in seam cells of daf-2(e1370) (B), daf-2(e1370); atg-7(bp411) (C), and daf-2(e1370); rab-10(ok1494) (D) treated with RNAi. Error Bars ± S.E. of the mean. Statistical analysis was done using an unpaired, two tailed t-test, p-values ** ≤ .01 and *** ≤ .001. # of seam cells quantified is ≥29 from at least 6 animals per strain. Data shown represents the average from a single trial.
A4. The GTPase cycling activity of RAB-10 is required for autophagy

Given that *rab-10* is a GTPase that cycles between inactive and active states to facilitate its function, it may act as a switch regulatory element in the autophagy pathway. One way to approach this question was to determine whether the GTPase activity of RAB-10 is required for normal autophagy. As discussed in chapter 2, we found that GTPase cycling was required for RAB-10 activity in autophagy. In addition, we analyzed whether mutant forms of RAB-10 rescued or enhanced the changes in mCherry::LGG-1 expression observed in *daf-2(e1370); rab-10(ok1494)* mutants. We created *daf-2(e1370); rab-10(ok1494); Ex[Pnhx-2::mCherry::LGG-1]* double mutants that express the GFP::RAB-10(Q68L) or GFP::RAB-10(T23N) mutant forms (Fig. A4). Q68L is a mutation in which the GTPase is constitutively bound to GTP, while T23N is a mutation in which the GTPase is constitutively bound to GDP (Li and Zhang 2004). As described in chapter 2 (Fig. 2.7), *daf-2(e1370); rab-10(ok1494)* L3 animals had an increase in the number of intestinal mCherry::LGG-1 foci, with no change in size, when compared to *daf-2(e1370)* L3 animals. *daf-2(e1370); rab-10(ok1494)* dauer L3 animals had an increase in the size, but no change in the number of intestinal mCherry::LGG-1 foci. Compared to controls, we found that the GTP-locked version of RAB-10 was able to rescue changes in the number of mCherry::LGG-1 foci in *daf-2(e1370); rab-10(ok1494)* nondauers, but failed to rescue the size of mCherry::LGG-1 foci in *daf-2(e1370); rab-10(ok1494)* dauers (Fig. A4A and A4D). Moreover, we found that the GDP-locked version of RAB-10 exacerbated the number and size of mCherry::LGG-1 foci in *daf-2(e1370); rab-10(ok1494)* non-dauer L3 animals (Fig. A4A). Interestingly, GFP::RAB-10(T23N) failed to increase the size of mCherry::LGG-1 foci in *daf-2(e1370); rab-10(ok1494)* dauers (Fig. A4D). Finally, expression of GFP::RAB-10(wt) rescued the number and size of mCherry::LGG-1 foci in *daf-2(e1370); rab-10(ok1494)* L3s and dauers, respectively (Fig. A4A and A4D). In all, these results confirm that the RAB-10 GTPase cycling activity is required in autophagy.

We evaluated whether expression of the GDP-locked version of RAB-10 would result in a dominant negative phenotype for GFP::ATG-9 localization in intestinal cells (Fig. A5). Expression of the GDP-locked RAB-10 in *rab-10(ok1494)* mutants resulted in a significant decrease in the number of intestinal GFP::ATG-9 foci (Fig. A5A and A5B). Unexpectedly, these animals displayed an increase in the size of GFP::ATG-9 foci, greater than that of wild type or *rab-10* mutants lacking the RAB-10(T23N) transgene (Fig. A5A and A5C). Indeed, although GFP::ATG-9 foci were rarely observed in *rab-10(ok1494)* mutants expressing
**Figure A4: RAB-10 GTPase Activity is needed to rescue the altered localization of mCherry::LGG-1 in daf-2 mutants. A-D** Quantification of the average number (A and C) and size (B and D) of mCherry::LGG-1 foci in posterior intestinal cells of *daf-2(e1370)* single and *daf-2(e1370); rab-10(ok1494)* double mutants expressing GFP fusion constructs for either wild type, GTP-locked, or GDP-locked RAB-10 in the intestine. Animals were analyzed at the L3 stage (A and B) or dauer larval stage (C and D). Data shown represents the average from a single trial. n# ≥ 10 animals. Error bars ± SE of the mean. Statistical analysis was done using an unpaired, two tailed t-test, p-value *≤ .05 ** ≤ .01 and ***≤.001.
Figure A5: RAB-10 GTPase cycling is needed to rescue the altered localization of GFP::ATG-9 in intestinal cells. A) Representative confocal images of wild type and rab-10(ok1494) mutants with or without the transgene, Ex[Pvha-6::tagRFP::RAB-10(T23N)]. rab-10(ok1494); RFP::RAB-10(T23N) animals scarcely have GFP::ATG-9 puncta (A''''), but do occasionally contain enlarged GFP::ATG-9 foci (A'''). B)-C) Quantification of the average number (A) and size (B) of GFP::ATG-9 intestinal puncta in wild type and rab-10(ok1494) single mutants with or without the transgenic array, Ex[Pvha-6::tagRFP::RAB-10(T23N)]. Animals analyzed were at the L3 stage at 20°C. Magnification 630X. n# = 10 animals/strain. Data shown is a representative of a single trial. Error bars ± SE of the mean. Statistical analysis was done using an unpaired, two tailed t-test, p-value **≤.01 and ***≤.001.
RFP::RAB-10(T23N); we did occasionally see enlarged GFP::ATG-9 positive foci (Fig. A5A, A‴‴). These enlarged foci may represent the sequestration of GFP::ATG-9. Future studies will be needed to investigate this further.

Because the GTP-locked and GDP-locked versions of RAB-10 did not rescue the mCherry::LGG-1 or GFP::ATG-9 phenotypes in daf-2(e1370); rab-10(ok1494) or rab-10(ok1494) mutants, respectively, we conclude that RAB-10 GTPase cycling between GTP-bound and GDP-bound states is required for autophagy function. Similar observations have been documented for the GTPase Arf6 (Radhakrishna and Donaldson 1997; Claing et al. 2001; Klein et al. 2006). The observation that RAB-10 GTPase cycling is required for autophagy suggests that a GEF(s) and GAP(s) are needed to coordinate the recruitment of effector proteins that allow RAB-10 to promote autophagic flux. In addition, that RAB-10 GTPase cycling is required for autophagy, suggests that RAB-10 acts as a switch to promote autophagic flux, and therefore RAB-10 may act upstream of atg-7 and unc-51. Further studies will be needed to confirm this.

A5. Identification of RAB-10 effector proteins involved in autophagy

GTPases are evolutionarily conserved molecular switches that cycle between active/GTP-bound and inactive/GDP-bound conformational states (Hutagalung and Novick 2011). In their active state, GTPases facilitate specific cellular processes by recruiting downstream effector proteins (Grosshans et al. 2006b; Schwartz et al. 2007; Gillingham et al. 2014). For example, to promote endosome to endosome fusion events, GTP-bound RAB5 recruits the effectors, EEA1 and RABENOSYN-5 (Patki et al. 1997; Lawe et al. 2000). To identify RAB-10 effector proteins that function in autophagy, we conducted an RNAi screen against known RAB-10 interactors. We screened for genes that may encode a potential RAB-10 effector, by RNAi depletion, and screened for the formation of enlarged GFP::LGG-1 foci in daf-2(e1370) dauers (Fig. A6). Interestingly, RNAi against the known RAB-10 effector proteins, EHB-1, CNT-1, HUM-2, GCK-2, and TBC-2, did not result in the formation of enlarged GFP::LGG-1 foci compared to rab-10 depleted animals (Fig. A6B); however, future studies should thoroughly investigate changes in the number and size of GFP::LGG-1 foci for the depletion of the above genes. In addition, RNAi against rab-8 did not result in the formation of enlarged GFP::LGG-1 foci in daf-2(e1370) dauers (Fig. A6B), suggesting that rab-8 is not functionally redundant with rab-10 for autophagy function. We also evaluated the localization of GFP::LGG-
A. List of known RAB-10 effector proteins that facilitate RAB-10-specific functions.

<table>
<thead>
<tr>
<th>RAB-10 effector proteins</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT-1</td>
<td>-homolog of the Arf6 GTPase activating protein (GAP), Acap1/2 (Shi et al., 2012)</td>
</tr>
<tr>
<td>EHBP-1</td>
<td>-homolog of human EHBP-1, which contains a CH actin-binding domain (Shi et al., 2010)(Wang et al., 2016)</td>
</tr>
<tr>
<td>HUM-2</td>
<td>-ortholog of human myosin Va/Vb/Vc heavy chain (Barth Grant, personal communication)</td>
</tr>
<tr>
<td>GCK-2</td>
<td>-Member of the serine/threonine kinase MAP4K family (Barth Grant, personal communication)</td>
</tr>
<tr>
<td>TBC-1</td>
<td>-Orthologue of mammalian GAP, TBC1D23, and the yeast GAP, Gy11p (Alper et al., 2008; Barth Grant, personal communication)</td>
</tr>
<tr>
<td>TBC-2</td>
<td>-Orthologue of the mammalian GAP, TBC1D2B, and yeast GAP, Mdr1p (Albert and Gallwitz, 1999; Li et al., 2009)</td>
</tr>
</tbody>
</table>

B. Representative epifluorescent images of hypodermal seam cells in daf-2(e1370) dauers, 25°C.

Figure A6: Depletion of RAB-10 effector proteins does not result in enlarged GFP::LGG-1 foci in daf-2 dauer seam cells. A) List of known RAB-10 effector proteins that facilitate RAB-10-specific functions. B) Representative epifluorescent images of hypodermal seam cells in daf-2(e1370); Is[Pli:GFP::LGG-1 + rol-6(su1006)] animals treated with RNAi against RAB-10 effector genes. Animals shown represent dauers grown at 25°C. Magnification 630X.
1 in *daf-2(e1370); rab-10(ok1494)* mutants treated with RNAi specific to the exocyst subunit, *sec-15* (Fig. A7). The exocyst complex was shown to function with RAB-10 in facilitating the formation of hTAC endosomal tubules in intestinal cells (Chen et al. 2014). We found that depletion of *sec-15* significantly disrupted seam cell morphology and GFP::LGG-1 foci formation; however, it was difficult to determine if a significant enlargement of GFP::LGG-1 foci had occurred (Fig. A7A). Further analysis of *sec-15* depleted animals was challenging due to the lethality observed with *sec-15* depletion. The severe disruption of seam cell morphology in *sec-15* mutants made it difficult to quantify changes in GFP::LGG-1-positive foci (Fig. A7A). Strong conclusions cannot be made from a negative result of an RNAi knockdown, as there are many reasons why an RNAi knockdown may be inefficient. It is difficult to ascertain whether the GFP::LGG-1 localization phenotype in *sec-15* RNAi animals is due to a direct role for *sec-15* in autophagy or its general role in plasma membrane trafficking (TerBush et al. 1996; Hsu et al. 2004). Indeed, in mammals, the exocyst acts as a scaffold for autophagy-specific complexes (Bodemann et al. 2011). Whether this is also true in *C. elegans* will require additional studies.

Mutations for some of the RAB-10 effectors (*gck-2, tbc-2, and tbc-1*) were available and tested for any alteration in GFP::LGG-1 localization in *daf-2(e1370)* dauer seam cells (Fig. A7B and A7C). We found that loss of *tbc-1* had no effect on GFP::LGG-1 localization, while *gck-2* had a mild effect on GFP::LGG-1 localization in *daf-2(e1370)* seam cells (Fig. A7B). Interestingly, we found that loss of *tbc-2* resulted in the formation of enlarged intestinal vacuoles labeled with GFP::LGG-1 in *daf-2(e1370)* dauers, as previously published (Fig. A7C) (Chotard et al. 2010; Liu and Grant 2015). Furthermore, seam cells were not visible in these mutants, which may be a result of the high levels of GFP::LGG-1 expression in the intestine obstructing the view of the seam cells (Fig. A7C). As described in chapter 1, RAB-10 recruits TBC-2 to early endosomes to facilitate early endosome to late endosome maturation (Chotard et al. 2010; Liu and Grant 2015). *tbc-2(tm2241)* deletion mutants have enlarged intestinal vacuoles labeled with RAB-7, LMP-1, and weakly with RAB-5 (Chotard et al. 2010). This is similar to what is found in the intestinal cells of *rab-10(ok1494)* mutants (Chen et al. 2006). However, the severe vacuole phenotype found in *rab-10* and *tbc-2* deletion mutants was described in animals analyzed as L4 and/or adults, when intestinal cells are larger in size (Chen et al. 2006; Chotard et al. 2010). In L3s or dauers, *daf-2(e1370); rab-10(ok1494)* mutants do not display significant vacuolization in the intestine. It would be interesting to investigate if *rab-10* mutant
Figure A7: Depletion and loss of the RAB-10 effector proteins, SEC-15 and TBC-2, alters GFP::LGG-1 expression in daf-2(e1370) mutants. A) Representative epifluorescent images of seam cells in daf-2(e1370) mutants treated with RNAi against L4440 empty vector control, bec-1, and the exocyst subunit, sec-15. B) Representative epifluorescent images of seam cells in daf-2(e1370), daf-2(e1370); atg-7(bp411), daf-2(e1370); gck-2(tm2537); and daf-2(e1370); tbc-1(tm2282) mutants expressing the transgenic array Is[Pgg-1::GFP::LGG-1 + rol-6(su1006)]. C) Representative epifluorescent images of GFP::LGG-1 in daf-2(e1370) and daf-2(e1370); tbc-2(tm2241) mutants. Notice that seam cells are not visible in daf-2(e1370); tbc-2(tm2241) animals. Magnification 630x.
adults accumulate intestinal vacuoles labeled with GFP::LGG-1, similar to that found in tbc-2(tm2241) mutants. Future studies will be needed to investigate this possibility.

In all, we find that depletion and/or genetic loss of various potential RAB-10 effector genes failed to emulate the phenotype of GFP::LGG-1 in daf-2; rab-10 mutants. sec-15 RNAi and the tbc-2 loss of function mutation may be an exception. Based on published data showing a role for both sec-15 and tbc-2 in rab-10 mediated processes, one would predict that disruption of these genes disrupt autophagy; however, additional experiments will be needed to elucidate how SEC-15 and TBC-2 act in the autophagy pathway.

A6. RAB-10 promotes the degradation of SQST-1::GFP foci in the pharynx and intestine

As explained in chapter 2, SQST-1 is the C. elegans ortholog of the autophagy cargo adaptor protein, p62/SQSTM1, which binds to LC3/LGG-1 to facilitate the autophagic degradation of poly-ubiquitinated cargo (Vadlamudi et al. 1996; Pankiv et al. 2007; Tian et al. 2010). Compared to controls, we found that throughout the body of daf-2(e1370) mutants, the loss of rab-10 slightly increased the number of SQST-1::GFP puncta/foci in L3 animals (grown at the permissive temperature), and decreased the number of SQST-1::GFP foci in dauers (grown at the restrictive temperature) (Fig. 2.5A and 2.5B). However, in both daf-2(e1370) L3s and dauers, rab-10 loss increased the size of SQST-1::GFP foci throughout the body, compared to controls (Fig. 2.5A and 2.5C). These results supported a role for rab-10 in promoting normal autophagy flux.

We noticed that the loss of rab-10 also affected SQST-1::GFP puncta in the pharynx of daf-2(e1370) animals (Fig. A8). Quantification of SQST-1::GFP pharyngeal foci revealed that changes in the number and size of foci found in the pharynx of daf-2(e1370) and daf-2(e1370); rab-10(ok1494) L3 and dauer animals nearly matched that found in the body (Fig. A8A, A8B, and Fig. 2.5). One important observation was the increase in the size of pharyngeal SQST-1::GFP foci in daf-2(e1370); rab-10(ok1494) L3s and dauers, compared to controls (Fig. A8A and A8B). These results confirmed that rab-10 function normally prevents the accumulation of SQST-1::GFP cargo, and therefore promotes flux.

Because, our data supported a role for rab-10 in promoting autophagy flux, we investigated whether rab-10(ok1494) single mutants would have a greater number of GFP::LGG-1 foci compared to daf-2(e1370)
Figure A8: Loss of rab-10 blocks the degradation of the SQST-1::GFP cargo adaptor protein in the pharynx of L3 animals. A and B) Quantification of the average number (A) and size (B) of SQST-1::GFP foci in the pharynx of daf-2(e1370) and daf-2(e1370); rab-10(ok1494) mutants. C and D) Quantification of the average number (C) and size (D) of GFP::LGG-1 foci in the seam cells of wild type, daf-2(e1370), and rab-10(ok1494) mutants grown at 15°C. Data shown is an average from at least two independent trials. n ≥ 19 animals/strain (A and B), n ≥ 32 animals/strain; # of seam cells ≥ 80/strain (C and D). Error bars represent ± SE of the mean. Statistical analysis done using an unpaired, two tailed t-test, p-value *≤.05, **≤.001.
single mutants, which have increased autophagy (Fig. A8C and A8D). Therefore, we analyzed the number of GFP::LGG-1 foci in the seam cells of wild type, daf-2(e1370), and rab-10(ok1494) single mutants grown at the permissive temperature (Fig. A8C and A8D). To our surprise, we found that both rab-10(ok1494) and daf-2(e1370) mutants had a similar increase in the number of GFP::LGG-1 foci compared to wild type controls (Fig. A8C and A8D). The size of GFP::LGG-1 foci was similar between wild type, daf-2(e1370), and rab-10(ok1494) mutants. We hypothesized that the similarities in the number of GFP::LGG-1 foci found in daf-2(e1370) and rab-10(ok1494) mutants was due to the combined effects on autophagy; as rab-10(ok1494) increased GFP::LGG-1 foci due to blocked flux, and daf-2(e1370) increased GFP::LGG-1 foci as autophagy is induced.

To test this hypothesis, we conducted one more additional experiment that evaluated SQST-1::GFP foci (Fig. A9). RPL-43 encodes a 60S ribosomal subunit and a loss of function mutation in rpl-43 results in the formation of enlarged SQST-1::GFP puncta in the intestine, due to the accumulation of misfolded proteins (Guo et al. 2014c). The loss of function for a gene that normally inhibits autophagy, suppresses the rpl-43 phenotype, while the loss of function for a gene that normally induces autophagy results in either a greater accumulation or no change in SQST-1::GFP foci (Guo et al. 2014c). We therefore treated rpl-43(bp399) mutants that express SQST-1::GFP with dsRNA against rab-10 and found that depletion of rab-10 increased both the number and size of SQST-1::GFP puncta in intestinal cells (Fig. A9B and A9C), as would be expected for a block in flux (Guo et al. 2014c). We had also treated rpl-43(bp399); Is[Psqst-1::SQST-1::GFP] mutants with RNAi specific to daf-2; however, the RNAi failed since it did not remove the SQST-1::GFP puncta as was previously published, but found no increase in the number of SQST-1::GFP foci (data not shown)(Guo et al. 2014c). To confirm that our RNAi clone for rab-10 was functional, we treated wild type animals expressing tagRFP::RAB-10 in intestinal cells with rab-10 RNAi. We saw that rab-10 RNAi completely knocked down tagRFP::RAB-10, compared to control animals (Fig. A9D). In all, these results validate those found in chapter 2, and show that rab-10 is required for autophagy flux.

A7. Elucidating the mechanism by which RAB-10 promotes autophagy flux

We determined that RAB-10 promotes autophagy flux, but the mechanism by which RAB-10 promotes autophagic flux is still unknown. As described in chapter 2 and appendix A4, loss of rab-10
Figure A9: Depletion of rab-10 enhances the formation of enlarged SQST-1::GFP foci in rpl-43(bp399) mutants. A) Representative epifluorescent images of L4 rpl-43(bp399); Is[Psqst-1::SQST-1::GFP + unc-76] mutants treated with rab-10 RNAi at 20°C. Magnification 630x. B) Quantification of the average number of SQST-1::GFP foci per unit area in rpl-43(bp399) mutants treated with RNAi. C) Quantification of the average size of SQST-1::GFP foci in rpl-43(bp399) mutants treated with RNAi. Magnification 630x. D) Representative epifluorescent images of wild type animals expressing Is[Pvha-6::tagRFP::RAB-10 + unc-119(+)] at 20°C, and treated with L4440 or rab-10 dsRNA. Magnification 630x. Data shown is an average from at least two independent trials. n ≥ 19 animals/strain. Error bars represent ± SE of the mean. Statistical analysis done using an unpaired, two tailed t-test, p-value *≤ .05, **≤ .001.
reduced the number and size of GFP::ATG-9 intestinal foci. From these results, we postulated that RAB-10 may function at an early step of the autophagy pathway, where it delivers ATG-9 to the site of autophagosome biogenesis. Similar results were shown for the yeast RAB-10 ortholog, Sec4 (Geng et al. 2010). In yeast and mammals, Atg9/ATG9 is localized to various endocytic compartments, such as early and late endosomes, in addition to unique vesicles referred to as Atg9/ATG9 reservoirs (Young et al. 2006; Mari et al. 2010a; Longatti et al. 2012; Orsi et al. 2012; Popovic and Dikic 2014). We hypothesized that RAB-10 would facilitate the transport of ATG-9 from these compartments to pre-autophagosomes.

We therefore assessed whether ATG-9 localized to early and late endosomes, and evaluated whether a loss of rab-10 would disrupt that localization, or resulted in the sequestration of ATG-9 to those compartments. We evaluated colocalization events between the early endosome reporter, tagRFP::RAB-5, and GFP::ATG-9 in intestinal cells, and found that colocalization was rarely observed in wild type L3 larvae grown at 15°C or 25°C, and this was also true for rab-10(ok1494) and daf-2(e1370) mutants (Fig. A10A and A10B). We also evaluated tagRFP::RAB-5 and GFP::ATG-9 colocalization events in the intestinal cells of 1-day old adults for both wild type and rab-10 mutants (Fig. A11). In adults, which contain larger intestinal cells and more tagRFP::RAB-5 positive foci, we observed colocalization between tagRFP::RAB-5 and GFP::ATG-9 in wild type animals, but this colocalization was not significantly changed in a rab-10(ok1494) mutant (Fig. A11A and A11B). Additionally, we looked at colocalization between the late endosomal reporter, tagRFP::RAB-7, and GFP::ATG-9, and also found that both reporters rarely colocalized in wild type L3 larvae, and again observed no significant change in the colocalization of the two reporters in rab-10(ok1494) or daf-2(e1370) mutants (Fig. A12A and A12B). It should be noted that although in some instances we observed statistical differences between the colocalization of the ATG-9 reporter and endocytic reporters, for wild type, rab-10, and daf-2 mutants animals, we did not interpret those as reliable differences, since the overall correlation coefficient remained negative (Fig. 2.10, A10, and A12). From these results we concluded that similar to yeast and mammals, ATG-9-positive foci may represent a distinct endocytic/vesicular compartment in C. elegans.

Overall, these results, along with the results described in chapter 2 (Fig. 2.9 and 2.10), suggest that ATG-9-positive foci may represent distinct endocytic/vesicular compartments in C. elegans, and that due to the transient nature of ATG-9 trafficking to and from autophagosomes and endocytic compartments,
Figure A10: ATG-9 does not localize to RAB-5-positive early endosomes. A) Representative deconvolved epifluorescent images of wild type, rab-10(ok1494), and daf-2(e1370) animals that coexpress GFP::ATG-9 and tagRFP::RAB-5 in intestinal cells. B) Quantification of the Pearson’s correlation coefficient for colocalization between GFP::ATG-9 and tagRFP::RAB-5. A-C) Animals were analyzed at the L3 or dauer larval stages at 15°C and 25°C. n# ≥ 10 animals/strain; # of units per area analyzed/animal = 2. Statistical analysis was done using an unpaired, two tailed t-test. Error bars = ± S.E. Arrows point to representative tagRFP::RAB-5 vesicles and arrow heads point to representative GFP::ATG-9 vesicles. Magnification 630X.
Figure A11: Loss of rab-10 does not change the colocalization between ATG-9 and RAB-5-positive early endosomes in 1-day old adults. A) Representative confocal images of wild type and rab-10(ok1494) 1-day old adults that express GFP::ATG-9 and tagRFP::RAB-5 in intestinal cells show colocalization. B) Quantification of the Pearson’s correlation coefficient for colocalization between GFP::ATG-9 and tagRFP::RAB-5 in wild type and rab-10 mutant animals. A-B) Animals were analyzed as 1-day old adults at 20°C. Data shown represents the average from a single trial. n = 6 animals; # of units per area analyzed/animal = 3. Statistical analysis was done using an unpaired, two tailed t-test. Error bars = ± S.E. Magnification 630X.
Figure A12: ATG-9 does not localize to RAB-7-positive late endosomes. A) Representative deconvolved epifluorescent images of wild type, rab-10(ok1494), and daf-2(e1370) animals that coexpress GFP::ATG-9 and tagRFP::RAB-7 in intestinal cells. B) Quantification of the Pearson’s correlation coefficient for colocalization between GFP::ATG-9 and tagRFP::RAB-7. A-C) Animals were analyzed at the L3 or dauer larval stages at 15°C and 25°C. n# ≥ 10 animals/strain; # of units per area analyzed/animal = 2. Statistical analysis was done using an unpaired, two tailed t-test. Error bars = ± S.E. Arrows point to representative tagRFP::RAB-7 vesicles and arrow heads point to representative GFP::ATG-9 vesicles. Magnification 630X.
we were unable to observe significant changes in the colocalization of ATG-9::GFP with the endosomal reporters for RAB-5 or RAB-7, in either wild type or *rab-10(ok1494)* mutants.
Appendix B

B. The Atg6/Vps30/Beclin1 ortholog BEC-1 mediates endocytic retrograde transport in addition to autophagy in C. elegans

A. Ruck¹,², J. Attonito¹, T. Garces¹, L. Núnez¹, N. Palmisano¹, Z. Rubel¹, Z. Bai³, K. Nguyen⁴, L. Sun⁴,⁵, B.D. Grant³, D. H. Hall⁴, and A. Meléndez¹,²

¹Queens College, Department of Biology, 65-30 Kissena Boulevard, Flushing, NY 11367
²The Graduate Center, The City University of New York, 365 Fifth Avenue, NY 10016,
³Rutgers University, Department of Molecular Biology and Biochemistry, Piscataway, NJ 08854,
⁴Center for C. elegans Anatomy, Albert Einstein College of Medicine, Bronx, NY 10461
⁵Center for Biological Imaging, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101,

China

Corresponding author: Alicia Meléndez
718 997 4580
Fax 718 997 9236
Alicia.Melendez@qc.cuny.edu

Running Title: BEC-1 in endocytosis

Subject categories: membrane and transport; development

Keywords: C. elegans; autophagy; endocytosis; lysosomes
Abstract

Autophagy and endocytosis are dynamic and tightly regulated processes that contribute to many fundamental aspects of biology including survival, longevity, and development. However, the molecular links between autophagy and endocytosis are not well understood. Here, we report that BEC-1, the C. elegans ortholog of Atg6/Vps30/Beclin1, a key regulator of the autophagic machinery, also contributes to endosome function. In particular we identify a defect in retrograde transport from endosomes to the Golgi in bec-1 mutants. MIG-14/Wntless is normally recycled from endosomes to the Golgi through the action of the retromer complex and its associated factor RME-8. Lack of retromer or RME-8 activity results in the aberrant transport of MIG-14/Wntless to the lysosome where it is degraded. Similarly, we find that lack of bec-1 also results in mislocalization and degradation of MIG-14::GFP, reduced levels of RME-8 on endosomal membranes, and the accumulation of morphologically abnormal endosomes. A similar phenotype was observed in animals treated with dsRNA against vps-34. We further identify a requirement for BEC-1 in the clearance of apoptotic corpses in the hermaphrodite gonad, suggesting a role for BEC-1 in phagosome maturation, a process that appears to depend upon retrograde transport. In addition, autophagy genes may also be required for cell corpse clearance, as we find that RNAi against atg-18 or unc-51 also results in a lack of cell corpse clearance.

Introduction

Macroautophagy (hereafter autophagy) is a cellular bulk degradation process conserved in all eukaryotes (Melendez and Neufeld 2008; Mizushima et al. 2008). In autophagy, a portion of the cytoplasm is sequestered by a double membrane organelle called the autophagosome, which then fuses with the lysosome to degrade the materials inside. Endocytosis is the vesicle-mediated process to internalize plasma membrane, and/or macromolecules from the outside environment. The endosomal system sorts the internalized molecules to their proper intracellular destination. In yeast, the vacuole is functionally equivalent to the lysosome in higher eukaryotes. In the yeast Saccharomyces cerevisiae, mutations in vacuolar protein sorting (Vps) genes result in secretion of proteins normally localized to the vacuole. Characterization of the VPS genes has provided insight into the mechanism of protein sorting and vesicle–mediated intracellular transport.
Genetic studies in the yeast *S. cerevisiae* have identified many genes that function in autophagy (ATG) (Klionsky et al. 2003; Suzuki et al. 2007). Many of the ATG genes encode proteins that are required for the early formation of the autophagosome, and are thus localized to a perivacuolar structure called the preautophagosomal structure (PAS) (Kim et al. 2001; Suzuki et al. 2001). Additional regulators of autophagy have been identified in screens for mutants in the cytoplasm-to-vacuole (Cvt) pathway (Harding et al. 1995; Harding et al. 1996). The Cvt pathway delivers vesicle-bound proteins to the vacuole from the cytoplasm. However, unlike autophagy which is degradative and mainly non-selective, the Cvt pathway is biosynthetic, and selects specific cargo from the cytoplasm to be delivered to the vacuole (Teter and Klionsky 2000).

Yeast Atg6/Vps30 and its orthologs in multicellular organisms play a pivotal role in autophagy. The mammalian ortholog of Atg6/Vps30 is Beclin 1. In addition to regulating autophagy, Beclin 1 has been shown to be a tumor suppressor protein and the antiapoptotic protein Bcl-2 negatively regulates autophagy through binding with Beclin 1 (Liang et al. 1998; Liang et al. 1999; Qu et al. 2003; Yue et al. 2003; Pattingre et al. 2005). *C. elegans* affords another genetic system for studying the role of Beclin 1. The *C. elegans* Beclin 1 ortholog, *bec-1* mediates autophagy and is essential for morphogenesis of the dauer larva and for lifespan extension mediated by insulin signaling (Melendez et al. 2008) or dietary restriction (Jia and Levine 2007; Hansen et al. 2008b; Melendez and Levine 2009). We have studied the role of *C. elegans* BEC-1 in autophagy and endocytosis.

In yeast, distinct roles of Atg6/Vps30 protein in autophagy and vacuolar protein sorting reflects two related but functionally different phosphatidylinositol 3-kinase (PI3K) complexes: the type I complex functions in autophagy and the type II complex functions in vacuolar protein sorting (type II complex), respectively (Kametaka et al. 1998). The difference between the complexes is that complex I contains Atg14, whereas complex II contains Vps38, instead of Atg14. Vps38 is present on endosome and vacuolar membranes, and it is required for the targeting of Atg6/Vps30 and Vps34 to the endosome (Obara et al. 2006). In contrast, Atg14 localizes to the PAS and vacuolar membrane, and generates the autophagosome by recruiting Vps34 and Atg6/Vps30 to the PAS (Obara et al. 2006).

Vps34, which corresponds to the mammalian class III PI3-kinase, is important for various membrane trafficking pathways such as the Golgi to lysosome pathway, internal vesicle formation in late
endosomes, and autophagy (Brown et al. 1995; Davidson 1995; Petiot et al. 2000; Futter et al. 2001; Kihara et al. 2001; Johnson et al. 2006). Although it has been well established that the mammalian Beclin 1/Vps34 complex is required for autophagy, its involvement in other membrane trafficking pathways has been controversial in higher eukaryotes. Expression of C. elegans BEC-1 or human Beclin 1 in atg6-depleted yeast restores autophagy but not vacuolar protein sorting function in yeast (Meléndez et al. 2003). Although Beclin 1 and hVps34 were shown to co-localize in the trans-Golgi network (TGN) in HeLa cells, the role of Beclin 1 in vesicular trafficking in mammalian cells may be limited. RNA interference (RNAi) mediated suppression of beclin 1 cells impairs autophagy but does not affect fluid-phase endocytosis, endocytic sorting of the epidermal growth factor receptor (EGFR) or cathepsin D transport from TGN to lysosomes (Zeng et al. 2006). In addition, the overexpression of a mutant version of human Beclin 1 that cannot bind to human Vps34 does not inhibit the ability to process cathepsin D, but significantly reduced autophagy activity and tumor suppressor functions in MCF7 cells (Furuya et al. 2005). In contrast, expression of plant Atg6 in yeast atg6 mutants restores both autophagy and vacuolar protein sorting function (Fujiki et al. 2007). Furthermore, C. elegans BEC-1 has been shown to bind to VPS-34 and affect PI3P localization and general uptake of an endocytic marker (Takacs-Vellai et al. 2005). Biological functions of atg6/beclin 1 in higher eukaryotes suggest a role outside of autophagy. Arabidopsis atg6 mutants exhibit defects in autophagy and pollen germination, but the latter was not observed in other autophagy mutants, suggesting that Atg6 confers some autophagy–independent function (Fujiki et al. 2007). In mice, Beclin 1 knockout animals die at approximately embryonic day 7.5,(Yue et al. 2003) whereas Atg5 and Atg7 knockout mice can survive until birth,(Kuma et al. 2004; Komatsu et al. 2007) thus Beclin 1 might have a more complex function in mice. Recently, orthologs of Atg14 and Vps38 have been identified in mammals,(Itakura and Mizushima 2009; Zhong et al. 2009) and shown to form distinct PI3-kinase complexes with different intracellular localization, suggesting that Vps34-Beclin 1 complexes may have different functions in metazoans similar to that in yeast cells.

Although C. elegans bec-1 had been previously implicated in endocytic trafficking,(Takacs-Vellai et al. 2005) the nature of the contribution had not been described. Here, we show that bec-1 mutant animals display defects in recycling of cargo molecules from the endosome to the late-Golgi. We observed that maternally rescued bec-1 mutants accumulate large vacuoles of mixed endosomal and lysosomal identity.
Furthermore, we show that \textit{bec-1} functions in endosome to Golgi retrograde transport. In particular, \textit{bec-1} mutant animals display defects in the recycling of MIG-14/Wntless cargo protein from the endosome to the trans-Golgi network. Recycling of the MIG-14/Wntless cargo is mediated by the retromer complex, a conserved cytoplasmic coat recycling complex that mediates the endosome-to-Golgi retrieval of vacuole/lysosome hydrolase receptors in yeast and mammals (Bonifacino and Hurley 2008; Collins 2008). We further show that RME-8, a subunit of the retromer complex, and BEC-1 colocalize in endosomes that are found adjacent to lysosomes in wild-type animals, and that RME-8 is redistributed to the cytoplasm in \textit{bec-1} mutants, indicating a role for \textit{bec-1} in retrograde transport. Finally, we report a lack of cell corpse clearance as observed by electron microscopy and an increased number of CED-1::GFP positive cells in \textit{bec-1} mutant gonads, similar to the phenotype in animals lacking the retromer subunit rme-8 (Chen et al. 2010b).

\textbf{Results}

\textit{bec-1} has maternal and zygotic activity

The \textit{C. elegans} Gene Knockout Consortium generated two deletion mutations in \textit{bec-1}, \textit{ok691} and \textit{ok700}. The \textit{ok691} allele deletes the first six out of seven exons, including the ATG start codon (Fig. B1) and is thus a predicted molecular null allele (Takacs-Vellai et al. 2005). The \textit{ok700} allele deletes amino acids 168 to 314 of the “evolutionarily conserved domain” (ECD) in BEC-1 and also causes a frameshift that removes the remainder of the protein (Materials and Methods); this allele appears to be a strong hypomorph.

Both \textit{ok691} and \textit{ok700} mutations were reported to display a highly penetrant embryonic lethal phenotype when segregating from a balanced heterozygote (Takacs-Vellai et al. 2005). In contrast, using a balancer marked with \textit{unc-5(e53) jcls[ajm-1:gfp]}, which enables us to unambiguously identify \textit{bec-1} mutant animals during both embryonic and adult stages (see Materials and Methods), we found that \textit{ok691} and \textit{ok700} homozygous individuals derived from a \textit{bec-1(mutant)/unc-5(e53) jcls1} heterozygous parent can reach adulthood (Table B1). However, these adults are sterile and die after the first day of adulthood. These results confirm that \textit{bec-1} activity is required for viability (Table B1). Further analysis suggests that \textit{bec-1} mutants segregating from heterozygous mothers reach adulthood because of maternal rescue.
Both \textit{bec-1(ok691)} and \textit{bec-1(ok700)} homozygous mutants that segregate from the \textit{bec-1(mutant)/unc-5(e53) jcls1} heterozygous parent, appear superficially wild-type during early larval stages but become increasingly uncoordinated during later larval stages and display a low penetrance molting defect. In addition, mutants accumulate vacuoles that become first visible during the L3 stage [\textbf{Table B2}: 70\% of \textit{ok691} (n=37), and 30\% of \textit{ok700} (n=40) contain at least one vacuole, \textbf{Fig. B1B}, middle panel]. These vacuoles are present in the intestine, hypodermis, pharynx, and coelomocytes. Both the number and size of the vacuoles increases with age in larvae (\textbf{Table B2}). By L4 stage, 80\% of the \textit{ok691} animals (n=39), and 52\% of \textit{ok700} (n=40) contain at least one vacuole, and approximately 42\% of \textit{ok691} mutants (n=39), and 5\% of \textit{ok700} mutants (n=40) contain more than 10 vacuoles (\textbf{Table B2}). Lastly, both \textit{ok691} and \textit{ok700} mutant animals display growth retardation and reach the adult stage up to 1.5 days later than wild-type animals.

To confirm that the observed phenotypes are due to a mutation in \textit{bec-1}, we injected a genomic sequence containing wild-type sequences of \textit{bec-1} into \textit{ok691} mutant animals (\textit{Ex [bec-1(+), SUR-5::GFP]}) and obtained rescue of both the developmental defects and vacuolar phenotypes (\textbf{Table B2, Fig. B1B}, bottom panel). Both the \textit{ok691} and \textit{ok700} \textit{bec-1} deletion alleles are likewise rescued by a functional BEC-1::RFP fusion construct (kindly provided by B. Bamber (Rowland et al. 2006))(\textbf{Table B3}). Interestingly, a significant number of transgenic embryos failed to hatch (\textbf{Table B3}). Only 15.1\% of the embryos carrying the \textit{Ex[BEC-1::RFP, rol-6(d)]} transgene, and 38.6\% of the embryos carrying the \textit{Ex [bec-1(+), SUR-5::GFP]} transgene hatch. Since multicopy transgenic arrays are not efficiently expressed in the hermaphrodite germline, (Mello et al. 1991; Kelly et al. 1997) this observation suggested that \textit{bec-1} is maternally contributed.

To directly test this possibility, we analyzed the phenotype of \textit{bec-1} mutant embryos that originated from fertile homozygous \textit{ok691} mutant mothers carrying the rescuing transgene, \textit{Ex [bec-1(+), SUR-5::GFP]}. The lack of the transgene in embryos can be visualized by the lack of GFP (\textbf{Fig. B1C}, right bottom panel). These embryos likely lack both maternal and zygotic expression of \textit{bec-1}. We find that maternal and zygotic loss of \textit{bec-1} results in complete early embryonic lethality. As these embryos fail to hatch, we infer that they were not rescued maternally. These embryos display classic morphogenesis defects with unattached cells floating outside the embryo (\textbf{Fig. B1C}, right top panel) and visible apoptotic cell corpses (Takacs-Vellai et al. 2005).
**bec-1 mutation disrupts the localization of PI3P**

We analyzed the phenotype of *bec-1(ok691)*, an unambiguous molecular null allele in detail. To understand the relationship of *bec-1* and *vps-34*, and to test whether there is any dysregulation of phosphoinositides in *bec-1* mutants, we monitored the expression and localization of PI3P-, PI(4,5)P2-, and PI(3,4,5)P3-binding reporters (Bae et al. 2009) in wild-type and *bec-1(ok691)* mutant animals. *vps-34/let-512* encodes the class III phosphoinositide 3-kinase, a protein that regulates multiple steps in endocytosis (Roggo et al. 2002). Since BEC-1 associates with VPS-34, (Takacs-Vellai et al. 2005) we expected that the localization of PI3P might be affected in the *bec-1(ok691)* null mutant. The product of the PI3P was detected with a transgene encoding a tandem repeat of human Hrs FYVE domain, 2XFYVE::GFP. As shown previously, (Takacs-Vellai et al. 2005) lack of *bec-1* activity disrupts the localization of PI3P. In *bec-1(ok691)* mutants we observed a more diffuse localization of the 2XFYVE::GFP reporter (Fig. B2A') as compared to the same marker in control animals (Fig. B2A). The membrane of the enlarged vacuoles in mutant intestines was not labeled with the PI3P marker. Localization of PI(4,5)P2 was determined with a reporter that expresses the PH domain of phospholipase C delta. PI(4,5)P2 in the worm intestine is normally enriched apically with a less pronounced localization on basolateral membranes (Fig. B2B). The PI(3,4,5)P3 reporter consisted of the PH-domain of Akt fused to GFP, and was localized apically and basolaterally in the intestine (Fig. B2C). In *ok691* mutants, the expression of the PI(4,5)P2 and PI(3,4,5)P3 reporters was largely unaffected when compared to that of wild-type animals (Fig. B2B' and B2C').

**bec-1 mutants have defects in endocytosis**

The endocytic nature of the vacuoles and defects in PI3P localization observed in *bec-1* mutant animals prompted us to directly test whether *bec-1* is required for endocytic trafficking in *C. elegans*. We concentrated our analysis on coelomocytes, which are large scavenger cells, situated in the pseudocoelomic space that continuously endocytose fluid from the pseudocoelom (Fares and Greenwald 2001a). To define more precisely the defect in *bec-1* endocytosis, we performed fluid uptake assays by injection of Texas Red-coupled BSA (TR-BSA) into the body cavity of wild-type and *bec-1* mutant individuals. We used LMP-1::GFP to visualize lysosomes and late endosomes. LMP-1 is similar to the
vertebrate lysosome-associated membrane protein LAMP/CD68 (Kostich et al. 2000). LMP-1::GFP has been widely used as lysosomal marker (Treusch et al. 2004; Hermann et al. 2005). Injection of TR-BSA into the pseudocoelom (body cavity) can be monitored for its uptake, which is mediated by endocytosis at the basolateral cell membrane bordering the pseudocoelom (Grant et al. 2001). In wild-type animals, the fluorescent marker enters the coelomocytes by fluid phase mediated endocytosis and is transported through the different endocytic compartments to the lysosomes (Treusch et al. 2004; Nicot et al. 2006). After injection of wild-type animals, the fluid-phase marker is present in early endocytic compartments. After 15 minutes, it begins to accumulate in endosomes (LMP-1::GFP negative), and by one 1 hour it accumulates in lysosomes (LMP-1::GFP positive in Fig. B2D) (Zhang et al. 2001; Treusch et al. 2004; Nilsson et al. 2008). In bec-1 mutants, the endocytosis of the fluid phase marker was found to be abnormal (Fig. B2D'). Texas Red BSA does not localize to LMP-1 positive endosomes even after 24 hours. After 1 hour of soaking the animals with fluid markers (or allowing them to feed on it), the Texas Red BSA marker is clearly endocytosed into wild-type coelomocyte lysosomes (LMP-positive in Fig. B2D), but not into the bec-1(ok691) mutant coelomocytes (Fig. B2D'). These results indicate a role for BEC-1 in an early step of endocytosis.

The vacuoles in bec-1(ok691) mutants are of endocytic origin

To characterize the abnormal vacuoles in bec-1 mutant animals and their origin, we used a combination of fluorescent and electron microscopy. In electron micrographs, the vacuoles are clearly surrounded by a membrane and, are either empty, filled with membrane whorls, or electron dense deposits, features typical of late endosomes and lysosomes (Fig. B2H and I). We also often observed aggregation of endocytic structures (Fig. B2I (black arrow head)).

To better define the origin of the vacuoles, we crossed ok691 mutants with transgenic animals expressing markers for a variety of specific endocytic compartments. Wild-type intestines contain a large number of small GFP::RAB-5 positive punctate structures (Fig. B3A) near the basolateral and apical plasma membrane corresponding to early endosomes (Apodaca et al. 1994). Similarly a number of GFP::RAB-7 positive punctate structures, presumably corresponding to maturing endosomes are observed near the plasma membrane (Chen et al. 2006) in wild-type intestines (Fig. B3B). A number of larger ring-like
GFP::RAB-7 positive structures localize deeper in the cytoplasm and are presumed to be mature late endosomes (Hermann et al. 2005; Chen et al. 2006). We find that in ok691 mutants, the GFP::RAB-5 early endosomal marker labels the membrane of the abnormal vacuoles (Fig. B3A’). The GFP::RAB-5 is often specifically localized to selected regions of the abnormal vacuoles. However, the number and the intensity of GFP::RAB-5 punctate dots did not change in ok691 mutants when compared to wild-type (Fig. B3I). In contrast, the late endosomal markers, GFP::RAB-7 and LMP-1::GFP, label the membrane of the enlarged abnormal vacuoles in ok691, but only rarely the membrane of small abnormal vacuoles. These results suggest that the vacuoles are aberrant endosomes and can be distinguished as two classes, perhaps representing different stages in the endosome maturation process (Fig. B3B’). Unlike RAB-5-positive puncta, the small RAB-7-positive puncta over-accumulated in bec-1 mutant intestines, (Fig. B3B, B’ and I). The accumulation of the small RAB-7-labeled structures appeared to be at the expense of the larger RAB-7-labeled rings that decreased in number (Fig. B3I). Markers for other endosome types such as apical recycling endosomes (GFP::RAB-11) and basolateral recycling endosomes (GFP::RAB-10) appeared normal (Fig. B6, and data not shown). Moreover, the abnormal vacuoles in bec-1 mutant animals were very faintly labeled or not at all labeled by GFP::RAB-10 (Fig. B6). Similarly, we note that the expression of ALX-1::GFP in bec-1 mutants was not significantly different to that of wild-type animals (Fig. B6). ALX-1 is an Alix/Bro1p ortholog in C. elegans, a protein that together with RME-1 regulates recycling from endosomes to the plasma membrane (Shi et al. 2007). Taken together these results suggest a requirement for BEC-1 at a specific stage of endosome maturation.

As another control, we assayed the distribution of the human transferrin receptor (hTfR::GFP), transmembrane cargo that is recycled through the recycling endosome and not the Golgi (Burack et al. 2000; Lin et al. 2001b; Shi et al. 2009). An equivalent version of this GFP-fusion protein was shown to be functional and traffic normally in mammalian cells or C. elegans (Chen et al. 2006). We find that the abnormal vacuoles in bec-1 mutants did not accumulate hTfR::GFP (Fig. B3D and B3D’), and its steady state localization was unaffected by a mutation of bec-1, indicating that hTfR transit through the recycling endosome is unaffected. From this and the experiment described using the RAB-10 and RAB-11 markers, we conclude that bec-1 is not required for the function of recycling endosomes.
Endosome to TGN retrograde transport is defective in \textit{bec-1} mutants

In yeast, the identification of the yeast VPS34 gene encoding the PI3-kinase in a screen for vacuolar sorting mutants has demonstrated a direct role for PI3P in vesicular trafficking (Apodaca et al. 1994). Specifically, PI3P was shown to be required for the efficient sorting of proteins from the late-Golgi to the vacuole (Schu et al. 1993). Given the disruption in early endosomal trafficking that we see in \textit{bec-1} mutants, we next asked whether the loss of \textit{bec-1} could affect endosome to Golgi retrograde trafficking.

In \textit{C. elegans}, SNX-1 is the only ortholog of mammalian Sorting Nexin 1 and Sorting Nexin 2 proteins that function in endosome-to-Golgi retrograde transport along with Vps-26, Vps-29, and Vps-35, as part of the retromer complex (Bonifacino and Hurley 2008; Collins 2008). RME-8, a large protein with a DNA-J domain, binds to SNX-1 (Shi et al. 2009). \textit{C. elegans} GFP::SNX-1 and RME-8::GFP fusion proteins colocalize with early endosomal markers in living animals (Shi et al. 2009). In \textit{C. elegans}, like all invertebrates, the Golgi network appears as dispersed ministacks throughout the cell rather than in one large juxtanuclear stack (Sato et al. 2009). To date, the only retromer dependent cargo protein known in \textit{C. elegans} is MIG-14/Wntless (Pan et al. 2008; Yang et al. 2008). In the absence of retromer function, MIG-14/Wntless is depleted from the Golgi and missorted to the late endosome and lysosome (Belenkaya et al. 2008; Franch-Marro et al. 2008; Pan et al. 2008; Port et al. 2008; Yang et al. 2008; Shi et al. 2009). Endosome to Golgi transport and sorting of cargo protein MIG-14/Wntless depend on RME-8 and SNX-1 (Shi et al. 2009). Thus, we reasoned that if BEC-1 functions in retrograde transport, \textit{bec-1} mutants should abnormally transport the MIG-14/Wntless cargo and have a similar phenotype to \textit{snx-1} or \textit{rme-8} mutants.

When compared to wild-type control animals, \textit{bec-1(ok691)} mutants displayed an approximately 4-fold reduction in the number of MIG-14::GFP- positive puncta. A similar reduction was observed in animals treated with RNAi against \textit{bec-1} and \textit{vps-34} (\textbf{Fig. B8}). These results are consistent with the idea that BEC-1 functions with VPS-34 in retrograde transport (\textbf{Fig. B3E, B3E’}; \textbf{Fig. B8}). However, we observed an increase in the total intensity of the MIG-14::GFP signal in contrast to what has been previously reported for MIG-14::GFP in \textit{rme-8}, or \textit{snx-1} mutants (Pan et al. 2008; Yang et al. 2008; Shi et al. 2009). In \textit{bec-1} and \textit{vps-34} mutants, MIG-14::GFP is found in ring-like and intralumenal structures (\textbf{Fig. B8}). The reasons for this difference are unclear, but one possibility is that MIG-14::GFP localizes to the membrane of the abnormal and enlarged vacuoles that accumulate in \textit{bec-1} mutants (\textbf{Fig. B3E, B3E’}; \textbf{Fig. B8}), similar to
the localization of GFP::RAB-7, and LMP-1::GFP. Another possibility is that the discrepancy between bec-1 and rme-8 phenotypes is due to the hypomorphic nature of the rme-8 mutation we used. These results suggest that there is a defect in the retrograde transport of MIG-14::GFP from the endosome to the Golgi in bec-1 mutants and that in bec-1 mutants, a substantial amount of MIG-14 is degraded by the lysosome, similarly to what has been described in animals with defective retromer subunit activity.

To determine if the decrease in MIG-14::GFP in bec-1 mutants is the result of missorting and lysosomal-mediated degradation, we monitored MIG-14::GFP levels in bec-1 mutants after RNAi depletion of cup-5/mucolipin1, a transmembrane protein required for normal lysosome biogenesis and normal levels of hydrolytic activity (Treusch et al. 2004; Schaheen et al. 2006; Shi et al. 2009). Depletion of CUP-5 by RNAi increased the number of MIG-14::GFP positive puncta to nearly normal levels in the bec-1 mutant background (quantitation shown in Fig. B3J; Fig. B9). In addition, a significant increase in the pixel intensity of MIG-14::GFP was observed in bec-1 mutant that were depleted for CUP-5 by cup-5 RNAi (Fig. B3J; Fig. B9). These results confirm that lysosomal degradation of MIG-14::GFP indeed occurs in bec-1 mutants. A similar result was previously reported for retromer mutants, such as rme-8. In rme-8 mutants, CUP-5 depletion by RNAi restores the levels of MIG-14::GFP pixel intensity to nearly normal levels. Thus, loss of BEC-1 activity results in a similar phenotype to the retromer mutants, where the degradation of MIG-14 occurs through the missorting into the lysosomal pathway (Fig. B3J).

The increased degradation of MIG-14::GFP suggested that multivesicular body (MVB)-mediated transport of membrane proteins to the lysosome does not require BEC-1. To examine this point further, we assayed the degradation of CAV-1::GFP in early embryos (Sato et al. 2006; Shi et al. 2007; Shi et al. 2009). CAV-1 is a known transmembrane cargo protein that is degraded in the one cell embryo, right after fertilization, after the metaphase to anaphase transition (Sato et al. 2006; Bembenek et al. 2007; Sato et al. 2008a). Degradation of CAV-1::GFP requires endocytosis and the endosomal sorting complex required for transport (ESCRT) machinery. CAV-1::GFP degradation was unaffected in animals treated with bec-1 RNAi (Fig. B7), even after animals were grown as the second generation in RNAi plates, further suggesting that BEC-1, just like RME-8 and SNX-1,(Shi et al. 2009) is not required for ESCRT-mediated degradation of integral membrane proteins. Similarly, we do not see a defect in CAV-1::GFP degradation after vps-34 RNAi (Fig. B7).
**Localization of RME-8, SNX-1 and VPS-35 in bec-1 mutant animals**

Since we observed a retrograde trafficking defect in bec-1 mutants, we considered that *C. elegans* BEC-1 may act similarly to Atg6/Vps34 in the yeast retrograde transport. In yeast, mutations in ATG6/VPS30 lead to a selective sorting and maturation phenotype of the soluble vacuolar protease CPY. Redistribution of Vps5p and Vps17p, two subunits of the retromer complex, was observed in strains deficient for Atg6/Vps30 (Burda et al. 2002). To determine if the localization of retromer complex proteins is altered, we examined the localization of RME-8::GFP (**Fig. B3F, F'**), GFP::SNX-1 (**Fig. B3G, G'**) and GFP::VPS-35 (**Fig. B3H, H'**). In bec-1 mutants, we found that the intensity and number of RME-8::GFP-positive puncta is decreased (**Fig. B3 compare F to F'; Fig. B10**). Although RNAi against bec-1 did not have as dramatic an effect on RME-8::GFP as the bec-1 mutant, knockdown of vps-34 by RNAi also affected the number of RME-8::GFP positive puncta significantly (**Fig. B10**).

RME-8 and SNX-1 have been shown to physically associate *in vivo* and this association is functionally relevant for the regulation of endosomal clathrin (Shi et al. 2009). We observed some increase in the intensity of clathrin positive endosomes after RNAi treatment against vps-34, visualized with a functional clathrin reporter, GFP::CHC-1 (Sato et al. 2009; **Fig. B11**), and no obvious changes upon knockdown of bec-1 activity. However, consistent with the decrease in number and intensity of RME-8::GFP positive puncta observed in bec-1 mutants, we observed a significant increase in GFP::SNX-1 and GFP::VPS-35 positive puncta in bec-1 mutants when compared to wild-type animals (**Fig. B3G' and H'**; quantitation in **Fig. B3I**). Moreover, the size of the GFP::SNX-1 positive puncta was significantly increased in bec-1 mutant animals (**Fig. B12**). This increase in the size and distribution of SNX-1 positive endosomes in bec-1 mutants is similar to that observed in rme-8 mutants (Shi et al. 2009).

**Colocalization of BEC-1 and RME-8**

The retromer complex, including RME-8 and SNX-1, is found primarily on early endosomes, and many of the RME-8/SNX-1 positive endosomes are closely juxtaposed to the Golgi apparatus (Bonifacino and Hurley 2008; Shi et al. 2009).
We found that in *C. elegans* BEC-1::RFP colocalizes well with a subset of RME-8::GFP labeled endosomes in the intestine, consistent with a direct role of BEC-1 in retrograde transport (Fig. B4C'). Interestingly, we note that BEC-1::RFP and RME-8::GFP double-positive endosomes are clearly adjacent to autofluorescent lipofuscin-positive lysosome-like organelles (in blue, Fig. B4C' and C’).

**bec-1 has a role in germ cell corpse clearance**

Loss of *bec-1* leads to an increase in the number of visible apoptotic cell corpses (Takacs-Vellai et al. 2005). This phenotype could result from increased apoptosis or clearance defects in the execution of the cell death pathway, or both. To distinguish between these possibilities, we analyzed germ cell corpses and their surroundings using transmission electron microscopy. Wild-type sheath cells are so efficient in clearing apoptotic germline cells that corpses are virtually never seen in a wild-type gonad by electron microscopy, although they have been observed in other endocytic mutants ((Gumienny et al. 2001). In contrast, in *bec-1(ok691)* mutants, we found several examples where dying germ cells were engulfed by the sheath cell but not digested (Fig. B5A). We analyzed serial sections that span the entire diameter of each cell corpse to determine whether it is totally inside a sheath cell. In *bec-1(ok691)* mutants, all germ cell corpses were engulfed by gonadal sheath cells. These results suggest that the dying germ cell is still able to trigger the sheath cell to encircle it, but that *bec-1* activity is required to promote the delivery of other components to the phagosome for the completion of cell degradation. To further corroborate these findings we followed individual apoptotic nuclei in animals with reduced *bec-1* function and in wild-type animals and determined the time required for clearance. We find that all apoptotic nuclei in wild-type animals are cleared in less than 50 min, most within 30 minutes, whereas nuclei persist in animals with reduced *bec-1* function for over 1 h (Fig. B5B). These observations indicate that the apparent increase in apoptotic nuclei reflects a defect in cell corpse clearance rather than an increase in the number of cells undergoing apoptosis.

To investigate whether inactivation of other autophagy genes has an effect in cell corpse clearance, we counted apoptotic nuclei in the gonads of animals that had been treated with dsRNA against various autophagy genes. The phagocytic receptor cell death abnormal CED-1 is an engulfing cell-specific marker that recognizes cell corpses and clusters to the growing phagocyte (Zhou et al. 2001b). Using CED-1::GFP, we find an accumulation of germ cell apoptotic nuclei in the gonad vps-34 mutant animals (Fig. B5C),
consistent with previous reports (Zhou et al. 2001b; Kinchen et al. 2008; Zou et al. 2009). In addition, we find that RNAi mediated knock down of unc-51/ATG1, and atg-18 result in an increase of CED-1::GFP positive nuclei (Fig. B5D). Although we did observe an increase in CED-1::GFP positive nuclei in animals treated with RNAi against atg-7, after analysis of variance (ANOVA) for multiple comparisons, we found this result not to be significant.

Discussion

C. elegans bec-1 has been shown to have a role in organismal size, dauer morphogenesis, resistance to pathogens and the longevity of daf-2 (the insulin-like receptor) mutants, as well as the longevity of dietary restricted animals (Melendez et al. 2008). daf-2 constitutive mutants at the restrictive temperature were shown to have an increase in autophagy, as marked by the GFP::LGG-1 autophagosome marker (Meléndez et al. 2003). GFP::LGG-1 localization in daf-2 insulin-like receptor mutants after bec-1 RNAi was observed to form large aggregates, rather than small punctate structures which represent “normal” autophagosomes in daf-2 mutants with wild-type bec-1 activity. This may be due to the lack of clearance of the GFP::LGG-1 protein due to a defect in the autophagolysosomal pathway, (Hansen et al. 2008b; Melendez et al. 2008) but awaits further clarification. In addition to confirming a role for bec-1 in endocytic trafficking,(Takacs-Vellai et al. 2005) we used a combination of endocytic markers to show that BEC-1 is involved in various steps of endocytosis. In particular, we have identified a role for BEC-1 in retrograde trafficking that resembles defects associated with loss of retromer activity, including defective recycling of MIG-14, known cargo for the endosome to Golgi retromer transport and a high number of CED-1::GFP positive cell corpses in bec-1 mutant gonads, as also observed in gonads from animals lacking retromer subunits (Chen et al. 2010b).

bec-1 has a maternal effect lethal phenotype

Animals that completely lack maternal and zygotic BEC-1 activity die during embryogenesis. These embryos display a high incidence of apoptotic nuclei(Takacs-Vellai et al. 2005). Animals that segregate from a heterozygous parent, and have the maternally derived wild-type BEC-1 function, live to early adulthood. These homozygous bec-1 mutant animals display a striking accumulation of vacuoles in different
tissues including the intestine and hypodermis. They are also uncoordinated in their movement.

Studies in mice have shown that lack of beclin 1 activity is also lethal. beclin 1 mutants die at day 7.5 of development,(Qu et al. 2003; Yue et al. 2003) whereas atg5 and atg7 mutant mice die after birth in the period of starvation before the animals begin to breastfeed (Kuma et al. 2004; Komatsu et al. 2005). Therefore, it is possible that the difference between the phenotype of beclin 1 and other autophagy genes is due to beclin 1 having additional defects in endocytosis in mammals.

**bec-1 mutants display endocytic defects**

The lack of accumulation of endocytic tracers in coelomocytes indicates that the internalization step of endocytosis is defective in bec-1 mutants. This is consistent with previous reports of a pronounced defect in bec-1 mutants when assaying for the uptake of vitellogenin by oocytes,(Takacs-Vellai et al. 2005) a process that requires endocytosis. Thus, bec-1 is required at an early step in endocytic trafficking.

We have analyzed the number and morphology of all major endocytic compartments in bec-1 mutant intestinal cells *in vivo* using a set of GFP-tagged markers that label each class of compartment. Our goal was to determine if there was a block in a particular transport step caused by the lack of bec-1 activity by measuring specific changes in endosome morphology, and intensity or number of GFP markers. We would expect that an endocytic compartment that normally receives cargo in a BEC-1 dependent fashion would be smaller in size or be lacking completely in intestinal cells lacking BEC-1 activity. We would also expect an accumulation or an enlargement of BEC-1 dependent endosomes or BEC-1 donor compartments in the bec-1 mutant intestines. Trafficking from the cell membrane to the lysosome requires the activity of Rab5 and Rab7 GTPases (Stenmark 2009). Rab GTPases regulate intracellular trafficking by controlling the transport of vesicles between membrane compartments along endocytic transport. Rabs alternate between an "active" guanosine triphosphate (GTP)-bound state and an "inactive" guanosine diphosphate (GDP)-bound state. Active Rab5 localizes to early endosomes whereas Rab7 localizes to late endosomes. Rab5 and Rab7 regulate trafficking of cargo from the plasma membrane to the lysosomes (Stenmark 2009). In bec-1 homozygous mutants, we find an accumulation of RAB-7 positive puncta structures and a decrease in RAB-7 ring-like structures. As RAB-7 positive puncta are presumed to be maturing endosomes, and the ring-like structures to be late endosomes, our results suggest a defect prior to full maturation of the...
late endosome. In addition, we found that the membrane of the abnormal vacuoles seen in bec-1 mutant animals were positive for RAB-5, RAB-7 and LMP-1, consistent with accumulation of incompletely matured endosomes. In yeast and mammalian cells, Ypt7p and Rab7 can mediate homotypic fusion of vacuoles and late endosomes/lysosomes (Papini et al. 1997; Bucci et al. 2000). Thus, it is possible that the accumulation of RAB-7 and LMP-1 positive large vacuoles is the result of an enhancement of homotypic fusion consistent with an increase in RAB-7 activity. Distorted endosomal compartments were also observed by electron microscopy. These data demonstrate that the wild-type BEC-1 activity is required for the endolysosomal pathway. The fact that we see a lack of PI3P localization, the product of VPS-34, in the bec-1 null mutants indicates that the bec-1 phenotype may be due to the mislocalization of PI3P on endosomes.

In this study we show that the enlarged vacuoles in bec-1 null mutants result from a defect in maturing endocytic compartments. This phenotype has been previously documented for C. elegans intestines with defects in the recycling of endosomes as in rme-1, or rab-10 mutants, or when recycling is blocked by pharmacological agents, in certain mammalian cell types (Apodaca et al. 1994; van Weert et al. 2000). Similar vacuoles or enlargement of endocytic compartments have also been observed in phosphatidylinositol phosphate kinase 3 mutants, ppk-3, the C. elegans ortholog of the yeast PIKfyve/Fab1p (Nicot et al. 2006). In these animals, enlargement of RAB-7-positive, and LMP-1 positive endocytic compartments occurs.(Nicot et al. 2006) Thus, there are multiple steps in endocytosis for which disruption may result in enlarged endocytic compartments. However the recycling marker RAB-10::GFP did not localize to the bec-1 mutant vacuoles, and the number of RAB-10::GFP puncta was not affected in bec-1 mutants, suggesting that the lack of bec-1 does not affect the recycling of endosomes.

**BEC-1 functions in the retrograde transport**

Yeast Atg6/Vps30 has been implicated in early endosome to Golgi retrograde transport (Seaman et al. 1997; Burda et al. 2002). We find that C. elegans bec-1 function is also required for retromer function. We show that BEC-1 and VPS-34 are required to rescue MIG-14 from degradation after its endocytosis. Our results show that aberrant sorting of MIG-14 in bec-1 and vps-34 mutants is similar, although not as dramatic as that in rme-8 or snx-1 mutants (Shi et al. 2009). It is not known whether MIG-14 may utilize any other alternative route of retrograde transport in C. elegans or in mammals (Shi et al. 2009). LMP-1 and
MIG-14, two different transmembrane cargo proteins, labeled the abnormal vacuoles that accumulate in \textit{bec-1} mutant intestinal cells, whereas hTfR::GFP did not, indicating that LMP-1 and MIG-14 require BEC-1 for transport, while hTfR does not.

\textit{bec-1} mutants have a marked decrease in number of RME-8::GFP puncta expression. A similar phenotype was observed in animals with knockdown RNAi mediated against \textit{vps-34}. Conversely, the number of GFP::SNX-1 and GFP::VPS-35 positive puncta increases in \textit{bec-1} mutants when compared to wild-type animals. For SNX-1, the size of the puncta is larger in \textit{bec-1} mutants. These findings are similar to those made in \textit{rme-8} mutants,(Shi et al. 2009) although \textit{bec-1} and \textit{vps-34} mutants accumulate aberrant MIG-14::GFP positive vacuoles. We conclude that \textit{bec-1} and \textit{vps-34} function in retrograde transport, possibly in concert with \textit{rme-8}. Since it is not known how RME-8 is recruited to the endosome, we hypothesize that BEC-1 and the product of VPS-34, PI3P, may facilitate the endosomal recruitment of RME-8. Further studies will be required to better understand the relationship between BEC-1, VPS-34 and RME-8, and the role of BEC-1 and VPS-34 in early endosome maturation and transport to the Golgi.

\textbf{BEC-1 is required for cell corpse clearance}

We find that \textit{bec-1} loss of function mutant animals display a lack of cell corpse clearance in the hermaphrodite gonad rather than an increase in the incidence of apoptosis (Takacs-Vellai et al. 2005). Instead, we find that there is a significant lack of germ cell corpse degradation in \textit{bec-1} mutant animals when compared to wild-type. In addition, using transmission electron microscopy we do not see a major effect on engulfment, implying that loss of \textit{bec-1} activity does not appear to affect the signal from the dying cell. We cannot rule out a low-penetration effect, but our \textit{in vivo} results contrast with the previously reported requirement for autophagy genes such as \textit{beclin 1} and \textit{atg5} as an energy source to facilitate signaling from the dying cells to the phagocytic cell in embryoid bodies derived from ES cells (Qu et al. 2007).

We also find that animals deficient in \textit{vps-34} (which is required for RAB-5 recruitment to the nascent phagosome (Zhou et al. 2001b) show an increase in the number of germ cell nuclei. The degradation of cell corpses has been shown to require the sequential enrichment of early endosomes, late endosomes and lysosomes to the nascent phagosome (reviewed in (Zhou and Yu 2008)). RAB-7 and PI3P have been proposed to function as downstream effectors of the CED-1 pathway to mediate phagolysosome
formation. (Kinchen and Ravichandran) Since we show that RAB-7::GFP as well as PI3P localization is not normal in bec-1 mutants, the cell clearance defect of bec-1 mutants may also be due to the mislocalization of PI3P at the phagosome. Interestingly, we observe that knockdown of other autophagy genes by RNAi results in a similar accumulation of CED-1-positive engulfed germ cells in the hermaphrodite gonad. This result suggests that autophagosomes are also required in the process of phagosome maturation. Since a defect in cell clearance has also been reported for retromer subunit mutants, rme-8 and snx-1, (Chen et al. 2010b) at this point we do not know whether the lack of cell clearance defect in bec-1 mutants reflects a requirement for autophagy or for a bec-1-mediated retrograde transport function, or both. This point should be addressed in future experiments.

**Comparisons with mammals and yeast**

In yeast, two different Atg6/Vps30 complexes were found to function in autophagy and vacuolar protein sorting (Seaman et al. 1997; Kametaka et al. 1998). The two complexes differed only in two proteins: Atg14 and Vps38. The complex consisting of Atg14, Atg6/Vps30, Vps15, and Vps34 has been shown to have autophagy function, and the complex consisting of Vps38, Atg6/Vps30, Vps15, and Vps34 was shown to be specific for vacuolar protein sorting, more specifically endosome to Golgi retrograde trafficking (Burda et al. 2002). Thus, all the subunits except for Atg14 and Vps38 are shared between the two complexes. The counterparts for these two complexes had not been found in other organisms, and orthologs for either Atg14 or Vps38 in higher eukaryotes were seemingly lacking, leading to the assumption that these molecular complexes only existed in yeast. Recently, using highly sensitive methods of purification, mammalian proteins were identified that interact with Beclin 1, the mammalian ortholog of Atg6/Vps30. In fact, three distinct Beclin1 complexes have now been described and orthologs of yeast Atg14, and Vps38 have been found (Matsunaga et al. 2009; Zhong et al. 2009). The human ortholog of yeast Atg14 is Atg14L, and the human ortholog of Vps38 has been proposed to be Uvrag, since Uvrag primarily localizes to late endosomes, and shows weak homology with yeast Vps38 (Itakura and Mizushima 2009). A complex consisting of Beclin1, hVps34, hVps15, and Atg14L, functions in early autophagosome formation. Another complex consisting of Uvrag, Beclin1, hVps34, and hVps15 appears to act in endosome to Golgi retrograde trafficking as well as in the fusion of lysosomes and autophagosomes. This is consistent with our findings.
A third complex consists of Rubicon, Uvrag, Beclin 1, hVps34, and hVps15, where Rubicon negatively regulates the autophagosome maturation process, as well as endocytic trafficking (Matsunaga et al. 2009; Zhong et al. 2009). As Rubicon protein localizes to endosomes and lysosomes, it may be directly involved in the regulation of membrane fusion processes of endosomes/lysosome and autophagosomes. Uvrag has the capacity to bind to the Class C VPS complex, which is involved in the fusion process of autophagosomes and in endocytosis (Liang et al. 2008a; Liang et al. 2008b). In tissue culture experiments, the knockdown phenotypes of Atg14L and Rubicon are different, indicating that Beclin 1 has multiple roles in autophagy through the formation of different complexes. Orthologs to Uvrag and Rubicon have yet to be found in *C. elegans* by sequence homology, although an ortholog to Uvrag does exist in Drosophila (Melendez and Neufeld 2008). Our results support the idea that at least two BEC-1 complexes exist in *C. elegans* which function in autophagy and retrograde transport. Significant future work will be required to better understand the mechanisms by which BEC-1 activity functions in development, longevity, and resistance to pathogens, and if these require BEC-1 autophagy function or its endocytic function, or both.

**Materials and Methods**

**C. elegans strains:** Standard procedures were used to culture *C. elegans* worms (Brenner, 1974). All strains were grown at 20°C, unless otherwise stated. The wild-type *C. elegans* strain N2 and the following mutant alleles were used: LGIV, *bec-1(ok691)* and *bec-1(ok700)* provided by the Knockout consortium, *unc-5(e53) jcls1 (Is[jam-1::GFP])*(gift from T. Schedl, University of St. Louis). Strains QU and QU were constructed by crossing *unc-5(e53) jcls1/+ males to bec-1;Ex[the The transgenes used: *pwls50[imp-1::gfp unc-119(+)]* (Treusch et al. 2004), *pwls72[P_vha-6::gfp::rab-5, unc-119(+)], pwls87[P_vha-6::gfp::rme-1, unc-119(+)]* (Hermann et al. 2005; Chen et al. 2006), *pwls170[P_vha-6::gfp::rab-7, unc-119(+)]* (Chen et al. 2006) *P_vha-6::gfp::rab-10, unc-119(+)], pwls69[P_vha-6::gfp::rab-11, unc-119(+)]* (Chen et al. 2006), *pwls90[P_vha-6::hTIR::gfp], pwls112[P_vha-6::hTAC::gfp, unc-119(+)]* (Chen et al. 2006), *izEx1[lgg-1_promoter::RFP::LGG-1]* (Meléndez et al. 2003), *izEx5[pAy39.1, bec-1_promoter::BEC-1::RFP]* (Rowland et al. 2006), *izEx6[pBEC-1, pTG96], P_vha-6::RME-8::GFP, unc-119(+)], p[snx-1::GFP::SNX-1], izEx [P_vha-6::MIG-14::GFP, unc-119(+)]* (Shi et al. 2009), *pwls61 [GFP::cav-1, unc-119(-)]* (Sato et al. 2006), and *bcls39[P(lim-7)ced-1::GFP, lin-15(+)]* (Zhou et al. 2001b).
**Molecular analyses:** To characterize the nature of the *bec-1* transcript present in *bec-1(ok700)* mutant animals, total RNA was isolated and subjected to RT-PCR using oligo dT primers. Determination of the DNA sequence for the RT-PCR product revealed a short transcript containing exons 1-4 resulting in a frameshift that removes the remainder of the open reading frame after exon 4.

**Transgenes:** For rescue experiments of *bec-1* mutants, a 14.0 Kb PCR fragment was amplified using wild-type genomic DNA as a template. This fragment contains the entire *bec-1(+)* gene plus 10 Kb of flanking 5’ DNA and 970 bp of downstream 3’ DNA. This PCR fragment was directly injected into *bec-1(ok691)/nT1* mutant animals at 40µg/ml together with the ubiquitously expressed cotransformation marker pTG96 (SUR-5::GFP) at 100 µg/ml. The rescuing BEC-1::RFP, pAR 39.1, (Rowland et al. 2006) was injected at 20 µg/ml along with the cotransformation marker pRF4 (rol-6(d)) at 100 µg/ml, and Rol lines were established.

**Endocytosis assays:** To investigate endocytosis at the basolateral membrane of the intestine, young adult hermaphrodites were injected into the pseudocoelomic space with 0.1 mg/ml Texas Red-conjugated BSA (TR-BSA from Sigma, St. Louis, MO), as previously described (Zhang et al. 2001). Briefly, TR-BSA is dissolved in egg salts (118mM NaCl, 48 mM KCl, 2mM MgCl2, 2 mM CaCl2, 10mM HEPES, pH 7.4). We injected TR-BSA into the pseudocoelomic space in the pharyngeal region of adult worms that were immobilized on a dried agarose pad immersed in oil. Injected animals were rehydrated with M9 buffer and transferred to seeded NGM plates. At different time points, animals were mounted on agarose pads with a 1-2 µl drop of M9 containing 25mM sodium azide to anesthetize them and view them on the confocal microscope. Animals were soaked in the same solutions to investigate endocytosis at the apical membrane.

**RNA interference:** dsRNA-mediated gene interference experiments were performed by feeding bacteria expressing the dsRNA to larval L4 stage individuals, and scoring their progeny, unless described otherwise. L4 larvae were placed on plates containing NGM agar with 5mM IPTG and HT115 (DE3) bacteria carrying double stranded RNA expression constructs and allowed to lay eggs for 24 hours at 20°C, except in the
case of GFP::CAV-1 expressing animals, which were incubated at 25°C. P0s were transferred every day for 3 days. F1 progeny were raised in the plates containing dsRNA bacteria and scored as 1-day old adults. To determine if there is a lack of GFP::CAV-1 degradation, the second generation of RNAi animals were also assayed. In all experiments with bec-1 and vps-34 RNAi, the accumulation of vacuoles was monitored as the most obvious phenotype to determine that RNAi had been successful. RNAi clones were obtained from the Ahringer and Vidal genomic RNAi libraries (Kamath and Ahringer 2003) (a gift from Dr. Malene Hansen).

**Epifluorescence microscopic analyses:** We used a Leica TCS-SP5 laser-scanning confocal microscope to analyze the subcellular localization of fluorescent markers of endocytic and lysosomal compartments as well as endocytosis markers in mutant and wild-type animals. Images on the confocal were collected by a PMT (photomultiplier tube) detector, converted to Tiff format, and cropped using Adobe Photoshop CS3. Quantifications were performed by counting punctate-positive structures in the wild-type and mutant intestines using Image J Software (National Institutes of Health, Bethesda, MD). To quantify intensity of images, Metamorph software ver 6.3r2 (Universal Imaging) was used.

Live worms were mounted on 2% agarose pads with 10 mM sodium azide. (Shaham 2006) For GFP::RAB-5, and RME-8::GFP puncta quantification, only structures smaller than 0.3 microns were counted in either wild-type or mutant animals. For MIG-14::GFP- positive puncta quantification, only structures smaller than 0.2 microns were counted in either wild-type or mutant animals. For quantification of GFP::SNX-1 and GFP::RAB-7, GFP-positive puncta are counted manually. Images taken with the DAPI filter were used to identify broad-spectrum intestinal autofluorescence caused by lipofuscin-positive lysosome-like organelles. (Clokey and Jacobson 1986; Hermann et al. 2005)

**Electron microscopy:** Animals were prepared for electron microscopic analysis as described previously. (Meléndez et al. 2003; Melendez et al. 2008) Briefly, L4 animals were packed into the metal planchette using an excess of bacteria to avoid any empty space surrounding the animals. The animals were then frozen using a Bal-Tec HPM 010 high pressure freeze apparatus Bal-Tec HPM 010 and freeze substituted in 1% osmium tetroxide in acetone beginning at -90°C. The samples were then infiltrated with epoxy resin
and heat cured. These plastic blocks were thin sectioned, post-stained with uranyl acetate and lead citrate, and then examined by electron microscopy.

**Quantitation of Cell Corpses**

The increase in the number of cell corpses was measured using the CED-1::GFP marker. Adult hermaphrodites that 36h post larval L4 stage animals were scored. Quantification of germ cell corpses in wild-type animals after treatment with control RNAi, *unc-5/Atg1, bec-1, vps-34,* and *atg-18* RNAi animals. Data are derived from three experiments observing adults, 36 hours post larval L4 stage. Data were compared by Analysis of variance (ANOVA) and five two-sample t-tests between control strain and the five treatments; 30 animals were analyzed for each treatment and control. Analysis of variance (ANOVA) indicated that there are significant differences between treatments and the t-tests found significant differences between the control treatment and all other treatments except *atg-7* RNAi (*P* < 0.001). Threshold for significance (alpha) in the t-tests was *P* < 0.01 using a "Bonferroni correction" for multiple corrections.

**Apoptotic Cell Clearance Assay**

Germ cell corpses in the adult hermaphrodite gonads were scored under the Nomarski DIC microscope by their highly refractile appearance. (Yu et al. 2006) A Zeiss ApoTome microscope equipped with Time Lapse software was employed to capture apoptotic nuclei. In addition, serial Z-section images of regions of the hermaphrodite gonad were recorded at 0.5 μm intervals every 5 minutes. Movement of cells and focus were closely monitored to ensure that the region of the gonad being recorded did not change. Animals were closely monitored for viability during the recording. Adult hermaphrodites 36h- 48h post larval L4 stage animals were scored.
**Acknowledgements:** We thank Drs. Johnny Fares, Hannes Bülow, Malene Hansen, Tim Schedl, as well as the *C. elegans* Knockout Consortium and CGC, for strains and reagents. We are grateful to Dr. Areti Tsiola and Dr. Nathalia Holtzman for assistance with the imaging and Image J program, and to the Core Facility for Imaging, Cellular & Molecular Biology at Queens College. We are thankful to Drs. Cathy Savage-Dunn, Hannes Bülow, and Iva Greenwald for critical reading of this manuscript and for helpful discussions. This work was supported in part by grants from the NIH/NIA (AG024882-04S1) to Dr. Monica Driscoll (Rutgers University) and A.M., and from the NSF, Research Initiation Grant 0818802 to A.M. A.M. is an Ellison Medical Foundation New Scholar in Aging (AG-NS-0521-08). D.H.H is supported by NIH RR 12596. B.D.G. is supported by NIH Grant GM067237.

**Figure Legends:**

**Figure B1: bec-1 gene, protein, mutations and phenotypes** (A) At the top, the genomic structure of the bec-1 gene encompassing 7 exons, and encoding a 375 amino acid protein, shown below. In the middle, two deletions of bec-1 as provided by the *C. elegans* Gene Knockout Consortium. *ok691* deletes most of the bec-1 open reading frame including the start ATG and exons 1-6, thus it is a molecular null. *ok700* deletes exons 4-6 of bec-1 and renders the mRNA out of frame for exon 7. A cDNA was sequenced after RNA was extracted from an *ok700* lysate, which shows that there is bec-1 mRNA transcribed in *ok700* mutants that lacks the evolutionarily conserved domain of BEC-1 required for binding VPS-34. (B) Both deletions display an adult lethal phenotype with a striking accumulation of vacuoles (marked by black arrows in B, middle, right panel). *ok691* mutants (B, middle panel) segregating from a heterozygous parent (B, top panel). bec-1 homozygous mutant, either *ok691* or *ok700*, appear superficially wild-type during early larval stages but become increasingly uncoordinated during later larval stages and display molting problems (white arrow in B, middle, left panel). Rescue of the bec-1 phenotypes including uncoordination, the accumulation of vacuoles, and lethality was observed with a transgene containing the bec-1 wild-type genomic sequences and a ubiquitously expressed SUR-5::GFP marker (B, bottom, left panel) or a BEC-1::RFP construct (B, bottom, right panel). (C) Animals that carry the rescuing bec-1 genomic extrachromosomal array (*Ex[bec-1(+), SUR-5::GFP]*) (left bottom panel) are rescued for the bec-1 phenotype. Animals that have lost the array early in development are completely devoid of the GFP marker.
expression (C, right bottom panel) and lack the maternal and zygotic bec-1 expression. A hatched line delineates the periphery of the embryo (C, right bottom panel). These animals die during embryogenesis with an increase in the number of visible apoptotic nuclei (marked by black arrow heads, C top right panel).

**Figure B2: bec-1 mutants display defects in endocytosis.** The detection of the phosphoinositides PI3P, PIP2, and PIP3 in the intestines of wild-type (A, B, C), bec-1(ok691) mutants (A′, B′, C′), animals is shown. Scale bars indicate 10 µm. In wild-type animals (A), PI3P is enriched in the apical membrane with weak basolateral labeling. In bec-1(ok691) (A′) mutants very low and diffused expression of PI3P was observed. PIP2 expression is not affected in bec-1(ok691) mutants (B′) when compared to wild-type animals (B). The PIP3 reporter labels apical membrane and strong basolateral expression in wild-type animals (C). The localization of PIP3 in bec-1(ok691) animals (C′) was similar to that of wild-type animals (C). Micrographs of LMP-1::GFP positive lysosomes in coelomocytes 24 hours after the injection of Texas Red BSA (a fluid phase marker for endocytosis) into the body cavity (pseudocoelom). A hatched line demarcates the periphery of the coelomocytes. The red dye accumulates in lysosomes (LMP-1 positive) localized in the coelomocytes of the wild-type animals (D), but fails to accumulate in the lysosomes (LMP-1 positive) of bec-1(ok691) mutants (D′). Micrographs of LMP-1::GFP positive lysosomes in coelomocytes after one hour of soaking in Texas Red BSA (a fluid phase marker for endocytosis). The red dye accumulates in lysosomes (green) localized in the coelomocytes of the wild-type animals (E), but fails to accumulate in the lysosomes (green) of bec-1 mutants (E′). Electron microscopy of an N2 wild-type (F, G) and bec-1(ok691) mutant young adults (H, I). (F) Low power electron micrograph of wild-type adult intestine in cross-section, fixed by high pressure freezing. Most of the basal membrane shows few signs of endocytosis, although there is some membrane infolding (black arrow in F) and a few small vesicles, particularly near the lateral membrane border between two intestinal cells on the extreme left edge of the tissue. Higher power view of adult wild-type intestine shows a few examples of membrane infolding (black arrows in G) representing a local zone where endocytosis is active. It is notable that the infolded membranes are rather large, and there are almost no vesicles nearby in the cytoplasm. In contrast, chains of vesicles (white arrows in I) and extensive infolded membranes seen along the basal pole in the bec-1 mutant (H and I). Thus endocytotic events must be cleared relatively quickly in the wild-type intestine. In bec-1 mutant intestines, defective
enlarged endosomes (marked by white arrow head in H) are seen that correspond to abnormal vacuoles of different sizes and contain either membranes or condensed material. Fig. 2(I) is a close up of the region marked in (H) and it shows enlarged vacuoles and a high number of early endosomes that appear to be fusing in some cases (black arrowhead in I). This phenotype is never seen in the intestines of wild-type animals (F or G).

**Figure B3: bec-1 mutants display a defect in retrograde transport.** Confocal images in a wild-type background are shown for GFP::RAB-5 (A), GFP::RAB-7 (B), LMP-1::GFP (C), GFP::hTfR (D), MIG-14::GFP (E), GFP::RME-8 (F), GFP::SNX-1 (G), GFP::VPS-35 (H). Confocal images of *bec-1(ok691)* mutants are shown for GFP::RAB-5 (A'), GFP::RAB-7 (B'), LMP-1::GFP (C'), GFP::hTfR (D'), MIG-14::GFP (E'), RME-8::GFP (F'), GFP::SNX-1 (G'), GFP::VPS-35 (H'). (B) GFP::RAB-7 positive puncta (marked by yellow arrow) labels maturing endosomes and late endosomes (marked by yellow arrowhead. *bec-1(ok691)* mutants accumulate maturing endosomes marked with GFP::RAB-7, and display a decrease in late endosomes marked with GFP::RAB-7 (compare B and B'; See Figure 2I). White arrowheads indicate enlarged intestinal endosomes (abnormal vacuoles) labeled by GFP::RAB-5 and GFP::RAB-7. In *bec-1(ok691)* animals, LMP-1::GFP also accumulates in the membrane of the enlarged endosomes (C'; marked by white arrowheads). In wild-type animals (D), the basolateral plasma membrane and basolateral endocytic compartments are labeled by hTfR::GFP (human transferrin receptor). The accumulation of hTfR::GFP is no affected in *bec-1(ok691)* mutants (D'), and the recycling endosome cargo hTfR::GFP does not accumulate in the enlarged endosomes (abnormal vacuoles) of *bec-1(ok691)* mutants. Confocal images of the worm intestine expressing the GFP-tagged endocytic transmembrane cargo marker MIG-14::GFP in wild-type (E), and *bec-1(ok691)* (E') animals. The retromer-dependent cargo protein Wntless MIG-14::GFP normally localizes basolaterally in the intestine and colocalizes with the early endosomal marker RAB-5. (Shi et al. 2009) In *bec-1* mutants, MIG-14::GFP transmembrane cargo protein accumulates in the enlarged endosomes (abnormal vacuoles; E'). We observe a decrease in the number of MIG-14::GFP positive puncta (I). The retromer subunit RME-8::GFP (F'), GFP::SNX-1 (G') and GFP::VPS-35 (H'), do not accumulate in the enlarged intestinal endosomes (abnormal vacuoles). A decrease in the number of GFP::RME-8 positive puncta is observed in *bec-1(ok691)* mutants (F'), and its intensity is markedly decreased. For all images,
scale bars represent 10 µm. Quantification of endosome number as visualized by the positive labeling with endocytic markers is shown in (I). In the quantification of endosomal compartments, we see no significant difference in RAB-5 early endosomes, we observe an increase in RAB-7 maturing endosomes, and a dramatic decrease in RAB-7-positive late endosomes. In addition, we observe a decrease in MIG-14 as well as RME-8 labeled compartments, and a slight increase in SNX-1 as well as in VPS-35 labeled compartments. Error bars represent standard deviation from the mean (n=30 each, 10 animals of each genotype were sampled in three different regions of the intestine). In J and K panels, we analyze whether lysosomal degradation of GFP::MIG-14 occurs in bec-1 mutant animals. RNAi mediated depletion of lysosome biogenesis protein CUP-5/mucolipin1 restored the number and the intensity of MIG-14::GFP positive puncta (J and Fig. S5). After cup-5 RNAi, we see a 4X increase in the number of MIG-14 positive puncta in bec-1 mutants, when compared to control RNAi, indicating that there is lysosomal processing of the MIG-14 cargo in bec-1 mutants (J, left panel). Similarly, we observe a significant increase in the intensity of MIG-14::GFP in bec-1 mutants after cup-5 RNAi (J, right panel). Asterisks indicate a significant difference in the one-tailed Student’s t-test (* P<0.05, ** P<0.005, *** P<0.0005, n.s.: not significant).

**Figure B4: BEC-1 colocalizes with RME-8.** Panels show representative images of intestinally expressed RFP-tagged BEC-1 (red, A, A’) and GFP-tagged RME-8 (green, B, B’) in wild-type intact living animals. Autofluorescent lysosome-like organelles are shown in blue (+ DAPI filter, D). White arrowheads indicate colocalization of the BEC-1::RFP and RME-8::GFP signals (C), observed as the yellow fluorescence. The blue autofluorescence is also shown with BEC-1::RFP (A’) and with GFP::RME-8 (B’) and in the merger of both in C’. The colocalized BEC-1::RFP and GFP::RME-8 signals are found adjacent to the autofluorescent lysosomes which can be visualized with the DAPI filter (C’). A close-up of C’ is shown in C”. Arrowheads in yellow show BEC-1::RFP that is not associated with RME-8::GFP. Magnification is 630x in panels A-C’.

**Figure B5: bec-1 mutants display lack of cell corpse clearance.** (A) Micrograph of transmission electron microscopy (TEM) where poor degradation of apoptotic germ cell corpses is observed in bec-1 mutants. This TEM image was taken in a cross-section throughout the midbody. A rounded dying germ cell is shown with very dark cytoplasm and several large round vacuoles near the nucleus, which contains clumped
chromatin. The dying cell is completely wrapped by the somatic sheath cell (tinted in purple) in an early phase of apoptosis. The sheath cell also wraps the normal germ-line (bottom two cells in the panel), separating this mesodermal tissue from the intestine (top cell in the panel). (B) Histogram indicating the distribution of the duration of germ cell corpses before they are completely degraded. The y-axis indicates the percentage of germ cell corpses that lasted for the period of time indicated in the x-axis before clearance. n is the number of cell corpses analyzed. (C) Panels show representative images of CED-1::GFP positive labeled cell corpses in wild-type animals after treatment with control RNAi and RNAi against bec-1 and vps-34. (D) Quantification of germ cell corpses in wild-type animals after treatment with control RNAi, and RNAi against unc-51/Atg1, bec-1, vps-34, atg-7 and atg-18. Data derived from observing adults, 36 hours post larval L4 stage. Data were compared by unpaired t tests; 30 animals were analyzed for each experiment. Asterisks indicate a significant difference as a result from an analysis of variance (ANOVA). This analysis indicated that there are significant differences between all treatments and control, except for atg-7 RNAi ($P < 0.001$). Threshold for significance (alpha) in the t-tests was $P < 0.01$ using a "Bonferroni correction" for multiple corrections.
Figure B1

A.

B.

C.
Figure B2

In the images, wild-type and ok691 strains are compared for three phosphoinositide modifications: PI(3)P, PI(4,5)P2, and PI(3,4,5)P3. The images show the distribution and intensity of these modifications across the cell membranes.

- Panel A: Wild-type PI(3)P
- Panel A': ok691 PI(3)P
- Panel B: Wild-type PI(4,5)P2
- Panel B': ok691 PI(4,5)P2
- Panel C: Wild-type PI(3,4,5)P3
- Panel C': ok691 PI(3,4,5)P3

Additional panels (D-D') and (E-E') show the distribution of these modifications at higher magnification. The scale bar in the images indicates 10 µm.

Panel F and G: Wild-type and ok691 tissue sections, respectively, showing cellular structures. Panel H and I: Enlarged views of the boxed areas from panels D and E, respectively, highlighting specific regions of interest.
Figure B5

A. [Image of a cell structure with labeling]

B. Bar graph showing % Germ cell corpses over Duration (min) for L4440(RNAi)(n=8) and bec-1(RNAi)(n=26).

C. Images showing CED-1::GFP expression in different RNAi conditions: wt(control RNAi), bec-1(RNAi), and vps-34(RNAi).

D. Bar graph showing No. of CED-1::GFP positive cells in the gonad for control, unc-51, bec-1, vps-34, alg-7, and alg-18 conditions.
### Table B1: Lack of zygotic \textit{bec-1} results in adult lethality

<table>
<thead>
<tr>
<th></th>
<th>Eggs</th>
<th>hatched</th>
<th>Reach adulthood</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{bec-1(ok691)/unc-5; jcls1}</td>
<td>325</td>
<td>97.8%</td>
<td>20.3% \textit{unc-5} homozygous</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>53.1% \textit{unc-5} heterozygous</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26.6% \textit{bec-1} homozygous</td>
</tr>
<tr>
<td>\textit{bec-1(ok700)/unc-5; jcls1}</td>
<td>270</td>
<td>94%</td>
<td>24.6 % \textit{unc-5} homozygous</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50 % \textit{unc-5} heterozygous</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25.4 % \textit{bec-1} homozygous</td>
</tr>
<tr>
<td>\textit{N2}</td>
<td>195</td>
<td>97%</td>
<td>100%</td>
</tr>
</tbody>
</table>

### Table B2: Lack of maternal and zygotic \textit{bec-1} results in embryonic lethality

<table>
<thead>
<tr>
<th>Aberrant Vacuole Phenotype</th>
<th>% of Animals that contain at least one vacuole</th>
<th>% of Animals that contain more than 10 vacuoles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{L3} (n=37)</td>
<td>\textit{L4} (n=39) n.s.</td>
</tr>
<tr>
<td>\textit{ok691}</td>
<td>70%</td>
<td>80%</td>
</tr>
<tr>
<td>\textit{ok700}</td>
<td>30% (n=40)</td>
<td>52% (n=40) n.s.</td>
</tr>
</tbody>
</table>

### Table B3: \textit{bec-1} mutants accumulate aberrant vacuoles

<table>
<thead>
<tr>
<th>\textit{ok691; Ex array}</th>
<th>+ GFP Hatched</th>
<th>-GFP Hatched</th>
<th>Adults</th>
<th>n= eggs counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>\text{Ex[BEC-1::RFP, pRF4]}</td>
<td>15.1%</td>
<td>0</td>
<td>100%</td>
<td>n=651</td>
</tr>
<tr>
<td>\text{Ex[T19E7.3, pTG96]}</td>
<td>38.6%</td>
<td>0</td>
<td>92%</td>
<td>n=185</td>
</tr>
</tbody>
</table>
Figure B6: Morphology of recycling endosomes is not affected in *bec-1* mutants. Confocal images of GFP::RAB-10 in wild-type (A) and *bec-1* animals (B), and GFP::ALX-1 in wild-type (C), and *bec-1* mutant animals (D). GFP::RAB-10 is normally localized to early endosomes and Golgi in the intestine (Chen et al., 2006) of wild-type animals (A). In *bec-1(ok691)* mutant animals GFP::RAB-10 does not label the enlarged endosomes and the GFP::RAB-10 expression and localization is similar to that of wild-type animals (B). GFP::ALX-1 is associated with recycling endosomes and multivesicular bodies, and promotes the recycling of basolateral cargo internalized independently of clathrin (Shi et al., 2007). In wild-type animals (C), GFP::ALX-1 is observed in small puncta in the basolateral region of the intestine. Although the puncta may appear slightly larger in *bec-1* mutant intestines (D), GFP::ALX-1 did not label the enlarged endosomes an the number of GFP::ALX-1 endosomes appeared similar to that of wild-type animals (C).
Figure B7: Degradation of CAV-1::GFP occurs in **bec-1** mutants. Localization of CAV-1::GFP was determined in **bec-1** (RNAi) (C) and **vps-34** (RNAi) (D) animals by confocal microscopy. CAV-1::GFP is normally degraded via clathrin and RAB-5-dependent endocytosis after fertilization (K. Sato et al., 2006). The degradation of CAV-1::GFP appeared normal in **bec-1** (RNAi), and **vps-34** (RNAi) treated animals, when compared to that of mock treated animals fed bacteria harboring the control empty RNAi vector (A and B). For each confocal CAV-1::GFP image (except **rab-5** (RNAi) animals) the Nomarski image is provided below (A’ for mock **L4440** RNAi treated, B’ for **bec-1** (RNAi) and C’ for **vps-34** (RNAi) treated animals. Other phenotypes associated with the knockdown of **bec-1** and **vps-34** function were observed (data not shown). We observed a lack of degradation in fertilized embryos of **rab-5** RNAi animals, an experiment performed in parallel (D). Magnification for images A-C is 200X, and for D, is 400X.
Figure B8: Lack of *bec-1* and *vps-34* affect the localization of MIG-14::GFP.
Representative images of the expression of the retromer cargo protein MIG-
14::GFP in wild-type animals (A,A',A''), *bec-1(ok691)* mutants (B,B',B''), *rme-
8(b1023)* mutants (C,C',C''), and animals that have been treated with RNAi against
*bec-1* (D,D',D'') and *vps-34* (E,E',E''). Left panels show the DIC Nomarski images,
center panels show the MIG-14::GFP, and the right panels show the merged image.
Scale bars represent 10µm. (F-G) Bar graphs indicate the average number of MIG-
14::GFP positive puncta, and MIG-14::GFP fluorescence intensity. The number of
MIG-14::GFP positive puncta is significantly decreased in animals treated with
RNAi against *bec-1* and similarly in *vps-34* RNAi. The fluorescence intensity of
MIG-14::GFP increased in animals treated with RNAi against *bec-1* or *vps-34*.
Asterisks indicate a significant difference in the on-tailed Student’s t-test (* P<0.05,
** P< 0.005, *** P<0.0005, n.s.: not significant).
Figure B9: RNAi to the lysosomal biogenesis *cup-5* increases MIG-14::GFP to nearly wild-type levels in the *bec-1* mutant animals. Representative images of animals that are wild-type (A,B) or *bec-1(ok691)* mutant (C,D). Animals were treated with control RNAi (A,C) or the lysosomal biogenesis gene, *cup-5*. Treatment with *cup-5* restores the levels of MIG-14::GFP in *bec-1* mutants to that of wild-type animals. Scale bars represent 10µm. The quantification for this experiment is on Fig. 3J.
Figure B10: RNAi against vps-34 decreases the number of RME-8::GFP positive puncta. Bar graphs indicate the average number of RME-8::GFP positive puncta. The number of RME-8::GFP positive puncta is significantly decreased in animals treated with RNAi against vps-34, but not affected in animals treated with RNAi against bec-1. However, a decrease in RME-8::GFP was observed in bec-1(ok691) and shown in Fig. 3I. We observed no effect in the number of RME-8 positive puncta in animals that were treated with RNAi against the autophagy genes unc-51/ATG1 or lgg-1/ATG8. Asterisks indicate a significant difference in the on-tailed Student’s t-test (* P<0.05, ** P<0.005, *** P<0.0005, n.s.: not significant).
Figure B11: The number clathrin GFP::CHC-1 positive endosomes is not affected in animals treated with RNAi against *bec-1* or *vps-34*. A significant increase in the intensity of GFP::CHC-1 was noted in animals that have treated with RNAi against *vps-34*, suggesting that clathrin accumulates in animals lacking *vps-34*. Asterisks indicate a significant difference in the one-tailed Student’s t-test (* P<0.05, ** P< 0.005, ***P<0.0005, n.s.: not significant).
Figure B12: *bec-1* loss of function increases the number and the size of GFP::SNX-1 positive puncta. Bar graphs indicate the average area of GFP::SNX-1 labeled puncta (A) and the number of GFP::SNX-1 positive puncta. The number of SNX-1:GFP positive puncta is significantly increased in *bec-1(ok691)* animals. A similar phenotype has been reported for *rme-8(b1023)* mutants (Shi et al., 2009). Representative images of this quantification are shown in Fig. 3G. Asterisks indicate significant difference in the one-tailed Student’s t-test (* P<0.05, ** P<0.005, *** P<0.0005, n.s.: not significant).
References:


Appendix C


Nicholas J. Palmisano\(^1,2\) and Alicia Meléndez\(^1,2\)*

\(^1\) Queens College-CUNY, Department of Biology, Flushing, NY, USA, \(^2\) The Graduate Center, The City University of New York, New York, USA

*, correspondence should be addressed to A.M., Alicia.Meléndez@qc.cuny.edu

Running title: Autophagy in *C. elegans*

**Acknowledgement:** This is a published chapter found in Cell Death Techniques: A Laboratory Manual, Courtesy of the Cold Spring Harbor Laboratory Press., available online Feb. 1, 2016, pdb.top070466. doi: 10.1101/pdb.top070466.
ABSTRACT

Autophagy is a dynamic and catabolic process that results in the breakdown and recycling of cellular components through the autophagosomal-lysosomal pathway. Many autophagy genes identified in yeast and mammals have orthologs in *C. elegans*. In recent years, gene inactivation, by RNAi and/or chromosomal mutations, has been useful to probe the functions of autophagy in *C. elegans*, and a role for autophagy has been shown in multiple processes such as, the adaptation to stress, longevity, cell death, cell growth control, clearance of aggregate prone proteins, degradation of P granules during embryogenesis, and apoptotic cell clearance. Here we discuss some of these roles and describe methods that can be used to study autophagy in *C. elegans*. Specifically, we summarize how to visualize autophagy in embryos, larva, or adults, how to detect the lipidation of LGG-1 by western blot, and how to inactivate autophagy genes by RNAi.

INTRODUCTION

Autophagy in *C. elegans*

Autophagy is a lysosomal-mediated pathway resulting in the degradation and recycling of long-lived proteins, protein aggregates, as well as damaged and old organelles (Klionsky 2004). It is highly conserved and has been shown to be a fundamental catabolic process in eukaryotes that is required for key developmental and pathological events. Autophagy was first described in mammals, through morphological studies of rat liver cells (Deter et al. 1967). However, it was in yeast where many autophagy genes (*atg*) were discovered, by screening for mutations that decreased the survival of yeast cells under starvation, as well as mutations that disrupted the cytoplasm-to-vacuole targeting (*cvt*) process (Tsukada and Ohsumi 1993b; Thumm et al. 1994; Harding et al. 1995; Harding 1996; Hutchins and Klionsky 2001; Klionsky et al. 2003).

The process of autophagy is composed of several distinct steps: formation of a phagophore (also referred to as an isolation membrane or preautophagosomal structure); elongation and closure of the phagophore to form the double membrane autophagosome; transport and fusion of the autophagosome with a lysosome; and finally, degradation of the autophagosomal contents, and recycling of degraded material (*FIG. 1*) (Mizushima 2007; Xie and Klionsky 2007; Nakatogawa et al. 2009a). In addition to fusing
with a lysosome, an autophagosome may also fuse with an endosome to form a hybrid organelle called the amphisome (Liou et al. 1997; Jing and Tang 1999a). When an amphisome or autophagosome fuses with a lysosome, it is referred to as an autophagolysosome (or an autolysosome).

The evolutionary conservation of autophagy genes between yeast and C. elegans allowed for the identification of genes that encode core components of the autophagic machinery in C. elegans, on the basis of genomic sequence homology (Table C1) (Meléndez et al. 2003; Melendez and Levine 2009). Genetic screens for mutations that disrupt the degradation of P granules, has recently discovered autophagy genes not previously identified in C. elegans on the basis of sequence homology, including: epg-1, the ortholog of yeast ATG13, and epg-8, the ortholog of yeast ATG14 (Table C1) (Tian et al. 2009b; Yang and Zhang 2011). Although, the similarities between S. cerevisiae, mammals, and C. elegans autophagy proteins suggest that the molecular mechanisms of autophagosome formation may be conserved (FIG. C1 and Table C1) (Melendez and Levine 2009), genes recently identified in C. elegans that do not have a yeast ortholog may indicate that autophagy involves more complex membrane dynamics in higher eukaryotes. It is important to uncover further details about the roles of autophagy genes in autophagosome formation and maturation in C. elegans, and the role of these genes in different settings where autophagy is required.

The role of autophagy genes in C. elegans development has emerged from studies using chromosomal mutations or RNA interference against autophagy genes. Chromosomal mutations exist for many of the autophagy genes found in C. elegans and many RNAi clones are available (Table C1).

**Autophagy in C. elegans Development and Aging**

**L1 arrest after starvation:** Autophagy plays a role mediating the developmental changes associated with survival during extracellular and/or intracellular stress, such as starvation (Levine and Klionsky 2004). In the absence of food, L1 larvae undergo a reversible developmental arrest and can survive for 1-2 weeks (Johnson et al. 1984). The insulin/IGF-1 signaling pathway, composed of the insulin-like/IGF-1 receptor daf-2 and the FOXO transcription factor daf-16, is involved in regulating L1 arrest triggered by starvation (Gems 1998; Baugh and Sternberg 2006; Fukuyama et al. 2006). Interestingly, reduced levels of autophagy have been shown to greatly decrease the survival of starved L1 larvae, emphasizing the importance of
Figure C1: Autophagy in *C. elegans*. A. The process of autophagy has been delineated by studies in yeast and mammalian cells. We presume that induction of autophagy begins with the activation of UNC-51. B. Autophagosome formation requires the integral protein ATG-9, thought to contribute membrane to the developing autophagosome. C. Nucleation requires the Class III PI3K complex, which recruits downstream autophagy proteins to the isolation membranes (IM) in mammals or pre-autophagosomal structure (PAS) in yeast, through the production of PI3P (light purple). D. Two conjugation complexes (LGG-1 and ATG-12) are required for elongation of the isolation membranes and completion of the developing autophagosome. LGG-1 conjugated to phospatidylethanolamine (PE, red) binds to both the inner and outer membranes of the autophagosome. LGG-1 also has the ability to bind to the autophagic adaptor proteins, such as SQST-1 which bind poly-ubiquitinated aggregates. E. The complete autophagosome eventually fuses with the lysosome leading to the degradation of cargo within the autophagosome.
<table>
<thead>
<tr>
<th>C. elegans Atg gene</th>
<th>Allele</th>
<th>Yeast/ Mammalian ortholog</th>
<th>Phenotype in C. elegans</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-363&lt;sup&gt;R&lt;/sup&gt;</td>
<td>h96</td>
<td>TOR1,2/mTOR</td>
<td>Let, LL</td>
<td>(Brown et al. 1994; Noda and Ohsumi 1998; Vellai et al. 2003; Jia et al. 2004; Hansen et al. 2007; Hansen et al. 2008a)</td>
</tr>
<tr>
<td>unc-51&lt;sup&gt;R&lt;/sup&gt;</td>
<td>e369</td>
<td>ATG1/ULK1/2</td>
<td>Unc, AbD, Pg, Egl</td>
<td>(Hedgecock et al. 1985; Ogura et al. 1994; Matsuura et al. 1997; Kuroyanagi et al. 1998; Meléndez et al. 2003; Zhang et al. 2009)</td>
</tr>
<tr>
<td>epg-1&lt;sup&gt;R&lt;/sup&gt;</td>
<td>bp414</td>
<td>ATG13/Atg13</td>
<td>Dv, Pg</td>
<td>(Funakoshi et al. 1997; Chan et al. 2009; Tian et al. 2009a)</td>
</tr>
<tr>
<td>bec-1&lt;sup&gt;R&lt;/sup&gt;</td>
<td>ok691</td>
<td>ATG6, VPS30/ beclin 1</td>
<td>Let, AbD, St, SL, Pg pQ</td>
<td>(Seaman et al. 1997; Kametaka et al. 1998; Kihara et al. 2001; Meléndez et al. 2003; Takacs-Vellai et al. 2005; Jia and Levine 2007; Hansen et al. 2008a; Zhao et al. 2009b; Ruck et al. 2011)</td>
</tr>
<tr>
<td>let-512/ vps-34&lt;sup&gt;R&lt;/sup&gt;</td>
<td>h797</td>
<td>VPS34/Vps34</td>
<td>Let, SL, Pg</td>
<td>(Per O. Seglen 1982; Volinia et al. 1995; Roggo et al. 2002; Zhao et al. 2009b; Ruck et al. 2011)</td>
</tr>
<tr>
<td>ZK930.1&lt;sup&gt;R&lt;/sup&gt;</td>
<td>ok3132</td>
<td>VPS15/p150</td>
<td>ND</td>
<td>(Panaretou et al. 1997; Kihara et al. 2001; Kovács et al. 2003)</td>
</tr>
<tr>
<td>epg-8&lt;sup&gt;R&lt;/sup&gt;</td>
<td>bp251</td>
<td>ATG14/Atg14L, Barkor</td>
<td>Dv, Pg</td>
<td>(Kihara et al. 2001; Obara et al. 2006; Sun et al. 2008); (Fan et al. 2011a; Yang and Zhang 2011)</td>
</tr>
<tr>
<td>epg-6</td>
<td>bp242</td>
<td>-/WIPI4</td>
<td>Dv, Pg</td>
<td>(Lu et al. 2011b)</td>
</tr>
<tr>
<td>epg-3&lt;sup&gt;R&lt;/sup&gt;</td>
<td>bp405</td>
<td>-/VMP1</td>
<td>Dv, Pg</td>
<td>(Tian et al. 2010)</td>
</tr>
<tr>
<td>epg-4&lt;sup&gt;R&lt;/sup&gt;</td>
<td>bp425</td>
<td>-/EI24, PIG8</td>
<td>Dv, Pg</td>
<td>(Tian et al. 2010)</td>
</tr>
<tr>
<td>atg-3</td>
<td>bp412</td>
<td>ATG3/Atg3</td>
<td>Pg</td>
<td>(Tanida et al. 2002a; Zhang et al. 2009)</td>
</tr>
<tr>
<td>atg-4.1*&lt;sup&gt;R&lt;/sup&gt;</td>
<td>tm3949</td>
<td>ATG4/Atg4</td>
<td>Pg</td>
<td>(Kirisako et al. 2000; Tanida et al. 2004; Zhang et al. 2009)</td>
</tr>
<tr>
<td>atg-4.2*&lt;sup&gt;R&lt;/sup&gt;</td>
<td>tm3948</td>
<td>ATG4/Atg4</td>
<td>ND</td>
<td>(Kirisako et al. 2000; Tanida et al. 2004; Zhang et al. 2009)</td>
</tr>
<tr>
<td>atg-5</td>
<td>bp545</td>
<td>ATG5/Atg5</td>
<td>ND</td>
<td>(Mizushima et al. 1998a; Mizushima et al. 2001; Tian et al. 2010)</td>
</tr>
<tr>
<td>atg-7&lt;sup&gt;R&lt;/sup&gt;</td>
<td>tm831</td>
<td>ATG7/Atg7</td>
<td>AbD, SL, Pg, pQ</td>
<td>(Kim 1999; Tanida et al. 2001; Meléndez et al. 2003)</td>
</tr>
<tr>
<td>lgg-1&lt;sup&gt;R&lt;/sup&gt;</td>
<td>bp500, tm3489</td>
<td>ATG8/LC3</td>
<td>Let, Dv, AbD, SL, Pg</td>
<td>(Kirisako et al. 2000; He et al. 2003a; Meléndez et al. 2003; Zhang et al. 2009; Alberti et al. 2010b)</td>
</tr>
<tr>
<td>lgg-2&lt;sup&gt;R&lt;/sup&gt;</td>
<td>-</td>
<td>ATG8/LC3</td>
<td>Let, Dv, AbD, SL, Pg</td>
<td>(Kirisako et al. 2000; He et al. 2003a; Meléndez et al. 2003; Zhang et al. 2009; Alberti et al. 2010b)</td>
</tr>
<tr>
<td>atg-10&lt;sup&gt;R&lt;/sup&gt;</td>
<td>bp588</td>
<td>ATG10/Atg10</td>
<td>ND</td>
<td>(Shintani et al. 1999; Mizushima et al. 2002; Meléndez et al. 2003; Tian et al. 2010)</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------</td>
<td>-------------</td>
<td>-----</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>lgg-3&lt;sup&gt;R&lt;/sup&gt;</td>
<td>gk1857</td>
<td>ATG12/Atg12</td>
<td>SL, Pg</td>
<td>(Mizushima et al. 1998a; Meléndez et al. 2003; Hars et al. 2007a)</td>
</tr>
<tr>
<td>atg-16.1&lt;sup&gt;R&lt;/sup&gt;</td>
<td>-</td>
<td>ATG16/Atg16L1</td>
<td>ND</td>
<td>(Kuma et al. 2002; Mizushima et al. 2003; Tian et al. 2010)</td>
</tr>
<tr>
<td>atg-16.2&lt;sup&gt;R&lt;/sup&gt;</td>
<td>ok3224</td>
<td>ATG16/Atg16L1</td>
<td>ND</td>
<td>(Kuma et al. 2002; Mizushima et al. 2003; Tian et al. 2010)</td>
</tr>
<tr>
<td>atg-2</td>
<td>bp576</td>
<td>ATG2/Atg2</td>
<td></td>
<td>(Shintani 2001; Wang et al. 2001a; Lu et al. 2011b)</td>
</tr>
<tr>
<td>atg-9&lt;sup&gt;R&lt;/sup&gt;</td>
<td>bp564</td>
<td>ATG9/Atg9</td>
<td></td>
<td>(Noda et al. 2000; Yamada et al. 2005; Reggiori 2006)</td>
</tr>
<tr>
<td>atg-18&lt;sup&gt;R&lt;/sup&gt;</td>
<td>gk378</td>
<td>ATG18/WIPI1/2</td>
<td>Let, AbD, Pg, pQ</td>
<td>(Barth 2001; Meléndez et al. 2003; Jia et al. 2007); (Polson et al. 2010; Tian et al. 2010)</td>
</tr>
<tr>
<td>epg-2</td>
<td>bp287</td>
<td>-/-</td>
<td>Pg</td>
<td>(Tian et al. 2010)</td>
</tr>
<tr>
<td>epg-5&lt;sup&gt;R&lt;/sup&gt;</td>
<td>bp450</td>
<td>-/ KIAA1632</td>
<td>Dv, Pg</td>
<td>(Tian et al. 2010)</td>
</tr>
<tr>
<td>sepa-1</td>
<td>bp402</td>
<td>-/-</td>
<td>Pg</td>
<td>(Zhang et al. 2009)</td>
</tr>
<tr>
<td>T12G3.1</td>
<td>ok2982</td>
<td>-/p62(SQSTM1)</td>
<td></td>
<td>(Tian et al. 2010; Lu et al. 2011b)</td>
</tr>
</tbody>
</table>

Let = Lethal; Unc = uncoordinated; Dv = Decreased viability of L1s during starvation; AbD = Abnormal Dauer; St = Sterile; LL = Long lifespan; SL = Short Lifespan; Pg = P granule accumulation; Egl = Egg laying defective; pQ = polyQ expansion susceptibility; ND = Not determined; * Paralogs in C. elegans; <sup>R</sup> RNAi clone available.
autophagy during early stages of development (Kang et al. 2007; Tian et al. 2009b; Alberti et al. 2010a; Tian et al. 2010; Lu et al. 2011b; Yang and Zhang 2011).

**Dauer development:** During the first larval molt, animals that are exposed to a limited food supply develop into an alternate L3 larval stage termed dauer (Albert et al. 1981). Dauer development is associated with morphological and behavioral changes that allow for survival under harsh conditions and stress (Cassada and Russell 1975b; Golden and Riddle 1984). The regulation of dauer development has been well characterized and requires the IGF-1/insulin-like, guanylyl cyclase, and TGF-β signaling pathways, as mutations in any of these pathways can result in a dauer constitutive phenotype (Daf-c) or a dauer defective phenotype (Daf-d) (Estevez et al. 1993; Thomas et al. 1993; Gottlieb and Ruvkun 1994; Ren 1996; Schackwitz 1996; Patterson et al. 1997b; Birnby et al. 2000; Inoue and Thomas 2000a; da Graca et al. 2004). Dauer development is associated with an increase in autophagy, which appears to be required for the cell remodeling associated with proper dauer formation (Meléndez et al. 2003).

**Longevity pathways:** In *C. elegans*, aging is controlled by multiple longevity pathways, such as insulin-like growth factor signaling, TOR signaling, dietary restriction, mitochondrial activity, and germline signaling (Antebi 2007). Recent genetic studies suggest that autophagy interacts with many of these longevity signals to regulate *C. elegans* aging (Meléndez et al. 2003; Hansen et al. 2008b; Toth et al. 2008; Lapierre et al. 2011). Insulin/IGF-1R/daf-2 mutants display an increase in autophagy, as detected by an increase in the number of punctate structures labeled by the autophagy marker, GFP::LGG-1, in hypodermal seam cells, a cell type commonly used to visualize autophagy in *C. elegans* (FIG. C2) (Meléndez et al. 2003; Hansen et al. 2008b). A reduction in autophagy during development, or only during adulthood, shortens the long lifespan of daf-2 mutants (Meléndez et al. 2003; Hars et al. 2007b; Hansen et al. 2008b). Reduced food intake without malnutrition, otherwise referred to as dietary restriction, occurs in eat-2 mutants (Avery 1993). These animals lack a nicotinic acetylcholine receptor specific to the pharynx, thereby exhibiting reduced pharyngeal pumping, and have an extended lifespan phenotype (Raizen et al. 1995; Lakowski and Hekimi 1998). Consistent with a role for TOR in dietary restriction, eat-2 mutants have reduced TOR signaling, display an increase in autophagy, and require autophagy for their long-lived phenotype (Jia and Levine
Figure C2: GFP::LGG-1 expression in hypodermal seam cells of daf-2(e1370) mutants. A. daf-2(e1370) mutants grown on OP50 E. coli, at 15°C, display a diffuse localization of GFP::LGG-1. B. daf-2(e1370) mutants grown on OP50 E. coli, at 25°C, display an increase in GFP::LGG-1 positive puncta (up to 12 GFP::LGG-1 positive puncta/seam cell) that represent early autophagic structures or autophagosomes. C. daf-2(e1370) mutants grown on control RNAi E. coli (transformed with empty vector, L4440), at 25°C, display the characteristic GFP::LGG-1 positive punctate structures. D. daf-2(e1370) mutants fed bec-1 RNAi, and raised at 25°C, display an increase in GFP::LGG-1 expression and large GFP::LGG-1 positive aggregates.
2007; Hansen et al. 2008b; Toth et al. 2008). The reduction in mitochondrial respiration in isp-1 mutants extends lifespan (Dillin et al. 2002; Lee et al. 2003a), and this phenotype is also dependent on autophagy (Toth et al. 2008). Finally, glp-1/Notch germline-less mutants induce autophagy, and require autophagy for lifespan extension (Lapiere et al. 2011). Interestingly, HLH-30, the ortholog of the mammalian TFEB transcription factor, is required for the lifespan extension associated with the longevity pathways described above, and also regulates autophagy (Lapiere et al. 2013a). In conclusion, autophagy is required as part of most longevity pathways in C. elegans, the only exception thus far being the longevity associated with a reduction in protein translation (Pan et al. 2007; Hansen et al. 2008b).

**Degradation of paternal mitochondria:** Directly after fertilization, autophagy is induced resulting in the elimination of spermatozoon specific organelles, including paternal mitochondria (Al Rawi et al. 2011; Sato and Sato 2011). Whether autophagy also acts in higher eukaryotes to degrade paternal mitochondria is not known, however, an increase in ubiquitination and the localization of LC3 near the sperm mid-piece at the point of entry, may suggest that this is the case in fertilized mouse zygotes (Al Rawi et al. 2011).

**Autophagy in apoptosis, necrosis and cell clearance:** Although autophagy has a role in homeostasis as an important pro-survival mechanism in response to stress, an excess in autophagy may result in cell death (Kang et al. 2007). Autophagy is also required for necrotic cell death, a type of cell death characterized by the loss of plasma membrane integrity (Toth et al. 2007; Samara et al. 2008). Additionally, similar to mammals, BEC-1, a component of the class III Phosphatidylinositol 3-Kinase (PI3K) complex (FIG. C1), interacts with the anti-apoptotic ortholog of Bcl-2, CED-9, suggesting cross-talk between autophagy and apoptosis (Takacs-Vellai et al. 2005; Erdelyi et al. 2011). Autophagy proteins have been shown to play a role in the proper degradation of apoptotic cell corpses in C. elegans, since in autophagy deficient animals, apoptotic cells are internalized, but not properly degraded (Ruck et al. 2011; Li et al. 2012a). Interestingly, rescue experiments indicate that autophagy genes are required within the engulfing cell to promote apoptotic cell degradation (Li et al. 2012a).
Detecting autophagy in C. elegans: Autophagy can be monitored by transmission electron microscopy (TEM), fluorescent image analysis of the GFP::LGG-1 reporter or other autophagy reporters (Table C2), and by western blot, evaluating LGG-1 lipidation. It should be noted that an increase in the number of autophagosomes does not necessarily reflect an induction of autophagy (Klionsky 2012), and is therefore important to distinguish between induction of autophagy, an increase in autophagic flux, and the accumulation of autophagosomes due to inefficient or blocked autophagy (Klionsky 2012). Usually, it is useful to infer the turnover of autophagosomes in the presence and absence of lysosomal degradation. In C. elegans, this may be achieved by RNAi knockdown of genes with lysosomal function, such as cup-5 (Kostich et al. 2000; Fares and Greenwald 2001b; Sun et al. 2011b), or the addition of inhibitors such as bafilomycin A1, or chloroquine, routinely used in mammalian cells, which have also been successful in C. elegans (Oka and Futai 2000; Ji et al. 2006; Pivtoraiko et al. 2010). Clearly, the use of multiple assays to verify an increase in functional autophagy is recommended. A comprehensive list of guidelines was recently reported (Klionsky et al., 2012). Here we describe four protocols for the basic study of autophagy in C. elegans: detection of autophagy using GFP::LGG-1, autophagy in embryos, western blotting to evaluate lipidation of LGG-1, and RNAi as a method to target the knockdown of autophagy genes.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Tissue Expression</th>
<th>Transgenes[^b]</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LGG-1</strong></td>
<td>Microtubule —associated protein-1/Ubiquitin-like protein</td>
<td>Intestine, Hypodermis, Muscle, Pharynx, Neurons, Vulva, Somatic Gonad, Germline</td>
<td>adls2122[Plgg-1::GFP::LGG-1; rol-6(su1006)]&lt;br&gt;izEx1[Plgg-1::GFP::LGG-1; rol-6(su1006)]&lt;br&gt;izEx5[Plgg-1::GFP::LGG-1; Podr-1::RFP]&lt;br&gt;vkEx1093[Pnxh-2::mCherry::LGG-1]&lt;br&gt;dkls399[Ppie-1::GFP::lgg-1, unc-119 (+)]&lt;br&gt;ls[Ppie-1::GFP::mCherry::LGG-1; unc-119(+)]&lt;br&gt;Ex[Plgg-1::DsRED::LGG-1; Pmyo-2::GFP]</td>
<td>(Meléndez et al. 2003; Kang and Avery 2007; Samara and Tavernarakis 2008; Gosai et al. 2010; Manil-Segalen et al. 2014b)</td>
</tr>
<tr>
<td><strong>LGG-2</strong></td>
<td>Ubiquitin-like protein</td>
<td>Hypodermis, Intestine, Vulva, Pharynx, Neurons, Muscle</td>
<td>RD108 Ex[Plgg-2::GFP::LGG-2; rol-6(su1006)]&lt;br&gt;RD217 unc119(ed3)III; Ex[unc-119(+); Ppie-1::gfp::mcherry::lgg-1]&lt;br&gt;VIG9 unc119(ed3)III; ls[unc-119(+); Plgg-2::gfp::lgg-2]</td>
<td>(Alberti et al. 2010b; Manil-Segalen et al. 2014b)</td>
</tr>
<tr>
<td><strong>DFCP1</strong></td>
<td>Double FYVE-Containing Protein</td>
<td>Head, Tail, Vulva, Neurons</td>
<td>bpl168[Pnfya-1::DFCP1::GFP; unc-76(+)]</td>
<td>(Derubeis et al. 2000; Cheung et al. 2001; Axe et al. 2008; Tian et al. 2010)</td>
</tr>
<tr>
<td><strong>ATG-16.1</strong></td>
<td>WD repeat-containing protein</td>
<td>Intestine, Head, Pharynx, Muscle, Neurons</td>
<td>[Patg-16.1::ATG-16.1::GFP; rol-6(su1006)]</td>
<td>(Zhang et al. 2013)</td>
</tr>
<tr>
<td><strong>ATG-16.2</strong></td>
<td>WD repeat-containing protein</td>
<td>Intestine, Head, Pharynx, Muscle, Neurons</td>
<td>[Patg-16.2::ATG-16.2::GFP; rol-6(su1006)]</td>
<td>(Zhang et al. 2013)</td>
</tr>
<tr>
<td><strong>ATG-9</strong></td>
<td>Integral Membrane Protein</td>
<td>Head, Tail, Vulva, Neurons</td>
<td>bpl1211[Pnfya-1::ATG-9::GFP; unc-76(+)]</td>
<td>(Noda et al. 2000; Lu et al. 2011b; Liang et al. 2012; Lin et al. 2013)</td>
</tr>
<tr>
<td><strong>EPG-1</strong></td>
<td>Atg13 homolog</td>
<td>Neurons, Pharynx, Muscle</td>
<td>bpl175[Pegp-1::EPG-1::GFP; rol-6(su1006)]</td>
<td>(Tian et al. 2009a)</td>
</tr>
<tr>
<td><strong>EPG-9</strong></td>
<td>Atg101 homolog</td>
<td>Intestine, Pharynx, Neurons</td>
<td>bpl1214[Pegp-9::EPG-9::GFP; unc-76(+)]</td>
<td>(Liang et al. 2012)</td>
</tr>
<tr>
<td><strong>BEC-1</strong></td>
<td>Coiled-Coil protein</td>
<td>Intestine, Hypodermis, Vulva, Neurons, Somatic Gonad</td>
<td>swEx520 [Pbec-1::BEC-1::GFP; rol-6(su1006)]&lt;br&gt;grEx129 [Pbec-1::BEC-1::mRFP; lin-15(+)]&lt;br&gt;Ex[Pced-1::mCherry::BEC-1; rol-6(su1006)]&lt;br&gt;Ex[Pegl1-::mCherry::BEC-1; rol-6(su1006)]</td>
<td>(Takacs-Vellai et al. 2005; Rowland et al. 2006; Ruck et al. 2011; Huang et al. 2012)</td>
</tr>
<tr>
<td>Gene</td>
<td>Function</td>
<td>Tissues</td>
<td>Strain/Transgene</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------</td>
<td>------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>SQST-1</td>
<td>p62/Autophagy adaptor protein</td>
<td>Hypodermis, Neurons, Intestine, Vulva, Muscle</td>
<td>bpl151[Psqust-1::SQST-1::GFP; unc-76(+)] (Hunt-Newbury et al. 2007; Pankiv et al. 2007; Tian et al. 2010)</td>
<td></td>
</tr>
<tr>
<td>SEPA-1</td>
<td>Autophagy adaptor protein</td>
<td>Intestine, Head, Tail</td>
<td>bpl131[Psepa-1::SEPA-1::GFP; unc-76(+)] (Zhang et al. 2009; Tian et al. 2010)</td>
<td></td>
</tr>
<tr>
<td>PGL-1</td>
<td>RNA-binding protein/P-granule component</td>
<td>P-granules, Intestine</td>
<td>bns1[Ppie-1::GFP::PGL-1; unc-119(+)] bns26[Pelt-2::PGL-1::GFP; Pmyo-2::mCherry] (Cheeks et al. 2004; Zhang et al. 2009; Updike et al. 2011)</td>
<td></td>
</tr>
</tbody>
</table>

¥ Tissue expression may vary depending on the specific promoter used
Φ Transgenes shown are those found in autophagy studies or those which may be beneficial in autophagy studies;
Additional transgenes may be available for each gene
Protocol 1

Detection of autophagy using GFP::LGG-1 as an autophagy marker

Nicholas J. Palmisano¹,² and Alicia Meléndez¹,²*

¹ Queens College-CUNY, Department of Biology, Flushing, NY, USA, ²The Graduate Center, The City University of New York, New York, USA

*, correspondence should be addressed to A.M., Alicia.Melendez@qc.cuny.edu, (718)997-4580

Abstract:

In yeast and mammals, Atg8/LC3 has been the marker of choice to detect autophagosomes (Mizushima 2004; Klionsky 2012). The phosphatidyl-ethanolamine (PE)-conjugated form of Atg8/LC3 is localized to the inner and outer membrane of the phagophore. During autophagosome maturation, Atg8/LC3 on the outer membrane is cleaved and recycled, whereas Atg8/LC3 bound to the inner membrane remains, as the autophagosome fuses with the lysosome (Huang et al. 2000; Tanida et al. 2005). As this is presumed to also occur in *C. elegans*, tagging LGG-1 with a fluorophore has become a widely accepted method to visualize autophagosomes. Fluorescent reporters of LGG-1 with GFP, DsRED, and mCherry have been used to monitor autophagosomes *in vivo* (Meléndez et al. 2003; Maiuri et al. 2007; Gosai et al. 2010). In *C. elegans*, two orthologs of Atg8/LC3 exist, LGG-1 and LGG-2. GFP::LGG-1 is expressed throughout development in multiple tissues such as the nervous system, muscle, intestine, pharynx, vulva, hypodermis, and somatic gonad (Melendez et al., 2003). Under normal growth conditions, GFP::LGG-1 displays a diffuse expression pattern throughout these tissues, while under conditions that induce autophagy, GFP::LGG-1 labels positive punctate structures and its overall expression increases (FIG. 2) (Meléndez et al. 2003; Kang et al. 2007; Lapierre et al. 2011).

An increase in GFP::LGG-1 positive punctate structures was observed in loss of function mutations in the insulin-like/IGF-1 pathway, using *daf-2(e1370)* (Melendez et al., 2003), and in germline-less animals using *glp-1(e2141)* (Meléndez et al. 2003; Lapiere et al. 2011). In addition, dietary restricted animals such as *eat-2(ad1116)* mutants also display an increase in the number of GFP::LGG-1 positive structures (Hansen et al. 2008b). The GFP::LGG-1 positive vesicles were shown, by electron microscopy analysis, to correlate with the formation of autophagosomes in *daf-2* mutants (Melendez, et al., 2003), making GFP::LGG-1 an acceptable marker for autophagosomes. Thus, we can test whether a gene under investigation is required for autophagy, by monitoring changes in the expression and localization of GFP::LGG-1 in animals that carry any of the above loss of function mutations (Meléndez et al. 2003; Hansen et al. 2008b; Lapiere et al. 2011). We can also test whether the gene under investigation affects any of the phenotypes noted above that have been shown to require autophagy, such as L1 survival during starvation, dauer development, longevity, and P granule degradation.
In addition to using the GFP::LGG-1 reporter, an antibody against LGG-1 has also been used to monitor endogenous levels of LGG-1 and the presence of autophagosomes in embryos (Chen et al. 2010a; Tian et al. 2010; Lu et al. 2011b; Yang and Zhang 2011). Wild-type embryos display a number of punctate structures that are positive for the LGG-1 antibody, starting at the 50-cell stage and lasting throughout embryogenesis. Prominent LGG-1 positive puncta formation occurs at the 100 cell stage (Tian et al. 2010). Loss of autophagy activity can result in diffuse staining or aggregation of endogenous LGG-1 as in atg-3 and epg-1/atg-13 mutants, respectively (Tian et al. 2010). Theoretically, an anti-GFP antibody may also be used to monitor autophagy in fixed embryos/cells expressing the GFP::LGG-1 transgene, as has been done in mammalian cells expressing GFP-LC3 (Tian et al. 2010; Klionsky 2012).

LGG-2, a paralog of LGG-1 important for the acidification and degradation of autophagosomes, has also been utilized to monitor autophagy in C. elegans (Alberti et al. 2010a; Manil-Segalen et al. 2014b). GFP::LGG-2 is expressed in similar tissues as GFP::LGG-1 and both lgg-1 and lgg-2 have been shown to act synergistically in mediating dauer formation and longevity (Alberti et al. 2010a). Although LGG-1 and LGG-2 proteins are similar in structure, under conditions that induce autophagy, they appear to localize differently. For example, L1 larvae display a punctate expression pattern for GFP::LGG-2, and yet a more ubiquitous expression pattern for GFP::LGG-1 under starvation conditions (Alberti et al. 2010a). However, lgg-1, but not lgg-2, was found to rescue the decreased survival associated with atg8–depleted yeast (Alberti et al. 2010a). Therefore, lgg-1 appears to be more functionally related to ATG8.

Materials:

Reagents:

2%-3% agarose (2-3 grams of agarose filled to volume with 100ml of sterile deionized water)
M9 Minimal Medium buffer <R>:
NGM agar plates <R>:
25mM Sodium Azide (NaN₃)

Equipment:

Fluorescent microscope (Zeiss AxioImager A1)
Microscope coverslips (1.22 mm X .13mm)
Microscope slides (75mm X 25 mm X .96mm)
Standard platinum wire pick

Strains:

QU1: izEx1 [P_{gg-1}::GFP::LGG-1] (Meléndez et al. 2003)
DA2123: adls2122[P_{gg-1}::GFP::LGG-1; rol-6(su1006)] (Kang et al. 2007)
bpls168[P_{nfy-1}::DFCP1::GFP; unc-76(+)] (Tian et al., 2010)
FR758: swEx520 [P_{bec-1}::BEC-1::GFP + rol-6(su1006)] (Takacs-Vellai et al., 2005)
bpls211[P_{nfy-1}::ATG-9::GFP; unc-76(+)] (Lu et al., 2011).

Graphic Flow Chart #1:
Method:

1. Allow ~30 gravid adults carrying the *Is[P_{gg-1::GFP::LGG-1}]* integrated transgene to lay eggs on standard OP50 seeded NGM agar plates and keep overnight at the desired temperature.

2. Following an overnight incubation that allows for many eggs to be laid, perform a wash off/hatch off protocol, where you remove all adults and larvae from each plate, using ~1 mL of sterile M9 buffer. At this point, only eggs will remain on the plates.

3. Using sterile M9 buffer, collect L1 progeny that have hatched within the first 1 - 2 hours, and transfer ~50 to 100 uL of synchronized L1 larvae to freshly seeded OP50 plates and incubate at the desired temperature. *The collection period may vary depending on how tightly synchronized you wish the animals to be. If using a dauer constitutive strain such as daf-2(e1370), incubate the L1 animals at 25°C for 48h to ensure all animals are able to enter the dauer stage.*

4. When animals reach the stage of interest, imaging of the animals can begin.

5. To image, prepare 2%-3% molten agarose. Apply a single drop of agarose onto a microscope slide and immediately place another microscope slide on top of the agarose drop to form a thin agarose pad.

5. Place ~2 uL of 25mM sodium azide (NaN₃) in the center of the agarose pad and submerge the desired number of animals into the solution. A cover slip should be applied immediately, to avoid the evaporation of the sodium azide solution. Wait until animals are completely anesthetized to image.

6. Using 63X or 100X magnification, quantify/analyze the number of fluorescent GFP::LGG-1 puncta found in the tissue of interest. In most cases, the quantification has been done in hypodermal seam cells. However, quantification can also be done in the intestine or pharyngeal muscles. *Animals should not be kept in NaN₃ for longer than 15 minutes.*

Data Analysis:

To evaluate an increase or decrease in the number of autophagosomes present in a sample, GFP::LGG-1 punctate structures can be quantified by manually counting fluorescent positive dots within a
cell, or by using image analysis programs such as ImageJ (Manil-Segalen et al. 2014a). These punctate structures have been counted in hypodermal seam cells (lateral row of cells arranged on each side of the body (FIG. C2)), as well as in 1-500 cell stage embryos (Manil-Segalen et al. 2014b). When analyzing the GFP::LGG-1 positive punctate structures, be very careful to note their size and frequency. Another tissue, where GFP::LGG-1 positive vesicles have been analyzed is in the pharynx and intestine (Kang et al., 2007; Lapierre et al., 2011). Manually counting GFP-positive dots can be more accurate, than using computer software, since imaging programs may inappropriately detect background fluorescence as positive punctate structures. However, manually counting GFP-positive punctate structures can provide challenges of its own, especially when large quantities are present in a cell, and/or if the fluorescent intensity of GFP is variable, making it difficult to distinguish each individual punctate structure. In such cases, it may be advisable to use both methods to acquire more accurate results, although, this may be somewhat tedious.

Whether deciding to use computer software programs or to manually quantify fluorescently labeled punctate structures, the parameters used during the quantification process should be clearly stated to ensure repeatability. For example, Alberti et al. 2010 used ImageJ to analyze punctate structures between .3 and 4 μm² in size and a circularity of 0 to 1. Additionally, if different sized positive puncta are observed within a sample, it may be best to group them according to their size. This is especially useful when dealing with the presence of GFP::LGG-1 positive aggregates, which may be associated with defective vesicles. Lastly, because cells display basal levels of autophagy, quantification of GFP::LGG-1 positive punctate structures should be noted on a per cell basis as opposed to the total number of cells containing puncta (Klionsky 2012). It is important to note, however, that the size of puncta should not be the only parameter used to measure autophagic activity (Klionsky 2012).

Although GFP::LGG-1 is a widely accepted marker for visualizing autophagy in C. elegans, caution should be used when evaluating changes in GFP::LGG-1 expression. For example, increased levels of GFP::LGG-1 puncta and/or their size may represent increased autophagic flux or decreased lysosomal activity (Klionsky 2012). Additionally, in mammalian cells, GFP::LC3 has been documented to associate with poly-ubiquitinated proteins both during autophagy and also independent of autophagy (Kuma et al. 2007; Pankiv et al. 2007; Shvets and Elazar 2008), which may also be true for GFP::LGG-1. Therefore, additional reporters (Table C2), alone or in combination with GFP::LGG-1, may be required to distinguish
between functional autophagosomes and cytosolic GFP::LGG-1 aggregates, which would be indicative of defective autophagosomes.

**Troubleshooting:**

**Problem:** Animals submerged in NaN₃ do not become anesthetized (step 6).

**Solution:** The concentration of NaN₃ may be too low. Remake the solution or increase the concentration as needed.

**Problem:** Sodium Azide evaporates before animals are anesthetized (step 6).

**Solution:** Use additional NaN₃ or prepare the slide using fewer animals. If animals are dehydrated, the anatomy will be affected.

**Problem:** L3 larvae hypodermal seam cells are not clearly visible at low and high magnifications during image acquisition (step 7).

**Solution:** Increasing the exposure time during acquisition may be required to clearly image seam cells that are difficult to visualize. It should be noted that the exposure time should be kept constant for all images acquired if planning to obtain quantifiable data.

**Related Techniques:**

GFP::LC3 fluorescence microscopy

Tandem mCherry-GFP-LC3 fluorescence microscopy

**Discussion:**

An increase in the number of autophagosomes may reflect autophagy induction, a block in the fusion between autophagosomes/amphisomes and lysosomes, or lack of degradation (Klionsky 2012). Therefore, additional reporters may be necessary to distinguish between all these possibilities (Table 2).

Other markers that can be used in *C. elegans* to monitor the various steps of autophagy include: BEC-1::GFP, DFCP1::GFP, and ATG-9::GFP (Table C2) (Takacs-Vellai et al. 2005; Tian et al. 2010; Lu et
Al. 2011b). ATG-9 is the only integral protein to date that is speculated to contribute membrane to the developing autophagosome (Noda et al. 2000). DFCP1 is a double FYVE domain-containing protein which binds to phosphatidylinositol 3-phosphate (PI3P) localized to ER derived early autophagosomes called omegasomes (Derubeis et al. 2000; Cheung et al. 2001; Axe et al. 2008). In conclusion, these additional markers may be more useful for visualizing and possibly quantifying early and late autophagosomes.

An alternative to using GFP::LGG-1 for monitoring autophagosomes includes the use of a tandem GFP::mCherry:: LGG-1 reporter, as was done previously with LC3 (Kimura et al. 2007; Pankiv et al. 2007; Manil-Segalen et al. 2014b). When analyzing a tandem reporter, colocalization of GFP and mCherry fluorescence may indicate a compartment that has not fused with the lysosome. As autophagosomes fuse with lysosomes, the GFP signal becomes sensitized to the acidic conditions of lysosomes, while the mCherry signal remains stable. Thus, the mCherry signal alone corresponds to amphisomes or autolysosomes (Kimura et al. 2007; Manil-Segalen et al. 2014b).

Recipes:

M9 Minimal Medium buffer:

22mM KH₂PO₄
22mM Na₂HPO₄
85mM NaCl
1mM MgSO₄

Autoclave for 15 minutes on liquid cycle

NGM agar plates (per 4L):

68g Bacto Agar powder
12g NaCl
10g Bacto peptone
4ml 5mg/ml of Cholesterol in 100% EtOH

Add 3.9L deionized H₂O

Autoclave for 70 minutes on liquid cycle, let cool, and add the following:
4ml 1M CaCl₂
4ml 1M MgSO₄
100ml 1M KPO₄
PROTOCOL 2

Detecting Autophagy in *Caenorhabditis elegans* Embryos Using Markers of P Granule Degradation.3

Nicholas J. Palmisano1,2 and Alicia Meléndez1,2*

1 Queens College-CUNY, Department of Biology, Flushing, NY, USA, 2The Graduate Center, The City University of New York, New York, USA

*, correspondence should be addressed to A.M., Alicia.Meléndez@qc.cuny.edu, (718)997-4580

Acknowledgement: This is a published chapter found in Cell Death Techniques: A Laboratory Manual, Courtesy of the Cold Spring Harbor Laboratory Press., available online Jan. 4, 2016, pdb.prot086504. doi: 10.1101/pdb.prot086504.
Abstract:

Autophagy has been shown to play an active role during the early stages of embryogenesis in *C. elegans* (Melendez and Neufeld 2008; Zhang et al. 2009; Kovacs and Zhang 2010; Al Rawi et al. 2011; Sato and Sato 2011). Although their exact function is unknown, P granules are ribonucleoprotein particles thought to play a part in germ cell specification (Strome and Lehmann 2007). The localization of P granules is restricted to the germline precursor cells in wild-type embryos, as a result of their degradation in the somatic cell lineage (Hird et al. 1996). Autophagy was shown to be required for the degradation of P granules, since mutations in various autophagy genes result in the accumulation of the P granule components, PGL-1 and PGL-3 (termed PGL granules), in the somatic cells of *C. elegans* embryos (Zhang et al. 2009; Zhao et al. 2009a; Tian et al. 2010).

SEPA-1 was discovered as an adaptor protein important for the degradation of PGL granules in somatic cells through its interaction with LGG-1, linking PGL granules to the autophagosome for removal (Zhang et al. 2009). In wild-type animals, SEPA-1 protein aggregates begin to form at the 16-cell stage embryo and peak at the 100-cell stage, but disappear significantly by the comma stage (Zhang et al. 2009). However, in autophagy mutants, SEPA-1 aggregates persist past the comma stage, confirming that SEPA-1 is targeted to the autophagosome for removal (Zhang et al. 2009; Tian et al. 2010; Lu et al. 2011b).

*sqst-1* encodes the *C. elegans* homolog of the adaptor protein p62/SQSTM1, which has also been investigated during embryogenesis (Tian et al. 2010; Lu et al. 2011b). In mammals, p62 binds to both LC3 and poly-ubiquitinated proteins, linking them to the autolysosomal pathway (Kihara et al. 2001; Bjorkoy et al. 2005; Pankiv et al. 2007; Tian et al. 2010). SQST-1::GFP expression is diffuse in the cytoplasm throughout embryogenesis; however, in autophagy mutants, the SQST-1::GFP protein localizes to positive punctate structures, similar to that of SEPA-1 (Tian et al. 2010; Lu et al. 2011b). Overall, SEPA-1, SQST-1, and PGL-1 fusion reporters have allowed for the identification of new autophagy genes, by screening for mutant animals that lacked the degradation of these autophagy substrates (Tian et al. 2010). Therefore, such reporters can be used to identify additional genes required for normal autophagy activity during embryogenesis.

Materials:
Reagents:

2%-3% agarose (2-3 grams of agarose filled to volume with 100ml of sterile deionized water)

M9 Minimal Medium buffer <R>:

NGM agar plates <R>:

25mM Sodium Azide (NaN₃)

Equipment:

Fluorescent microscope (Zeiss AxioImager A1)

Microscope coverslips (1.22 mm X .13mm)

Microscope slides (75mm X 25 mm X .96mm)

Standard platinum wire pick

Strains:

bpIs151[Psqst-1::SQST-1::GFP, unc-76(+)] (Tian et al. 2010)

bpIs131[Psepa-1::SEPA-1::GFP, unc-76(+)] (Tian et al. 2010)

bnIs1[Ppie-1::GFP::PGL-1, unc-119(+)] (Tian et al. 2010)

RD210: unc-119(ed3); Is[Ppie-1::GFP::mCherry::LGG-1; unc-119(+)]
Method:

**Standard Fluorescent Microscopy:**

1. Allow ~50 gravid adults to lay eggs on a single plate for 10-15 minutes at the desired temperature, and then transfer the gravid adults to fresh plates. This can be repeated as desired. *This will result in multiple plates containing tightly synchronized embryos.*

2. Calculate the time that recently laid embryos will require to reach the desired stage of embryogenesis (i.e. at 20°C, for ~100 cell stage embryos, wait an additional ~200 minutes). *The amount of time required to reach the desired stage of embryogenesis will vary according to temperature.*

3. Once the stage of interest has been reached, imaging of the embryos can begin. Apply a single drop of molten 2%-3% agarose onto a microscope slide. Apply another microscope slide on top of the slide containing the drop of agarose to form a thin agarose pad.

4. Take ~2.5 uL of M9 buffer and place it in the center of the agarose pad.
5. Using a platinum wire, first dab the pick into bacteria and then GENTLY pick embryos and submerge them into the M9 buffer on the slide.

6. Use 63X or 100X magnifications to quantify and/or take images of the fluorescent positive puncta found within the embryos. *Embryos should not be kept on slides for more than 15 minutes.*

**Data Analysis:**

Changes in autophagic activity during embryogenesis are usually visualized by monitoring changes in the expression pattern of a fluorescent reporter relative to the control. Such changes may be easily observed since, under wild-type conditions, some reporters may have a diffuse cytoplasmic expression, whereas, under conditions of defective autophagy, the reporter may accumulate into aggregate-like structures or positive punctate structures. An example of this can be found by comparing wild-type animals and autophagy defective embryos expressing SQST-1::GFP or GFP::LGG-1 (Tian et al. 2010; Lu et al. 2011b). Other changes may not be so prominent and may include an increase in the number or size of pre-existing protein aggregates, as is found when comparing wild-type animals and autophagy mutant embryos treated with anti-LGG-1 primary antibodies (Tian et al. 2010; Lu et al. 2011b).

To determine whether subtle changes in the expression of a reporter protein are significant will require additional methods. One method is to quantify the positive punctate structures, however, manual quantification of aggregates within embryos can be extremely difficult as autofluorescence is usually too high and the number of positive puncta too great. Image analysis programs may provide better quantification abilities; however, even with image analysis programs, one may not distinguish true punctate structures from autofluorescence. One option for measuring changes in the number of autophagosomes, or active autophagy, during embryogenesis, is to measure the fluorescence intensity of the autophagy reporter used (Morselli et al. 2011). Such changes in fluorescent intensity can be used to determine whether there is elevated or decreased expression of the reporter, which may be indicative of increased or decreased autophagy.

**Troubleshooting:**

**Problem:** Embryos have not reached the expected stage from the time of egg lay (step 2).
Solution: It is possible that the particular strain being used has a delay in development compared to wild-type animals. Calculate the time required to reach the developmental stage of interest.

Problem: Difficulties arise when attempting to separate embryos from the platinum wire pick (Step 5).
Solution: CAREFULLY press the platinum wire against the agarose pad several times, without tearing it, to release embryos.

Problem: Positive puncta found within embryos are too large to quantify (step 6)
Solution: Image processing and analysis software, such as ImageJ or MetaMorph, can be used to quantify puncta number and/or fluorescent intensity by following the instructions provided by the software.

Related Techniques:
Immunofluorescence with anti-LGG-1, anti-SQST-1, and anti-SEPA-1 antibodies

Discussion:
When evaluating reporter expression, to determine if a gene of interest plays a role in autophagy during embryogenesis, changes in the expression pattern of the reporter may not be apparent compared to controls. As a result, additional methods, such as image processing software, will be required to ensure that such changes are significant. If choosing to monitor GFP::LGG-1 or SQST-1::GFP expression, care should also be taken. In mammals, p62 can form cytoplasmic inclusions unrelated to autophagosomes (Zatloukal et al. 2002a; Bjorkoy et al. 2005). Furthermore, in mammals, LC3 can become incorporated into p62 cytosolic aggregates through its direct interaction with p62 (Shvets et al. 2008). Although, this has not been formally shown in C. elegans, if visualizing SQST-1::GFP or GFP::LGG-1 positive structures, additional reporters (Table C2) may be required to determine whether such aggregates are true autophagosomes or membrane free inclusion bodies. Overall, when analyzing autophagy activity in embryos, careful interpretation of results may require additional methods and/or techniques.
Recipes:

M9 Minimal Medium buffer:

22mM KH$_2$PO$_4$

22mM Na$_2$HPO$_4$

85mM NaCl

1mM MgSO$_4$

Autoclave for 15 minutes on liquid cycle

NGM agar plates (per 4L):

68g Bacto Agar powder

12g NaCl

10g Bacto peptone

4ml 5mg/ml of Cholesterol in 100% EtOH

Add 3.9L deionized H$_2$O

Autoclave for 70 minutes on liquid cycle, let cool, and add the following:

4ml 1M CaCl$_2$

4ml 1M MgSO$_4$

100ml 1M KPO$_4$
PROTOCOL 3

Detection of Autophagy in Caenorhabditis elegans by Western Blotting Analysis of LGG-1.

Nicholas J. Palmisano1,2 and Alicia Meléndez1,2*

1 Queens College-CUNY, Department of Biology, Flushing, NY, USA, 2The Graduate Center, The City University of New York, New York, USA
* correspondence should be addressed to A.M., Alicia.Melendez@qc.cuny.edu, (718)997-4580

Abstract:

A common way to measure the induction of autophagy is to compare the amount of Atg8/LC3-I with that of Atg8-PE/LC3-II using western blot analysis (Kabeya et al. 2000; Mizushima and Yoshimori 2007). This is because changes in the amount of LC3-II are closely associated with changes in the number of autophagosomes present in a cell (Kabeya et al. 2000). Atg8/LC3 is initially synthesized as an unprocessed form, which is proteolytically processed by Atg4 to form Atg8/LC3-I, and then modified into the phosphatidylethanolamine (PE)-conjugated Atg8-PE/LC3-II form (Kabeya et al. 2000; Kirisako et al. 2000). Atg8/LC3-II is the membrane bound form of Atg8/LC3, while Atg8-PE/LC3-I is cytosolic (Kabeya et al. 2000). Atg8-PE/LC3-II associates with both the inner and outer membrane of the autophagosome, thus Atg8-PE/LC3-II is the only autophagy reporter that is reliably associated with completed autophagosomes (Klionsky 2012).

As with mammalian LC3 and yeast Atg8, the C-terminus of LGG-1 appears to be conjugated to PE, with LGG-1-I and LGG-1-II being visible on a western blot as one major band and minor band, respectively (Kang et al. 2007; Alberti et al. 2010a; Tian et al. 2010). LGG-2 contains two minor bands as opposed to one; yet it is not clearly defined which minor band represents the lipidated form of LGG-2 (Alberti et al. 2010a). Under normal non-stress conditions, the protein levels of LGG-1-I are higher than that of LGG-1-II. Under conditions that induce autophagy, LGG-1-I levels still appear higher than that of LGG-1-II; however, an overall increase in the amount of LGG-1-II is apparent (Kang et al. 2007; Alberti et al. 2010a). A similar increase is also found for LGG-2 protein levels (Alberti et al. 2010a). Therefore, changes in LGG-1-II and LGG-2 protein levels may be used to monitor autophagy activity in C. elegans.

Although Atg8-PE/LC3-II is reliably associated with the autophagosome, its protein levels may not change in a predictable manner upon autophagy induction (Mizushima and Yoshimori 2007; Klionsky 2012). For example, upon autophagy induction in mammalian cells, the total levels of LC3 may not change; instead an increase in the conversion of LC3-I to LC3-II, or a decrease in the level of LC3-II relative to that of LC3-I, may result. The decrease in LC3-II can be due to the rapid lysosomal degradation of LC3-II (Huang et al. 2000; Klionsky 2012).

Therefore, although changes in the protein levels of LGG-1 can be used to monitor autophagy, caution should be used when evaluating such changes.
Materials:

Reagents:

Bleach Solution <R>:
ECL Detection Kit
M9 Minimal Medium Buffer <R>:
Ponceau S solution <R>:
Anti-GFP and/or anti-LGG-1 Primary Antibodies
Secondary Antibody
Protease inhibitor cocktail
1X SDS gel-loading buffer <R>:
10% SDS polyacrylamide gel (20% gel) <R>:
5% Stacking gel <R>:
1X Transfer Buffer <R>:
10X Tris-Buffered Saline (TBS) <R>:
TBST blocking buffer <R>:
Tris-Buffered Saline, 0.1% Tween-20 (TBST) <R>:
1X Tris-glycine electrophoresis buffer (running buffer) <R>:

Equipment:

Fiber pads and Filter paper
Gel electrophoresis cassette and power supply
Gel Transfer cell and sandwich cassette
Nitrocellulose or PVDF membrane
Method:

Note: The following Protocol has been adapted from Molecular Cloning: A Laboratory Manual Sambrook and Russell

Sample Preparation:

1. Wash L4 larvae or young adult hermaphrodites from feeding plates using 1 mL of M9 buffer and transfer animals to a 1.5 mL microcentrifuge tube. Rinse worms several times with M9 buffer and centrifuge worms for 1 minute at 2000 rpm in between washes. For embryo preparation, collect gravid adults by washing plates with sterile water. In a sterile 15mL conical centrifuge tube collect worms with bleach solution (should be made fresh every time). Shake well or vortex tube for a few seconds, and repeat a few times, for not longer than 10 min. Adults will dissolve while embryos remain intact. Wash embryos several times with M9 buffer and then transfer proteins from the polyacrylamide gel to a nitrocellulose membrane using a protein transfer apparatus.
buffer and centrifuge in between washes at 2000 rpm for 1 minute. After the final wash, remove the supernatant leaving only a pellet of embryos and continue with step 2 below.

2. After washing, centrifuge animals for 1 minute at 2000 rpm and remove supernatant leaving a pellet of animals. “Snap freeze” the animals in liquid nitrogen and add an equal volume of 1X SDS gel-loading buffer containing protease inhibitors to the sample and boil at 100°C for 3-10 minutes. It is important to also prepare marker proteins of known molecular weight for control purposes.

3. After boiling, spin down samples and cool on ice for 5 minutes. Load the samples onto the SDS polyacrylamide gel.

**SDS-PAGE Preparation (modified from Sambrook and Russel, 2001):**

4. Assemble the polyacrylamide gel apparatus by inserting two glass plates into the gel caster as described by the manufacturer.

5. Using an Erlenmeyer flask, prepare a 12%-20% polyacrylamide resolving gel. Swirl the mixture rapidly and pour it into the gap between the glass plates. Be sure to leave enough space for the stacking gel. Overlay the polyacrylamide gel with isopropanol. Allow the gel to polymerize for ~30 minutes. Pour the resolving gel immediately after adding TEMED.

6. Once polymerization is complete, pour off the isopropanol and rinse the top of the resolving gel a few times with deionized water. Remove all residual isopropanol and water.

7. Prepare a 5% stacking gel and pour the stacking gel solution onto the polymerized resolving gel. Carefully insert a clean gel comb into the stacking gel solution by avoiding air bubbles. Allow the stacking gel to polymerize at room temperature. Polymerization of the stacking gel will begin once TEMED is added, so the pouring step has to be done quickly.

8. After polymerization is complete, remove the gel comb and wash all wells with running buffer to remove any unpolymerized acrylamide. Mount the gel in the electrophoresis apparatus and add running buffer to the top and bottom reservoirs of the apparatus. Load ~15 uL of sample into each well, using gel-loading tips. One can add a higher volume on to the wells using a pipette with longer tips.
9. Attach the electrophoresis apparatus to the power supply as described by the manufacturer’s instructions. Run the gel at ~180 to 200 volts or until the bromophenol blue dye in the sample buffer reaches the bottom of the gel.

10. Once electrophoresis is complete, gently remove the glass plates from the gel apparatus and carefully release the polyacrylamide gel.

**Membrane Transfer (Modified from Bio-Rad Laboratories Mini-Trans-Blot instruction manual):**

11. Cool 1X transfer buffer at 4°C. Cut the nitrocellulose membrane (or PVDF membrane) and filter paper to the dimensions of the polyacrylamide gel. Soak the membrane, filter paper, and fiber pad in transfer buffer until the Western blotting preparation is ready to be made.

12. Prepare the Western blot sandwich in the following order: Start with the clear side of the case, followed by the sponge pad, Whatman filter paper, membrane, gel, Whatman filter paper, sponge pad, and the black side of the cassette. Close the cassette firmly and ensure that all bubbles are removed from the sandwich.

13. Place the transfer cassette in the transfer cell with the black side facing black, and fill the cell with cooled 1X transfer buffer. Transfer for 1h, at 4°C and ~90 volts. *Make sure that the cassette is positioned in the correct direction so that the proteins in the gel transfer to the membrane. The voltage required may vary according to the manufacturer’s instructions.*

**Immunoblotting (Modified from (You et al. 2006)):**

14. Once transfer is complete, remove the membrane from the transfer cassette and incubate the membrane in TBST blocking buffer (which contains 5% non-fat dry milk) for ~1 hour at room temperature. This can be done in a plastic bag or a Tupperware dish that can be placed on a shaker. *Ponceau S stain can be administered to the membrane to check for successful transfer of proteins before blocking.* Prepare the 1X Ponceau S solution as described by the manufacturer and incubate the blot for 1 to 5 minutes in the solution on a rocker. Rinse with distilled water to rid the blot of stain, until protein bands are clearly visible. *Wrap blot with plastic wrap and take a picture or a Xerox. Keep the membrane from drying.* After Ponceau S staining is completed, continue with the blocking step 14.
15. Dilute the primary antibody, (i.e. anti-LGG-1 (Tian et al. 2010) or anti-GFP (Kang et al. 2007; Alberti et al. 2010a), in 2ml TBST blocking buffer (which contains 5% non-fat dry milk) at the appropriate concentration and incubate the membrane in a Ziploc bag overnight at 4°C, placed on a rocker. For primary antibodies, use the dilution factor as suggested by the manufacturer; anti-GFP from Roche has been commonly used at a 1:500 dilution (Alberti et al. 2010a; Djeddi et al. 2012). As antibodies vary, and the protocols may vary, determine the concentration of the primary antibody empirically before the start of the experiment.

16. Remove the membrane from the Ziploc bag and wash it three times with ~50-100 mL of TBST for 5 minutes each, in a small Tupperware on a shaker.

17. Incubate the membrane with the secondary antibody at the correct concentration (as directed by the antibody manufacturer, or previously described (You et al. 2006; Alberti et al. 2010a), for 1 hour at room temperature, in a Ziploc bag, on a rocker.

18. Rinse the membrane three times with ~50-100 mL of TBST blocking buffer in 10 minute intervals. Then rinse with distilled water once. After the last wash, use an ECL detection kit as instructed by the manufacturer to visualize the protein bands.

**Data Analysis:**

Western blot analysis provides a convenient way to measure any changes in the levels of the different forms of LGG-1; however, caution is advised when interpreting the quantity of LGG-1-II using western blot analysis.

A direct way to measure changes in overall LGG-1-II levels is through quantification, by comparing the protein levels of LGG-1-II with the protein levels of a housekeeping gene (i.e. tubulin) or with LGG-1-I (Kang et al. 2007; Michelet et al. 2009; Alberti et al. 2010a; Barth et al. 2010). In addition, it is important to use appropriate standardization controls to ensure equal loading between samples, as this may change the amount of LGG-1-I and LGG-1-II protein between samples (Klionsky 2012). Furthermore, the stress condition of the animals prior to experimental manipulation should be at a minimum, to ensure unaltered levels of LGG-1-I and LGG-1-II protein at the start of an experiment.
Increased levels of LGG-1-II relative to LGG-1-I can reflect autophagosome accumulation, due to increased autophagy, or an accumulation of autophagosomes, as a result of defective lysosomal degradation (Michelet et al. 2009; Alberti et al. 2010a; Barth et al. 2010; Lu et al. 2011b; Klionsky 2012). Alternatively, based on mammalian studies, lower levels of LGG-1-II compared to LGG-1-I can represent defective autophagy, as a result of poor LGG-1-I to LGG-1-II conversion, or increased autophagic flux, resulting in the rapid degradation of LGG-1-II (Mizushima and Yoshimori 2007). The use of lysosomal inhibitors can be one way to distinguish between all these possibilities (Oka and Futai 2000; Ji et al. 2006; Mizushima and Yoshimori 2007; Pivtoraiko et al. 2010).

Alternatively, protein extracts isolated from mutant animals previously shown to alter the lipidation of LGG-1 can also be useful as positive and/or negative controls in blots that measure changes in LGG-1 protein levels. gpb-2 is a G-protein β subunit involved in the muscarinic signaling pathway, and gpb-2 mutants, following starvation, have elevated levels of autophagy in pharyngeal muscles, visualized by the expression of GFP::LGG-1, and also have a higher ratio of lipidated LGG-1 to non-lipidated LGG-1, compared to wild-type controls (Kang et al. 2007). lgg-2 mutants, which have defects in the acidification and degradation of autophagosomes, result in elevated levels of both lipidated and non-lipidated forms of LGG-1 (Manil-Segalen et al. 2014b). In addition, protein extracts isolated from animals fed dsRNA against the C. elegans ortholog of TOR, let-363 (Long et al. 2002), and rab-7, the small GTPase involved in endosome/lysosomal fusion events (Bucci et al. 2000), can also be used as controls to monitor changes in the levels of the different forms of LGG-1 protein (Alberti et al. 2010a). RNAi against let-363 induces autophagy, observed by elevated levels of GFP::LGG-1 in the hypodermis and intestine, and was reported to display a decrease in the levels of non-lipidated LGG-1, but an increase in the levels of lipidated LGG-1, compared to empty vector controls. On the other hand, RNAi against rab-7, which leads to increased levels of GFP::LGG-1 as a result of defective lysosomal fusion, resulted in elevated levels of both lipidated and non-lipidated LGG-1, compared to controls (Alberti et al. 2010a).

It is important to note that the loss of certain autophagy genes can inhibit autophagy without affecting LC3-II/Atg8-PE formation (Klionsky 2012), which appears to be also true for LGG-1-II in C. elegans (Tian et al. 2010; Lu et al. 2011b; Liang et al. 2012). Therefore, additional methods may be required to determine if autophagy is functional when observing changes in the protein levels of LGG-1.
Additionally, LGG-1-II levels can be influenced by the type of anti-LGG-1 antibodies used, as well as the type of membrane used during protein transfer (Barth et al. 2010; Klionsky 2012). In *C. elegans*, experiments that visualize the lipidated and non-lipidated forms of LGG-1 by western blotting may utilize anti-GFP or anti-LGG-1 primary antibodies, nitrocellulose membranes, as well as protein samples from larvae and embryos (Kang et al. 2007; Alberti et al. 2010a; Tian et al. 2010).

In mammalian cells, during the initial periods of starvation, the amount of LC3-I may be inversely proportional to that of LC3-II; however, as the starvation period is prolonged, the levels of both LC3-I and LC3-II have been shown to decrease (Mizushima and Yoshimori 2007). Although this has not been fully examined for LGG-1 in *C. elegans*, one should consider the appropriate length of time that animals are exposed to starvation. Lastly, LGG-1 has been shown to localize to phagosomes in cells that engulf and degrade apoptotic cells in *C. elegans* (Li et al. 2012a). Thus, conditions that induce apoptosis should be at a minimum when evaluating levels of LGG-1-II during autophagy.

Overall, a way to measure autophagy induction is to analyze the levels of LGG-1-II and compare them to the levels of LGG-1-I; however, several considerations should be made to ensure the proper interpretation of results.

Troubleshooting (Modified from Abcam site (url: http://www.abcam.com/index.html?pageconfig=resource&rid=11352)):

**Problem:** No signal detected on membrane (Step 17)

**Solution:** Consider the following:

1. Lysate preparation was not carried out correctly.
2. Incorrect primary or secondary antibody was used or not enough primary or secondary antibodies were used.
3. ECL detection kit was not used properly.
4. Poor transfer to membrane. See solution below.

**Problem:** High background intensity on membrane (Step 17)

**Solution:** Consider the following:
1. Concentration of primary and secondary antibodies is too high. Dilute and repeat.

2. Not enough washes were done to remove unbound antibodies in step 17. Include more washes and repeat.

3. Cross-reactivity between blocking buffer and primary and/or secondary antibodies. Substituting non-fat dry milk with BSA may circumvent this issue.

**Problem:** Poor transfer of protein to membrane (Step 11-13)

**Solution:** Consider the following:

1. Transfer buffer was prepared incorrectly.
2. Transfer Cassette and sandwich were not setup properly according to the manufacturer’s instruction
3. If a PDVF membrane was used, pre-soak in methanol before use

**Discussion:**

Western blot analysis may be used to monitor autophagy by evaluating the levels of lipidated and non-lipidated Atg8/LC3/LGG-1. However, an increase in the lipidated form of Atg8/LC3/LGG-1-II can reflect the induction of autophagy and/or inhibition of autophagy, and is therefore not a direct measure of autophagic flux without the use of additional methods (Klionsky 2012). In mammalian cells, the accumulation of LC3-II can result from an increase in autophagy activity or defective lysosomal degradation (Mizushima and Yoshimori 2007). The addition of lysosomal protease inhibitors, the introduction of a mutation, or RNAi treatment that results in defective lysosomal degradation, may differentiate between an increase in autophagy or the reduction of autophagic function. As described by Mizushima and Yoshimori, an additional increase in LGG-1-II levels, observed under conditions that block the fusion between autophagosomes and lysosomes, would be indicative of autophagy induction (Mizushima and Yoshimori 2007). In contrast, no change in LGG-1-II levels after treatment with agents or RNAi that decrease lysosomal degradation would indicate a block in the autophagic pathway. In conclusion, with careful interpretation of results, changes in the protein levels of LGG-1 can provide a way to measure changes in autophagy activity.
Recipes:

Bleach Solution (should be made fresh each time):

3.5 mL sterile H₂O
0.5 mL 5N NaOH
1 mL of household bleach (sodium hypochlorite)

M9 Minimal Medium Buffer:

22mM KH₂PO₄
22mM Na₂HPO₄
85mM NaCl
1mM MgSO₄

Autoclave for 15 minutes on liquid cycle

Ponceau S solution:

2g Ponceau S
30g trichloracetic acid
30g sulfosalicyclic acid
H₂O to 100 ml

1X SDS gel-loading buffer:

50mM Tris-Cl (pH 6.8)
100mM dithiothreitol
2% (w/v) SDS
0.1% bromophenol blue
10% (v/v) glycerol

10% SDS polyacrylamide gel (20% gel):
30% acrylamide mix
1.5M Tris (pH 8.8)
10% SDS
10% ammonium sulfate
TEMED

5% Stacking gel:
30% acrylamide mix
1.5M Tris (pH 8.8)
10% SDS
10% ammonium sulfate
TEMED

1X Transfer Buffer:
25mM Tris
192mM glycine
20% (v/v) methanol (or ethanol)
pH 8.3

10X Tris-Buffered Saline (TBS):
24.2g Tris Base
80g NaCl
pH 7.6
Add distilled water to final volume of 1L

TBST blocking buffer:
1X TBS
0.1% Tween-20
5% w/v non-fat dry milk

Tris-Buffered Saline, 0.1% Tween-20 (TBST):

1X TBS
0.1% Tween-20

1X Tris-glycine electrophoresis buffer (running buffer):

25mM Tris Base
192mM glycine
0.1% SDS
pH 8.8
PROTOCOL 4

RNAi-mediated inactivation of autophagy genes in *Caenorhabditis elegans*

Nicholas J. Palmisano$^{1,2}$ and Alicia Meléndez$^{1,2}$*

$^1$ Queens College-CUNY, Department of Biology, Flushing, NY, USA, $^2$The Graduate Center, The City University of New York, New York, USA

*, correspondence should be addressed to A.M., Alicia.Melendez@qc.cuny.edu, (718)997-4580

Abstract:

RNA interference (RNAi) is a process that results in the sequence-specific silencing of endogenous mRNA through the introduction of dsRNA (Fire et al. 1998). In *C. elegans*, RNA inactivation can be used at any specific developmental stage or only during adulthood, to avoid developmental requirements for a given target gene. *C. elegans* is unusual in that RNAi is systemic, meaning that the dsRNA can spread throughout the animal and affect virtually all tissues except neurons (Grishok et al. 2000; Winston et al. 2002). There are multiple ways to deliver dsRNA, however, the most common method used in *C. elegans* is to feed the animals bacteria that express dsRNA complementary to a specific target gene (Timmons and Fire 1998; Kamath et al. 2001). This method is advantageous due to the availability of libraries (Ahringer and Vidal libraries) with bacteria RNAi clones that target most genes (Kamath et al. 2003; Rual et al. 2004), the ability to treat animals at any developmental stage, and cost effectiveness.

The dsRNA can also be administered through injection or soaking, however, these methods require the *in vitro* preparation of dsRNA and are more time consuming (Ahringer 2006). One problem with the soaking method is that it may also introduce a level of stress, as *C. elegans* do not feed well in liquid media and can undergo starvation (Klass 1977). However, the injection method has been shown to be more efficient in the inactivation of certain target genes (Ahringer 2006).

RNAi has been greatly advantageous for the study of autophagy genes in *C. elegans* development. RNAi treatment against *unc-51*, *bec-1*, *atg-7*, *lgg-1*, and *atg-18* (by injection of dsRNA) was shown to block dauer morphogenesis of *daf-2* insulin/IGF-1 receptor mutants, and to result in the appearance of GFP::LGG-1 positive protein aggregates in hypodermal seam cells (Meléndez et al. 2003). Defects in dauer development and GFP::LGG-1 expression are also observed after feeding animals dsRNA specific to *bec-1* and several other autophagy genes in *daf-2* mutants, although the phenotypes found from feeding are not as penetrant as those found after injection of dsRNA (N. Palmisano and A. Meléndez, unpublished observations). Although the exact nature of the GFP::LGG-1 aggregates is not clear, they have been suggested to be poly-ubiquitinated proteins that accumulate as a result of ineffective lysosomal degradation (Szeto et al. 2006). Therefore, GFP::LGG-1 localization should be carefully interpreted when using RNAi.
Knockdown of bec-1 gene activity (by injection and feeding), and knockdown of atg-12 and atg-7 gene activity (by feeding only), showed that these autophagy genes are also required for the longevity phenotype of daf-2 insulin/IGF-1 receptor mutants (Meléndez et al. 2003; Hars et al. 2007b; Hansen et al. 2008b). RNAi by feeding against vps-34, atg-7, unc-51, atg-18, lgg-1, or bec-1 was also shown to decrease the lifespan extension of dietary restricted eat-2 mutants (Hansen et al. 2008b), and more recently of germline-less glp-1/Notch mutants (Lapierre et al. 2011). The decrease in lifespan is not due to the effects of RNAi treatment during development, which may lead to decreased longevity, since RNAi treatment was conducted during adulthood (Hansen et al. 2008; Melendez et al. 2008). In conclusion, RNAi specific to autophagy genes has proven to be an excellent method to study the role of these genes in autophagy, as well as other cellular and developmental processes. For a description of the methods on longevity assays, we refer you to (Melendez et al. 2008).

Materials:

Reagents:

- 2%-3% agarose (2-3 grams of agarose filled to volume with 100ml of sterile deionized water)
- Ampicillin (100mg/ml)
- Tetracycline (10mg/ml)
- Carbenicillin (50mg/ml)
- 2M isopropylthio-β-galactoside (IPTG)
- Luria-Bertani Broth (LB) <R>
- M9 Minimal Medium buffer <R>
- NGM plates <R>
- NGM RNAi agar plates <R>
- 25mM Sodium Azide (NaN₃)

Equipment:

- Fluorescent microscope (Zeiss AxioImager A1)
Microscope coverslips (1.22 mm X .13mm)  
Microscope slides (75mm X 25 mm X .96mm)  
Standard platinum wire pick

**Graphic Flow Chart #4:**

**Method:**

1. Inoculate and culture each bacterial RNAi clone of interest in 5 mL of LB/AMP liquid media at 37°C overnight in a shaker at 250 rpm. *A bacterial clone that is isolated from the RNAi library for the first time should be verified to ensure the presence of the correct insert in the plasmid. Contaminations or incorrect clones have been found in the past. Glycerol stocks can then be made from the isolated verified colony. This is not necessary once a clone has been verified.*
2. Experimental RNAi plates should be seeded with 500 uL of bacterial liquid culture (HT115 expressing double-stranded RNA against the target gene, under the IPTG-inducible T7 RNA polymerase promoter). Plates should be allowed to dry overnight before adding worms to ensure sufficient induction of T7 RNA polymerase activity.

3. Place ~30 L4 animals on RNAi plates and allow animals to ingest bacteria overnight at the desired temperature. The strains to be analyzed should be well fed on NGM plates (Normal Growth Media) seeded with OP50 E. coli bacteria, for at least two generations prior to the experiment. Additionally, it should be noted that some C. elegans strains are temperature sensitive, and therefore, care should be taken when deciding on the temperature to carry out the experiment.

4. On the following day, transfer the animals (now adults) to new RNAi plates and allow them to lay eggs overnight at the desired temperature. The first set of plates (step 3) is discarded, and the F1 progeny produced from the second set of plates can be utilized for the particular experiment of interest when they reach the desired stage. Only progeny from the second set of plates are analyzed, as this ensures that the F1 progeny to be analyzed are derived from mothers that ingested the dsRNA. Note that the adults can be transferred to fresh RNAi plates for two more days (as a third set or a fourth set), to obtain more F1 animals with knockdown of the specific target gene. In addition, F1 progeny treated with RNAi can be tightly synchronized using the wash off/hatch off method (described in protocol 1).

5. Once the F1 progeny have reached the developmental stage of interest, the experimental analysis can begin. Take into account the temperature of the experiment to predict the timing of this step.

6. To mount animals, apply a single drop of molten 2%-3% agarose onto a microscope slide. Apply another microscope slide on top of the slide containing the drop of agarose, to form a thin agarose pad. Take ~3 uL of 25mM of sodium azide (NaN₃) and place it in the center of the agarose pad.

7. Transfer 10-20 animals into the NaN₃ drop. A cover slip should be applied immediately to avoid the evaporation of the sodium azide solution. Wait until the animals are completely anesthetized before imaging, this should take approximately 30s. Imaging can be done as described in protocol 1 and 2.

Data Analysis:
Confirmation that gene knockdown has occurred can be easily determined by measuring a reduction in mRNA levels of the target gene by RT PCR, or by measuring a reduction in protein levels by western blot analysis (Ahringer 2006). In addition, success of an RNAi experiment can be confirmed through the use of appropriate positive and negative controls. For example, when using strains that contain a GFP fluorescent reporter, a positive control that can be used is to feed animals with RNAi expressing bacteria against GFP (Timmons et al. 2001). After treatment with GFP RNAi, the expression of any GFP reporter should decrease significantly. A proper negative control for RNAi, is to feed animals with bacteria transformed with the empty vector control \textit{L4440} (Timmons and Fire 1998). Results obtained after RNAi treatment against a gene of interest should be compared to the results obtained after RNAi against the negative control with empty vector, to determine if the RNAi clone tested is the direct cause for any observed phenotype.

The effects of RNAi can be further enhanced by using certain mutations that have been shown to increase the sensitivity of animals to RNAi, such as mutations in the \textit{lin-35}, \textit{lin-15b}, \textit{eri-1}, and \textit{rrf-3} loci (Simmer et al. 2002; Kennedy et al. 2004; Wang et al. 2005; Lehner et al. 2006; Schmitz et al. 2007). To investigate the specific tissue that requires the target gene, by RNAi, we can use several strains that carry mutations that confer RNAi resistance in particular tissues (Smardon et al. 2000; Sijen et al. 2001; Kumsta and Hansen 2012). As neurons are commonly known to be refractory to RNAi treatment, to treat neurons by RNAi we require a strain with neuronal expression of SID-1, a transmembrane protein required for systemic RNAi (Calixto et al. 2010). When expressed in neurons, SID-1 increases the response of those neurons to RNAi, however, neuronal expression of \textit{sid-1} has been shown to decrease the effects of non-neuronal RNAi (Calixto et al. 2010), which has to be considered when evaluating gene knockdown in both neuronal and non-neuronal tissues. Additionally, expression of \textit{sid-1(+)} from a cell-specific promoter in a \textit{sid-1(-)} mutant can be used as a cell-specific method of feeding RNAi and limiting the effect of gene knockdown to specific cell types (i.e. neurons) (Calixto et al. 2010).

In \textit{C. elegans}, \textit{ego-1} and \textit{rrf-1}, encode RNA-dependent RNA polymerases (RdRPs), with tissue-specific RNAi-processing function, \textit{ego-1} partially targets the germ line and \textit{rrf-1} targets the somatic tissues (Smardon et al. 2000; Sijen et al. 2001; Suzuki et al. 2004; Qadota et al. 2007; Kumsta and Hansen 2012). Thus, to inquire whether a particular gene acts in somatic tissues, or in the germ line, one can utilize a
strain containing a mutation in the rrf-1 locus (Sijen et al. 2001). Two mutations of rrf-1, pk1417 and ok589, are available and have been shown to have no effect on lifespan or thermotolerance (Kumsta and Hansen 2012). However, care should be taken when using rrf-1 mutants since these mutants have been found capable of processing RNAi in somatic tissues, particularly in the intestine and a subset of hypodermal cells (Kumsta and Hansen 2012). Another consideration is whether rrf-1 has any phenotype in the process being studied, as it was shown that the rrf-1 mutation induces the expression of several transgenes, and increased the expression of sod-2, a DAF-16/FOXO transcription target (Kumsta and Hansen 2012).

Another method that has been previously used to achieve RNAi in specific tissues is to use an rde-1 mutant strain that is resistant to RNAi (Tabara et al. 1999), and to express the wild-type rde-1 cDNA under the control of tissue-specific promoters, to achieve RNAi sensitivity in the tissues that express rde-1 (Suzuki et al. 2004; Qadota et al. 2007). However, several considerations have to be made when using the tissue specific promoters, as they may not rescue completely, and they may be expressed in other tissues. For genes that express and/or function in multiple tissues, the assay of tissue-specific RNAi may be very helpful in distinguishing the activity of that gene in a particular tissue or cell type.

There are multiple phenotypes that have been shown to depend on autophagy gene activity, and thus can be used to determine if a particular gene of interest functions in autophagy. We can treat daf-2 mutants, expressing the GFP::LGG-1 reporter that has been shown to label GFP-positive punctate structures corresponding to autophagosomes, with RNAi against the gene of interest, and evaluate if the RNAi treatment affects dauer formation or the induction of GFP::LGG-1 positive punctate structures in seam cells. When conducting such an experiment, RNAi specific to autophagy genes, such as bec-1, unc-51, or atg-18, should be used as positive controls. RNAi against bec-1, unc-51, or atg-18, has been shown to increase the diffused expression of GFP::LGG-1 and also result in the formation of GFP::LGG-1 protein aggregates (FIG. C2) (Meléndez et al. 2003). However, the nature of the GFP::LGG-1 aggregates that arise due to inhibition, or knockdown of autophagy gene activity, is not fully understood. Thus, the formation of GFP::LGG-1 protein aggregates, that arise as a result of knocking down the gene of interest, should be further examined to determine where the gene of interest acts in the autophagy pathway. A next step would be to investigate whether the specific RNAi treatment affects the formation of autophagosomes, lysosomal
degradation, or the accumulation of poly-ubiquitinated proteins that exceed the degradative capacity of the lysosome.

Trouble Shooting:

**Problem:** Animals submerged in NaN₃ do not become anesthetized (step 6).

**Solution:** The concentration of NaN₃ may be too low. Remake the solution or increase the concentration as needed.

**Problem:** Knockdown of a particular gene does not give a phenotype (step 7).

**Solution:** Consider the following:

1. The specific RNAi bacterial clone was contaminated with another clone. It is important to ensure that the plasmid contains the correct DNA sequence corresponding to the gene of interest. If so, streak a fresh LB plate (containing ampicillin and tetracycline) to isolate new bacterial colonies, and inoculate a new colony into 5 ml of LB medium, then proceed with step 1.

2. Animals were not exposed to the RNAi clone for an adequate amount of time. Allow animals to ingest the bacteria expressing the dsRNA for a longer period of time.

3. To strengthen the effect of the RNAi, seed the plates with the bacteria expressing the dsRNA, allow the bacterial seed to dry, and then add 0.5–1 mM IPTG directly to the bacteria to induce expression. For this change in the experimental method, the NGM RNAi plates should be made only with carbenicillin and not containing IPTG.

**Problem:** Exposure to a specific RNAi clone results in embryonic lethality.

**Solution:** The particular gene targeted by RNAi is required during embryogenesis. To bypass the requirement for the essential gene, treat L1 animals with the specific RNAi clone and analyze animals once the appropriate stage has been reached.
**Discussion:**

RNAi is sometimes more advantageous to study gene function than mutations, as pleiotropic effects during development can be avoided by treating animals as adults or at a specific developmental stage. For example, in the case of *bec-1*, deletion mutants that lack both maternal and zygotic *bec-1* activity are embryonic lethal, however, *bec-1* RNAi animals live until young adulthood, and display severe vacuolization and incoordination (Ruck et al. 2011). Therefore, RNAi provides a means to study gene function by circumventing some of the drawbacks associated with strong loss of function mutations.

When investigating if a particular gene functions in autophagy, *daf-2* mutants expressing GFP::LGG-1 can be treated with RNAi specific against the gene of interest and evaluated for changes in the expression pattern of GFP::LGG-1. Other mutant backgrounds, such as *eat-2* dietary restricted or *glp-1* germline-less mutants, can also be used for investigating if a particular gene functions in autophagy, however, as for *daf-2* mutants, appropriate controls are required to make accurate conclusions and comparisons from the results obtained.

**Recipes:**

Luria-Bertani Broth:

10g Tryptone  
5g Yeast Extract  
10g NaCl  
P\( \text{H} \)\( \text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\.text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{
1mM MgSO\textsubscript{4}

Autoclave for 15 minutes on liquid cycle

NGM agar plates (per 4L):

68g Bacto Agar powder

12g NaCl

10g Bacto peptone

4ml 5mg/ml of Cholesterol in 100% EtOH

Add 3.9L H\textsubscript{2}O

Autoclave for 70 minutes on liquid cycle, let cool, and add the following:

4ml 1M CaCl\textsubscript{2}

4ml 1M MgSO\textsubscript{4}

100ml 1M KPO\textsubscript{4}

**ACKNOWLEDGMENTS**

We thank all Meléndez lab members for helpful discussions and comments on the manuscript. Special thanks to Melissa Silvestrini and Kristina Ames for comments. The work in the Meléndez lab is supported a National Science Foundation Research Initiation Award, an NIH R15 award, and AM is an Ellison Medical Foundation New Scholar in Aging.
References:

Aalto MK, Ronne H, Keranen S. 1993. Yeast syntaxins Sso1p and Sso2p belong to a family of related membrane proteins that function in vesicular transport. The EMBO journal 12: 4095-4104.


-. 2009b. Epithelial system, seam cells. WormAtlas.


Cherfils J, Zeghouf M. 2013. Regulation of small GTPases by GEFs, GAPs, and GDIs. *Physiological reviews* **93**: 269-309.


Fader CM, Aguilera MO, Colombo MI. 2012. ATP is released from autophagic vesicles to the extracellular space in a VAMP7-dependent manner. *Autophagy* **8**: 1741-1756.


Kang C, Avery L. 2007. To be or not to be, the level of autophagy is the question: Dual roles of autophagy in the survival response to starvation. *Autophagy* **4**.


Kramer L, Ungermann C. 2011. HOPS drives vacuole fusion by binding the vacuolar SNARE complex and the Vam7 PX domain via two distinct sites. *Molecular biology of the cell* **22**: 2601-2611.


Wickner W. 2010. Membrane fusion: five lipids, four SNAREs, three chaperones, two nucleotides, and a Rab, all dancing in a ring on yeast vacuoles. *Annual review of cell and developmental biology* 26: 115-136.


