“Rab GTPase mediated regulation of the autophagic pathway and mTOR signaling in the larval fat body of Drosophila melanogaster”

A Thesis
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Carlos I. Ayala-Navarro

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Dr. Thomas P. Neufeld

July 2016

Carlos I. Ayala-Navarro

Year of Publication: 2016

Acknowledgements

I am very grateful to my advisor, Dr. Thomas Neufeld, for taking me in the laboratory in a leap of faith with just two weeks in my rotation. He has always fostered my curiosity and encouraged all my proposed experiments throughout these five years. He has showed me that productivity without understanding is empty science and is continuously teaching me something new after every one on one meeting we have. Tom thanks for always being interested in the unexpected results and the diversity of topics in the lab. It makes for a challenging place to keep up with all the literature topics but gives us different angles and ideas for our thesis projects. Thank you for all the time you have invested in my training from the moment you taught me how to bisect larvae to the never forgetting what is the question of the experiment. I am forever grateful.

I am thankful for the input, advice and guidance given by my committee: Dr. Mike O'Connor, Dr. Paul Letourneu, Dr. Tom Hays, Dr. Sean Conner, Dr. Harry Orr and Dr. Lisa Schimmenti.

I would like to thank my parents, Carlos Ayala and Grisselle Navarro. Thanks for raising me and providing me with the tools and values to become the man I am. Most importantly, thanks for giving me the independence and choice of freedom. Thanks for always trusting and supporting my decision. Los amo y los quiero!!

Most importantly, I will like to dedicate my thesis to the love of my life, Samairis, my wife. I cannot imagine achieving this professional goal without your support and help. Thanks for your unconditional love this past 13 years and for believing in me. Thanks for listening me complaint about experiments that went wrong even if you didn't fully grasp the reason why and laughed, at least we got a laugh out of them. Thanks for all those weekends and late nights when I stayed late and you appeared in the lab with dinner. Thanks you for giving the best present in my life, parenthood. Most importantly, thanks for always being there for me. Chiquita te dedico esta tesis con todo mi amor, Te amo.

Table of Contents

List of Figures and Tables

Chapter 1: Introduction

I. Autophagy Overview

a. Autophagy and cellular homeostasis

Macro-autophagy, hereafter referred as autophagy, is a catabolic housekeeping mechanism employed by cells to maintain quality control of proteins and organelles under basal conditions. Autophagy can be induced upon a variety of cellular stressors, such as starvation, and has been shown to regulate a diverse number of developmental processes in higher eukaryotes (Singh and Cuervo, 2011). Not surprisingly, deregulation of autophagy has been shown to lead to disease states ranging from cancer to neurodegeneration (Beau et al., 2011). Mechanistically, the process starts via the formation of an isolation membrane (IM) at the endoplasmic reticulum (ER) around cytoplasmic material to be degraded. Extension of the IM occurs via contribution of membrane sources from endosomes, the Golgi and the plasma membrane culminating in its closure to produce a double membrane autophagosome (AP). Afterwards, AP's continue in a maturation path to encounter lysosomes to form autophagolysosomes (AL), after fusion. Enclosed cargo inside AL is degraded by lysosomal hydrolases to produce reusable macromolecular building blocks to maintain cellular homeostasis in the cell and re-activate mTOR at the lysosome (Fig. 1) (Chen and Yu, 2013; Yu et al., 2010). However, how cells shift their constitutive housekeeping traffic routes to supply the autophagic pathway with lipids and regulatory proteins via vesicular carriers is not completely understood.

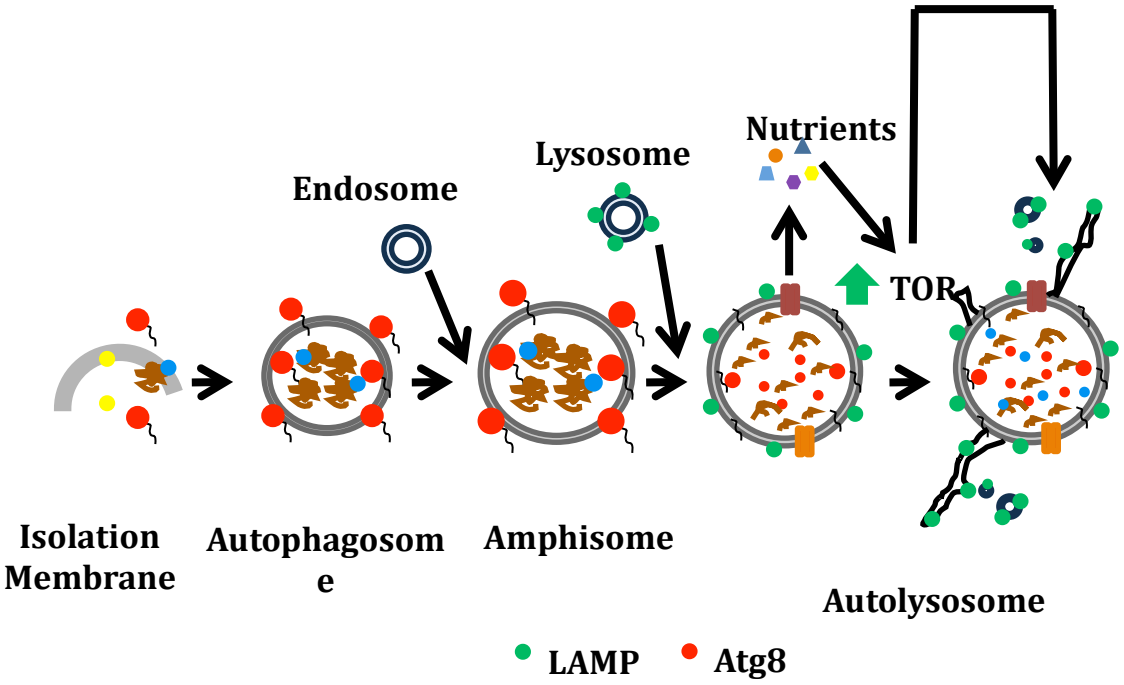


Figure 1. Brief schematic of the autophagic pathway.
The diagram highlights the extensive contribution of vesicular trafficking as a source of regulatory proteins and lipids. How the Golgi, ER, endosomes and mitochondria shift their constitutive roles to feed autophagy and the regulatory proteins mediating the shift allowing vesicular movement at the distinct steps in the autophagic pathway are not completely understood. In addition, mTOR a master regulator of growth is regulated via trafficking to alter its localization from an unknown cellular compartment upon amino acid and/or growth factor stimulation to the surface of the lysosome were it becomes activated. However, its still unknown how mTOR is recruited at the autolysosome, the regulatory proteins mediating its recruitment and where its coming from. Therefore, the identification of new regulators of trafficking might provide the answer.

b. Autophagy and disease

Autophagy is required to maintain quality control of mitochondria and misfolded proteins. In recent years autophagy has emerged as a major cellular pathway in cellular metabolism and a spectrum of disease states (Beau et al., 2011; Galluzzi et al., 2014). In addition, autophagy has been shown to have a critical role in the removal of aggregated proteins, to maintain neuronal health and prevent neurodegenerative diseases (Nah et al., 2015).

Neurodegeneration is characterized by loss of neurons or neuronal function in a specific region of the nervous system as a result of aggregated proteins and/or neuronal dysfunction. Parkinson's disease characterized by loss of substantia nigra and dopaminergic neurons can be caused by mutations in alpha-synuclein, Parkin and PINK (Lin and Farrer, 2014). PINK and Parkin have been shown to be required to promote a specialized type of autophagy called mitophagy ensuing neuron quality control of mitochondria (Pickrell and Youle, 2015). Meanwhile, alpha synuclein has been shown to regulate macroautophagy (da Fonseca et al., 2015; Pan and Yue, 2014). Additional examples of neurodegenerative diseases characterized by impaired autophagy include Alzheimer's disease, Amyotropic lateral sclerosis and Huntington's disease (Martinez-Vicente, 2015). Therefore, a commonality in many neurodegenerative disorders is the deregulation of autophagy and the loss of it quality control function. One of the strongest pieces of evidence to support a positive or neuroprotective role provided by autophagy is the administration of rapamycin to genetic models of neurodegeneration. Feeding of rapamycin results in inhibition of mTOR activity and induction of autophagy and has

been shown to promote degradation of aggregated proteins in disease models and promote longevity (Bjedov et al., 2010; Jiang et al., 2014b; Moskalev and Shaposhnikov, 2010; Ravikumar et al., 2004; Spilman et al., 2010; Tain et al., 2009).

Autophagy is a critical regulator of cell metabolism and energy homeostasis. The relevance has been best exemplified in the cancer field as autophagy has been shown to be dually regulated, positively and negatively during cancer in a tissue, type and stage dependent manner as a way to overcome diverse nutrient and growth factor availability states (Galluzzi et al., 2014; Jiang and Mizushima, 2014). Interestingly, we have come to appreciate that cancer cells can hijack autophagy as an alternative source of macromolecular building blocks to maintain cellular homeostasis under nutrient and growth factor limitation. This is achieved non-specifically via bulk sequestration of cytoplasmic content onto autophagosomes to replenish the cell with amino acids, simple sugars and lipids. Conversely, some cancers inhibit the tumorigenic protective role of autophagy in cells. Further evidence supporting a role for autophagy in cancer development is the finding of genes mutated in cancer that have a role in the regulation of autophagy, such as: Beclin/Atg6, PTEN, Bcl-2, class 1 Pi3K, PKB and TSC2 (Xie et al., 2015).

Deregulation of autophagy and its negative regulator mTOR (mechanistic target of rapamycin) have also been linked to metabolic diseases such as: Type 1 and 2 diabetes, obesity, hyperlipidemia, liver and cardiovascular diseases (Jia et al., 2014; Jiang et al., 2015; Singh and Cuervo, 2011). Therefore, understanding the mechanisms governing this pathway have gathered much attention with an interest of developing pharmacological

modulators to target the pathway in disease states ranging from cancer to neurodegeneration.

II. Regulation of Autophagy

a. Autophagy discovery and hierarchy of autophagy related genes

Autophagy was first described in yeast as a conserved cellular pathway dependent on the vacuole, the lysosome counterpart in mammals (Baba et al., 1997; Shibutani and Yoshimori, 2014; Takeshige et al., 1992). Genetic screens identified a core set of proteins conserved from yeast to mammals named autophagy related proteins (Atg) and studies in rat hepatocytes showed the pathway was induced by glucagon and inhibited by insulin (Klionsky et al., 2003; Shibutani and Yoshimori, 2014). Careful characterization and analysis of these Atg proteins has shed insight into their molecular function revealing that they act in a hierarchical cascade to regulate the autophagic pathway (Fig. 2)

Autophagy induction upon nutrient restriction results in activation of the serine/threonine kinase Atg1, in a molecular complex with Atg13-Atg17/FIP200-Atg101 (Jiang and Mizushima, 2014; Mehrpour et al., 2010; Shibutani and Yoshimori, 2014). Atg1 activation promotes synthesis of the isolation membrane (IM). In parallel, autophagy requires nucleation and expansion of the IM provided by the Class III PI3P kinase, Vps34, in a complex with Atg6/beclin-Vps15-Atg14 ensuing synthesis of PIP3 lipids. Formation of these lipids serves as a platform to recruit the PI3P binding proteins as the IM is formed at endoplasmic reticulum-mitochondria junction sites (Jiang and Mizushima, 2014). Expansion of the IM continues as Atg5 is conjugated to Atg12 by

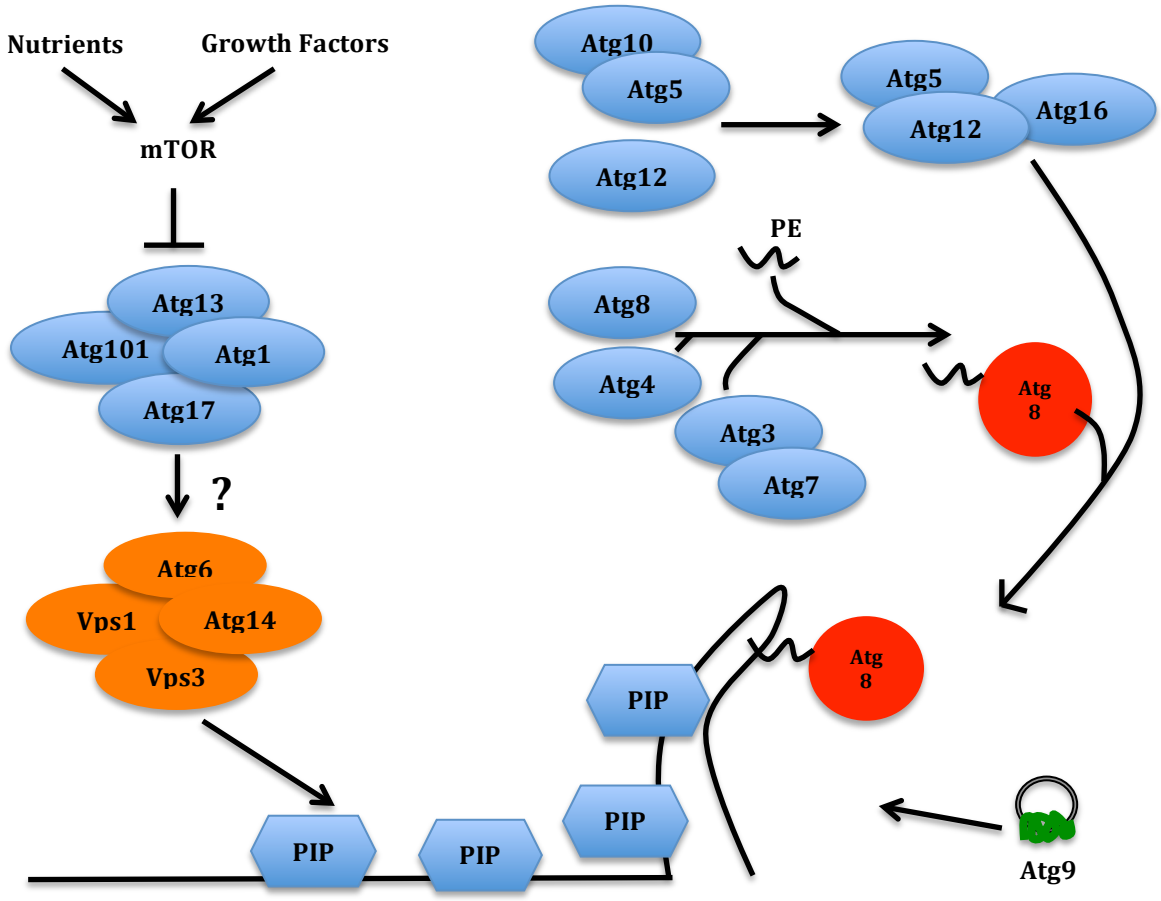


Figure 2. Hierarchy of Atg proteins required for the autophagic pathway.

Autophagy is negatively regulated by the mechanistic Target of Rapamycin (mTOR) kinase under basal states via direct phosphorylation and binding of the Atg1/Atg13 complex. Nutrient and growth factor limitation result in the activation of Atg1 and the Atg1/Atg13/Atg101/Atg17 complex that allow synthesis of the isolation membrane (IM). In parallel, the Vps34 complex (Vps15/Vps34/Atg14/Atg6) is activated to nucleate and expand the IM. Subsequently, two independent conjugation cascades allow the processing and lipidation of Atg8. The Atg12/Atg16/Atg5 recruits lipidated Atg8 is to the IM allowing its closure to form a double membrane autophagosome. The regulatory mechanisms coordinating the mobilization of Atg proteins from their constitutive compartments under basal states to supply the autophagic pathway under nutrient limitation are incompletely understood. In addition, the downstream targets of Atg1 coordinating the plethora of events unleashed upon autophagy induction have not been completely elucidated.

Atg5-Atg10 complex in parallel to the conjugation of Atg8/LC3 to phosphatidyl-enamine (PE) by the Atg3-Atg7 complex and recruited by Atg5-Atg12-Atg16 complex to the IM. At this point Atg8-PE is incorporated in the nascent IM and further carried until autolysosomes are turned over in cells at the completion of autophagy (Jiang and Mizushima, 2014). Atg9, the only trans-membrane Atg protein complements the hierarchy of Atg proteins by serving as a vesicular membrane donor allowing growth of autophagosomes and ensuing the stability of other Atg proteins at the IM (Ge et al., 2014; Jiang and Mizushima, 2014; Lamb et al., 2013). Interestingly, although the sequential flow of these Atg proteins has been exhaustively researched, the field is only starting to discover that some, if not all, of the Atg proteins interact and cross regulate each other to allow successful progression of autophagy. However, how these Atg proteins are traffic towards the autophagic pathway upon induction its still not completely understood.

b. Biogenesis of the autophagosomal membrane

The source of the autophagosomal membrane has been the most sought after research question in the field of autophagy. In yeast, the biogenesis of the phagopore (isolation membrane in higher eukaryotes) is believed to occur de novo as nucleation by Atg proteins takes place at the pre-autophagosomal structure (PAS), near the vacuole (Suzuki and Ohsumi, 2010). In contrast, the biogenesis of the isolation membrane in mammals has been a highly debated topic. To date organelles including: the Golgi network, endoplasmic reticulum (ER), the plasma membrane, mitochondria and endosomes have been invoked to be required for biogenesis of autophagosomes.

The first organelle believed to be used as a membrane donor source for the formation of the IM was the ER (Lamb et al., 2013). The association of the organelle was the result of electron microscopy observations of an electron dense cup shaped double membrane cisternae with unique lipid properties after osmium staining procedure. At the time and still today de novo synthesis has not been ruled out as an alternative lipid source for autophagosomes biogenesis.

The first studies that shed some insight onto the organelle responsible for formation of the IM came from live imaging analysis of the PIP3 biding protein Double FYVE Containing Protein 1 (DFCP1). The protein was localized to the ER and Golgi under basal states. Under starvation, the group noticed the protein enriched at the ER forming discrete punctate structures that co-localized with other Atg proteins. The localization of DFCP1 was dependent on its FYVE domain (PI3P binding domain), the activity of Vps34 and occurred near vesicles carrying the Vps34 complex components. This suggested that DFCP function during autophagy is dependent on PI3P binding and synthesis (Axe et al., 2008). These initial observations resulted in the incorporation of the omegasome in the autophagy nomenclature (due to it similarity to the Greek letter **Ω**) to describe the subdomain of the ER responsible for assembling a number of Atg proteins, like Atg1 and Atg14, required for autophagy initiation. Further supporting this notion was the finding that Atg14 null cells fail to form autophagosomes, as this protein is not recruited to the ER in response to starvation. These defects could be rescued by an Atg14 chimeric protein containing the DFCP ER targeting domain but not Atg14 lacking its ER

targeting domain indicating the relevance of ER recruitment of this Atg protein for IM synthesis and autophagy.

More direct evidence was provided when two independent groups evaluated the formation of autophagosomes under starvation using electron tomography (Hayashi-Nishino et al., 2009; Yla-Anttila et al., 2009). Both studies images revealed a direct and intricate relationship between the ER and the IM. Omegasome structures were observed forming from an ER subdomain around the IM. The most compelling evidence was provided when one of the groups took advantage of the Atg hierarchy of proteins and over-express a dominant negative version of Atg4 to slow the progression of the pathway to accumulate early precursors. Soon after this findings, an electron tomography study followed showing that an artificial mitochondrial marker was contributing to the biogenesis as it translocated from the outer membrane to the IM placing the mitochondria as an alternative source of membrane (Hailey et al., 2010).

Currently it is accepted that under starvation-induced autophagy the biogenesis of the IM occurs at ER-mitochondria contact sites. The study showed that Atg14 and Atg5, both markers found at the IM, accumulated at these contact sites referred as mitochondria-associated ER membrane (MAM). Purification of MAM sites revealed they contained DFCP1 and Atg5, both isolation membrane markers. Furthermore, disruption of the MAM sites severely impaired autophagosomes formation (Hamasaki et al., 2013). Collectively, this suggests that MAM sites are used during autophagy as a platform and membrane source for the formation of the IM.

c. Expansion and growth of autophagosomes

Formation of an autophagosome is followed by maturation to ensure its final size and functionality en route to fusion with lysosome to form an autolysosome (Ge et al., 2014; Singh and Cuervo, 2011). However, the membrane donor sources for the distinct steps of autophagy regulating the biogenesis and growth of autophagosomes have puzzled researchers for years. Studies have shown that formation of the IM can be the result of multiple donor events from diverse organelles. Similarly, autophagosomal growth has been shown to occur via contribution of multiple donor sources sites that include: the Golgi-network, ER exit sites (ERES), mitochondria, endosomes and the plasma membrane (Chan and Tang, 2013). In addition, expansion of the IM is a direct consequence of the recruitment of the Atg core proteins allowing the recruitment of donor membrane sources and obtain lipids (Lamb et al., 2013).

Alternatively, autophagosomes can grow once formed as a direct consequence of exchange and fusion with components of the endocytic pathway, for which ESCRT proteins, SNAREs, Rab 11 and Rab7 have been shown to have a regulatory role (Fader et al., 2008; Jager et al., 2004; Mehrpour et al., 2010). Other critical elements required for autophagosomal growth include the Vps34 complex, coat complexes (such as the exocyst), post-Golgi Sec proteins, Atg9 and the levels of Atg8 (mRNA and protein), among others (Chen and Klionsky, 2011; Jin and Klionsky, 2014; Yang and Rosenwald, 2014). Regardless of our knowledge on the organelles acting as membrane donors we still have no understanding of how traffic routes shift from their constitutive cellular traffic roles to meet the demands imposed by autophagy. Moreover, the traffic regulators

mediating these shifts from constitutive routes to supply autophagy under inductive states have not been fully elucidated.

A limitation in the elucidation of the biogenesis donor organelle in the field has been the lack of assays to discretely separate initiation of the IM and its expansion. Both are influenced and regulated by vesicle donor events (Lamb et al., 2013). Additionally, it is entirely possible that the membrane donor source are cell type and stimulus specific events. Discrimination between these variables may separate the contribution of organelles in future studies.

d. Autophagosome-lysosome fusion

Fusion between autophagosomes and lysosomes is a critical step in the formation of the degradative unit of autophagy, the autolysosome (Shen and Mizushima, 2014). Also, autophagosomes have been shown to alternatively fuse first with the endosomal network forming amphisomes en route to fusion with the lysosome to form the autolysosome. Achieving degradation capacity by autolysosomes is essential to carry a number of cellular tasks that include: quality control of organelles, turnover of aggregated proteins and combating bacterial infection in immune cells, among others (Deretic and Levine, 2009; Steele et al., 2015). For the most part, yeast and mammalian studies have shown that key regulators of autophagosome-lysosomes fusion include: Rab7, homotypic fusion and protein sorting (HOPS) complex, Vacuolar ATPases, SNAREs and the cytoskeleton. However, we will take this opportunity to distinguish the requirement of the V-ATPases in the coordination of autophagosome-lysosome fusion in Drosophila. Our laboratory has recently shown that in vivo knockdown of most of the subunits of the V0 and V1

vacuolar ATPase complex, required for the lysosomal and autolysosomal acidification, does not impair fusion of both compartments to allow autolysosome formation (Mauvezin et al., 2015). The data suggests that the pharmacological treatment routinely used in mammalian cell cultures studies to inhibit acidification might be acting trough a different or parallel mechanism to inhibit autophagosome-lysosome fusion. This highlights the importance of studying the regulation of autophagy in an in vivo context to elucidate novel regulators.

1. Rab7

Rab7 is a late endosome/lysosome associated protein. Among its cellular tasks it is required for endosomal maturation, lysosomal biogenesis and lysosomal function. Reduced function of Rab7 in mammalian cell culture studies, by RNAi or expression of a dominant negative Rab7-T121N, results in accumulation of Cathepsin D hydrolase and Mannose-6phosphate receptor at endosomes, failure of degradation of low density lipoproteins and impaired degradation of internalized viral components, among other phenotypic findings (Zhang et al., 2009). Altogether, these studies and similar studies in Drosophila and Ypt7, the yeast counterpart of Rab7, conclude that Rab7 is essential for the degradation of internalized cargo via endocytosis and at the lysosome. Among the regulators of Rab7, the Mon1-Ccz protein acts as a guanine nucleotide exchange factor (GEF) to promote activation of Rab7. Its function is conserved in yeast, flies and mammals (Nordmann et al., 2010; Yousefian et al., 2013).

Rab7 was the first Rab GTPase studied in the regulation of the lysosomal dependent pathway of autophagy. Two parallel studies showed that Rab7 was required for autophagy (Gutierrez et al., 2004; Jager et al., 2004). The Colombo group showed using a

dominant negative construct (Rab7-T121N) that inactivation of Rab7 resulted in formation of enlarged autophagosomes, impaired autolysosome formation and degradation of long-lived proteins. It should be noted that autolysosome formation was assayed using an old assay were dextran was used to label lysosomes and MDC (monodansylcadaverine) to label autophagosomes. Rather than the currently routinely used autophagosome marker Atg8 (LC3 in mammals) and the lysosomal marker Lysosome associated membrane protein (LAMP). In addition, they show that Rab7 is recruited to autophagosomes, even in a dominant negative state, in a starvation dependent manner. Interestingly, Jager et. al showed that knockdown of Rab7 resulted in a decrease in the number of Atg8 punctae while their quantification of electron microscope data showed an accumulation of late autophagic vesicles under starvation that failed to disappear after serum re-addition. These two opposite observations can be reconciled in a scenario were maturation of the autophagosomes is impaired resulting in their accumulation. However, quantification of the size of these compartments and combination of marker analysis for endocytic and autophagosomal structures would have helped in the interpretation of the role of Rab7 in these studies. Lastly, using LAMP1/2 deficient mouse embryonic fibroblasts (MEF) the group showed that the recruitment of Rab7 to autophagic vesicles was dependent on LAMP protein. The mechanistic relevance of these observation have not been elucidated to date but it suggests that the autophagic defects observed in LAMP deficient MEFs might be due to Rab7 recruitment defects at autophagic vesicles.

Recently, a Rab7 effector protein, PLEKHM1 was characterized using null fibroblast and cell culture studies (McEwan et al., 2015). Loss of this protein phenocopies the Rab7 associated phenotypes and results in impaired autophagosome-lysosome fusion, impaired maturation of autophagosomes, defective degradation of autophagosomal content and EGF receptor. Further analysis revealed that PLECKHM1 co-localizes with Rab7 and LAMP and is able to physically interact with LC3/Atg8 and the HOPS complex. This suggests that PKLEKHM1 coordinates with the HOPS and Rab7 the autophagosome-lysosome fusion event.

Lastly, Rab7 has been shown to be recruited at lipid droplets to mediate their degradation in Hep3b cells (Schroeder et al., 2015). The group led by MvNiven showed that Rab7 is required for the recruitment of multivesicular bodies and lysosomes at lipid droplets to allow their acidification. In addition, reduced Rab7 function resulted in degradation defects of autolysosomal content and accumulation of lipid droplets. Last, they showed that Rab7 mediated its autophagy-associated functions via the recruitment of its effector RILP.

2. SNARE Proteins

SNARE proteins belong to a family of proteins conserved in yeast, flies and mammals required for the fusion of vesicular compartments with target compartments. Functionally, they are categorized as v-SNARE and t-SNARE and are localized at the vesicular and target organelle, respectively. They perform their function by associating with tether complexes (such as the GARP, HOPS, COG and Exocyst), the Ras subfamily GTPases and SM proteins (Sly1, Vps33A/B, Munc18 and Vps45) to allow vesicular fusion events between all the cellular membrane compartments (Hong and Lev, 2014;

Shen and Mizushima, 2014). Their cellular roles include synaptic transmission, immune synapse function in response to pathogens, protein secretion and endosome-to-Golgi retrograde traffic used by plasma membrane and hydrolase sorting receptors.

SNARE proteins are essential during autophagy. It has been known for years that SNARE proteins Vam3, Vt1a and Vam7 mediate fusion and docking of autophagosomes at the yeast vacuole to allow autolysosome formation (Darsow et al., 1997; Fischer von Mollard and Stevens, 1999; Sato et al., 1998). However in higher eukaryotes a SNARE protein coordinating autophagosome-lysosome fusion was lacking until the recent discovery of syntaxin17 (Syx17). Parallel studies, in flies and mammalian cell culture revealed that Syx17 was required and necessary for autophagosome-lysosome fusion (Itakura et al., 2012; Takats et al., 2013). Both groups showed that loss of Syx17 lead to accumulation of mature autophagosomes that accumulated cytoplasmic cargo and failed to fuse with the endosome/lysosome compartment. Imaging analysis revealed that Syx17 is recruited at autophagosomes. Meanwhile, immuno-precipitation studies revealed that Syx17 mediates fusion via formation of a complex with SNAP-29 and Vamp8 in mammals and USNP/SNAP-29 and Vamp7 in flies. Vamp proteins localized at endosome/lysosomes allowing them to propose a model where Vamp SNAREs at endosomes and Syx17 on autophagosomes are linked via interaction with SNAP-29 to allow fusion and formation of autolysosomes. This opened a new door to evaluate the mechanisms regulating the movement of the Syx17 and Vamp containing compartments in the cell upon induction of autophagy. More recently, Atg14 was shown to bind the Syx17-SNAP-29 binary complex in HEK293 cells (Diao et al., 2015). Using in vitro

assays, they investigators showed that Atg14 promoted the fusion of protein-loaded liposomes containing Syx17-SNAP-19 and Vamp8, suggesting a role for Atg14 in the priming of autophagosomes preceding lysosomal fusion.

3. HOPS

The HOPS complex (composed of Vps11, Vps18 Vps16, Vps33A/B, Vps39 and Vps41) is a group of proteins required for endosomal biogenesis and homotypic fusion of vacuoles (Balderhaar and Ungermann, 2013). First identified as part of a comprehensive genetic screen evaluating the sorting of carboxypeptidase Y (CPY) and vacuolar morphology. The HOPS complex components and other candidates of this yeast screen were first named vacuolar protein targeting (Vpt), later changed to vacuolar protein sorting (Vps), due to the observed defects in CPY mis-localization towards the plasma membrane, CPY enrichment at the Golgi and vacuolar morphological changes in Vps mutants (Banta et al., 1988; Raymond et al., 1992; Robinson et al., 1988). In Drosophila, subunits of the HOPS complex were identified in eye color mutants due to their role in lysosome and lysosome related organelle biogenesis and function (Akbar et al., 2009; Pulipparacharuvil et al., 2005; Sevrioukov et al., 1999; Warner et al., 1998). Additional work in flies has shown a role for the HOPS complex in mTOR signaling regulation and phagolysosome and lysosomal function in hemocytes, flies immune system cells (Swetha et al., 2011; Takats et al., 2015). Importantly, the role of the HOPS complex in cellular processes such as lysosomal maturation and function, autophagy and mTOR regulation are conserved in yeast, flies, worm and mammals (Akbar et al., 2009; Flinn et al., 2010; Lindmo et al., 2006; Manil-Segalen et al., 2014; Ruan et al., 2010; Swetha et al., 2011; Wartosch et al., 2015).

The HOPS complex executes lysosomal maturation and fusion via interactions with SNAREs and Rab GTPase proteins (Balderhaar and Ungermann, 2013). Vps39 and Vps41 subunits of the HOPS complex are effectors of the late endosomal Rab7 GTPase in its GTP bound state. The Mon1-Ccz Guanine exchange factor (GEF) mediates the activation of Rab7 resulting in the recruitment of the HOPS complex to the endosomal pathway allowing its maturation and the tethering of juxtaposed membranes required for vesicle fusion with target membranes (Wang et al., 2011). Recently, in mammalian cell culture studies and in flies, this tether complex was shown to be required for autophagosome lysosome fusion (Jiang et al., 2014a; Takats et al., 2014). Immuno-precipitation studies showed that each protein of the HOPS interacted with the autophagosomal SNARE syntaxin17 (described above). Additionally, their depletion resulted in accumulation of autophagic vesicles enriched with syntaxin17 and impaired degradation under basal and starvation conditions suggesting impaired lysosomal function. Importantly, loss of syntaxin17 did not affect lysosomes like the HOPS subunits knockdown experiments, further supporting a high degree of specificity for these SNAREs in the regulation of autophagy. Imaging analysis revealed that the HOPS complex is localized in two separate pools, autophagosomes and lysosomes. Meanwhile, syntaxin17 was localized at autophagosomes under starvation. This suggests that the HOPS complex may coordinate the fusion event by physical interaction with syntaxin17 at autophagosomes from the lysosome or from autophagosomes via physical interaction with other HOPS proteins at the lysosome.

The discovery of these set of proteins coordinating maturation of autophagosomes and autophagosome-lysosome fusion opened a new door to research aimed at the understanding of regulation of the compartments containing syntaxin17, Rab7 and the HOPS complex under distinct nutrients states. Whether Ras GTPases, SM protein and other tether proteins regulate the shift of these proteins from basal states to starvation remains to be seen. Interestingly the HOPS complex has been shown to be an effector of Rab7, while argued by others to be a GEF for the GTPase function of Rab7. However in the light of this data, one should wonder whether Rab7 coordinates or mediates autophagosome fusion together with syntaxin17 and the HOPS complex.

e. Autophagic-lysosome reformation

Until recently, autophagy was believed to terminate upon formation of an autolysosome and the degradation of its cargo. However, researchers noted that the size of lysosomes, via monitoring of Lysosomal associated membrane glycol-protein (LAMP), was increased after four hours of amino acid deprivation and restored to basal levels after prolonged starvation in normal rat kidney (NRK) cells (Yu et al., 2010). Careful characterization of the LAMP marker revealed that after the first four hours of starvation lysosome started to form tubular structures. Subsequently, extrusion of vesicles from the tubular structures resulted in the production of new proto-lysosomes that lacked lysosomal hydrolases. Examination of the phosphorylation of S6 kinase, a downstream target of mTOR, revealed that the protrusion of these tubular structures correlated with re-activation of mTOR after its down-regulation upon four hours starvation. In addition Yu et al. showed that the re-activation of mTOR was dependent on autolysosomal

degradation, as protease inhibitors inhibit the tubule formation and resulted in size increase of autolysosomes under prolonged starvation. The protrusion of these tubular structures was also dependent on GTP-GDP nucleotide cycling on Rab7, as a GTP locked version blocked tubule formation and enlarged autolysosomes size (Yu et al., 2010). Altogether, the group showed a novel mechanism to promote the lysosomal biogenesis under prolonged starvation using autophagy as an alternative source of nutrients to re-activate mTOR and avoid cell death, they named the phenomena Autophagy-Lysosome Reformation (ALR).

Further insight into ALR came when the Yu laboratory carried a mass-spectrometry screen using NRK cells of purified tubules to uncover novel proteins regulating their formation and budding (Rong et al., 2012). Via elegant biochemical and imaging techniques, the group showed that conversion of PIP4 to PIP4,5 by the PIP5 isoform B kinase was important for tubule initiation and ALR, while isoform A of PIP5 kinase was required for the pinching of vesicles from tubules to make proto-lysosomes. In addition, they showed that clathrin is recruited to autolysosomes to coordinate ALR and promote budding of vesicles from tubules via recruitments of the adaptor proteins AP2 and AP4. However, evaluation of mTOR activity during ablation of these proteins was lacking in the study leaving unanswered whether structural integrity of the tubules is a requirement for mTOR activation. Whether structural stability and formation of these tubules is a downstream or upstream regulation of mTOR signaling remains to be seen.

Interestingly, a separate group working with Hep3B hepatocytes showed a requirement for dynamin2 in ALR during a specialized form of autophagy, called lipophagy (Schulze

et al., 2013). Mechanistically, the group led by McNiven showed that dynamin2 is physically recruited at autolysosomal tubules to regulate the scission of vesicles during the formation of proto-lysosomes. Inhibition or depletion of dynamin resulted in excessive tubulation of autolysosomes and a reduction in lipid droplet degradation supporting a role in ALR and autophagy. Interestingly, the fact that ablation of dynamin enhanced the accumulation of LD poses the question of whether ALR is a requirement for continuous degradation under prolonged starvation or if ALR regulates degradative capacity via an uncovered feedback mechanism.

The in vivo relevance of ALR was show when fibroblasts from patients with lysosomal storage diseases (LSDs) and the role for the spinster protein were used to study autophagy (Rong et al., 2011; Yu et al., 2010). Spinster is a lysosomal efflux permease that when mutated or ablated results in expansion of the lysosomal compartment and carbohydrate accumulation. These are similar to pathological findings found in LSDs when an enzyme or protein mutation results in the accumulation of complex sugars, proteins or lipids. In addition, ablation of spinster resulted in impaired re-activation of mTOR and defective ALR. This was shown to be dependent on its sugar transport activity, as an over-expression of a point mutation in the spinster channel impaired ALR. The findings evaluating spinster prompted the Lenardo group to evaluate fibroblasts derived from patients with Fabry's, Scheie and Aspartogluminuria LSDs (Yu et al., 2010). These patients' cells contained enlarged lysosomal compartments and defective mTOR re-activation under prolonged starvation, both findings consistent with impaired ALR. Given that lysosomal function is required for ALR and functional ALR is required

to maintain degradation under prolonged starvation. It is tempting to speculate that a common detrimental factor found in all LSDs is impairment of ALR. This might explain why it is not uncommon to find defects in degradative capacity regardless of the mutation responsible for the disease in LSDs.

These findings added a new step in the autophagic pathway and increased the complexity of roles mTOR plays in its regulation. The group showed that mTOR activation is required for ALR and imaging studies showed that mTOR is at the autolysosomes (Yu et al., 2010). However, the molecular mechanism responsible for the activation of mTOR and whether ALR uses the Rag or other Ras subfamily GTPase to activate mTOR or alter its localization under distinct nutrient states is still unknown and unexplored. Understanding the dynamics mTOR and other traffic regulators might be playing during ALR remains to be shown and may help in the understanding of genetic and metabolic diseases.

III. mTOR pathway

a. mTOR overview

The mechanistic target of rapamycin (mTOR) is a highly conserved negative regulator of autophagy and a positive regulator of cell growth and protein synthesis (Cornu et al., 2013). The positive cellular role mTOR exerts on cellular growth is essential for organismal development in response to environmental cues such as nutrients and growth factors (Fig. 3, briefly summarized). Additionally, mTOR regulates aging and lifespan in cells via modulation of autophagy and cellular metabolism. Therefore, it is not surprising that deregulation of mTOR has been linked to disease states such as cancer, obesity and

diabetes (Cornu et al., 2013; Dazert and Hall, 2011). In cells, Tor is found on two distinct complexes mTORC1 (hereafter mTOR) and mTORC2. Complex 1 is composed of Raptor, lst8, Deptor, Pras40 and Tor and is responsible for regulating cell growth via ribosomal and protein synthesis, nutrient uptake and inhibition of autophagy (Cornu et al., 2013; Takahara and Maeda, 2013). Meanwhile, complex 2 is composed of Rictor, lst8, sin1, Protor1/2, Deptor and Tor. The cellular functions of mTORC2 are less well understood and characterized but its dependency on growth factors, such as insulin, is well established (Cornu et al., 2013; Takahara and Maeda, 2013). Downstream targets include S6 kinase, 4E-BP and Atg1.

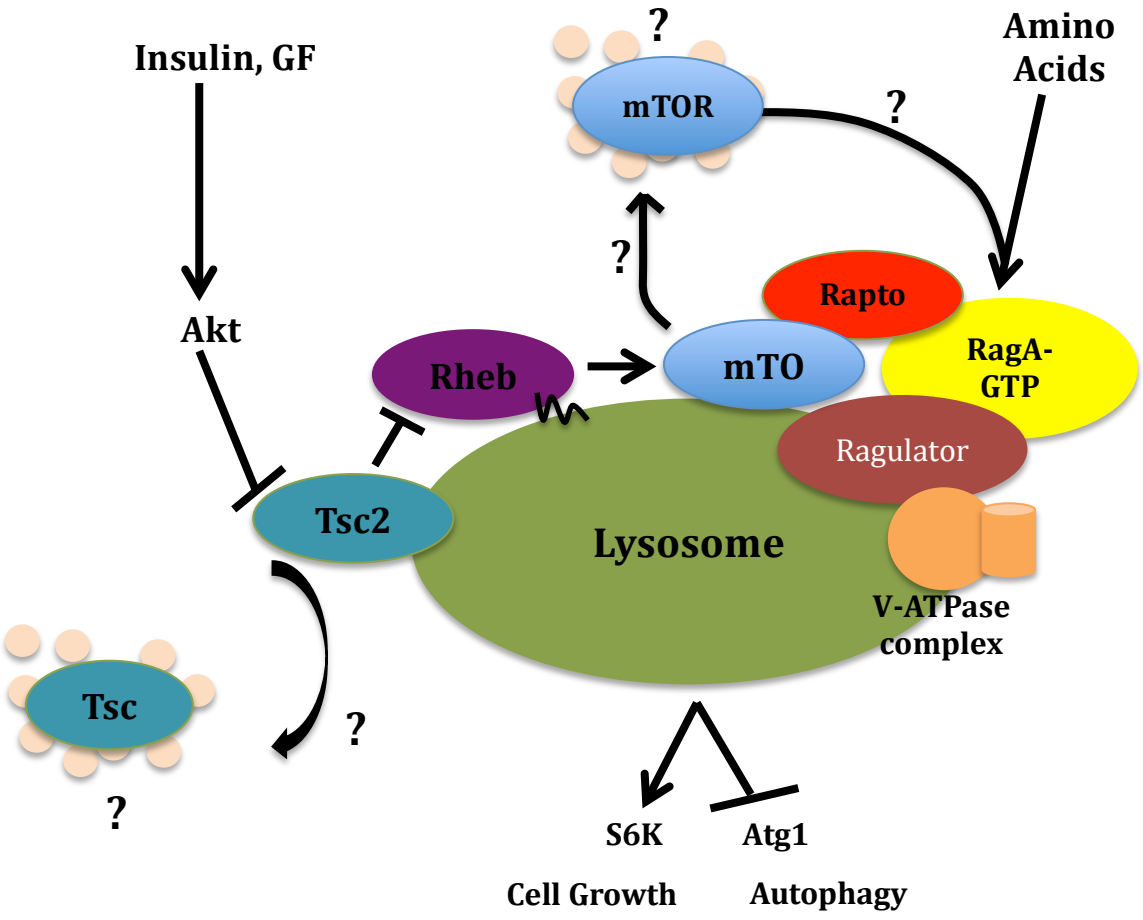


Figure 3. Insulin and amino acid dependent activation of mTOR is integrated at the lysosomal surface.

Amino acids and insulin positively regulate mTOR signaling to promote cell growth and inhibition of autophagy. Amino acids enter cells through transporters and are sensed via an inside-out mechanism dependent on the V-ATPase complex at the lysosomal surface. This results in the activation of the Rag GTPases, recruited at the lysosome by the Ragulator complex, and the recruitment of the mTOR binding partner Raptor by Rag GTPases. Ultimately, Raptor and Rag GTPases promote the recruitment of mTOR at the lysosomal surface to be activated by the GTPase Rheb. In parallel, insulin and/or growth factor dependent activation of mTOR results in the phosphorylation of Tsc2, by Akt, and the exit of Tsc2 from the lysosomal surface promoting mTOR activation by Rheb. However, the compartments Tsc2 is trafficked upon growth factor stimulation and the compartment mTOR is trafficked from upon amino acid stimulation are currently

unknown. In addition, the regulators of these traffic events, for Tsc2 and mTOR, remain a mystery.

b. Upstream regulators of mTOR

1. Rheb

Rheb (Ras homologue enriched in brain) is a member of the Ras subfamily of GTP-binding proteins required for cellular growth from yeast to mammals. In Drosophila, mutations resulting in loss of function of Rheb result in a cell-autonomous reduction in cell size and organismal lethality. Conversely, its over-expression results in a cell autonomous increase in cell mass and size and inhibition of autophagy (Aspuria and Tamanoi, 2004; Patel et al., 2003; Saucedo et al., 2003). Interestingly, the growth promoting effects exerted by Rheb are not the result of nutrient or glucose uptake, but rather the activation of mTOR to promote ribosome and protein synthesis (Hall et al., 2007). Mechanistically, Rheb can bind the catalytic domain of Tor and the mTOR complex 1 component Lst8 directly (Long et al., 2005a). Genetic epistasis analysis has positioned Rheb downstream of the TSC complex but upstream of Tor (Aspuria and Tamanoi, 2004; Patel et al., 2003; Saucedo et al., 2003). Together, these findings support a positive regulatory role of Rheb in the regulation of cellular growth via the activation of mTOR signaling.

Studies in mammals have supported and established a conserved role for Rheb in the regulation of growth. Rheb knockout animals are lethal and Rheb null cell lines show decreased activation of mTOR, as assayed by S6 kinase and 4E-BP phosphorylation. Conversely, over expression of Rheb results in hyper activation of mTOR and is sufficient to activate it independently of its cellular localization (Aspuria and Tamanoi,

2004). The functional consequences of these have not been explored. Importantly, Rheb conditional knockout mice have allowed the study of Rheb in developmental processes shedding light of its role in cardiac development, neuronal myelination, T-cell development and spermatogenesis, among others (Heard et al., 2014). Interestingly, some of these functions like the role of Rheb in T-cell development have been shown to be mTOR independent.

Rheb activity can be regulated via modulation of its nucleotide bound state (Aspuria and Tamanoi, 2004). Studies in flies and mammals have revealed that the GTP bound state of Rheb is negatively regulated via direct interaction with Tuberous sclerosis 2 (Gigas in flies) and its GTP activating protein (GAP) domain (Castro et al., 2003; Long et al., 2005a; Long et al., 2005b; Tee et al., 2003; Zhang et al., 2003). Conversely, a study in flies uncovered the guanine nucleotide exchange factor (GEF) positively regulating Rheb. The translationally controlled tumor protein (TCTP) promotes the exchange of GDP for GTP on Rheb resulting in its activation. Importantly, TCTP mutants' phenocopy Rheb mutants further supporting a role for this GEF in the regulation mTOR-signaling and growth (Hsu et al., 2007). Further characterization of TCCP established a direct interaction between Rheb-TCTP and genetic epistasis analysis positioned the protein upstream of S6 kinase and epistatic to TSC2 and Rheb.

Structurally, Rheb possess a C-terminal CAAX domain and an N-terminal GTPase region. The CAAX region is critical for Rheb to become farnesylated at a cysteine residue after cleavage of the –AAX region. Farnesylation of Rheb ensures its mobilization to the endomembrane system (Heard et al., 2014). For years, localization of

Rheb proved challenging and to date its localization remains controversial as different cell types, methods and tools have been used to detect it. However, in 2008, the Sabatini laboratory managed via biochemical and imaging methods to localize functionally Rheb at the lysosomal surface (Sancak et al., 2010; Sancak et al., 2008). The localization of Rheb at the lysosome is essential for mTOR activation, positioning the lysosome as a signaling platform in the cell for the integration of growth factor and nutrient signals.

Additional open questions remain regarding Rheb function and its cellular roles. For example, one of the earliest studies characterizing Rheb in flies in flies found the up-regulation of Rheb mRNA under starvation conditions (Saucedo et al., 2003). A paradoxical finding since Rheb established function is under basal states when growth factors and nutrient availability are available to activate mTOR. This unexplored finding suggests that Rheb might be playing a regulatory feedback role during starvation to activate mTOR, which would be supported by our knowledge that mTOR can be recruited to the autolysosome during starvation and that Rheb localizes at the lysosomal surface to activate mTOR. Alternatively, it could point to an mTOR independent role or a parallel mechanism to sustain mTOR activation to ensure cell viability under starvation conditions. It is well established that Rheb is required to activate mTOR, an event shown to occur at the lysosomal surface where both reside upon growth factor and amino acid stimulation. However, what is the functional consequence of Rheb activation on mTOR, apart from their physical binding, or downstream targets is unclear. In addition, whether the GTP/GDP bound ratio of Rheb has an impact on mTOR localization and activation remains to be elucidated.

2. Akt

Akt (also called Protein kinase B) has a wide array of cellular roles that include growth, cell survival, proliferation and cell migration (Li and Marshall, 2015). One of the earliest observations of Akt pro-survival roles was observed in flies, as mutation of the Akt gene resulted in apoptosis of embryos and was suppressed by parallel removal of caspases (Staveley et al., 1998). In wing discs Akt levels have been correlated with tissue growth and its inactivation resulted in a cell autonomous decrease in growth (Verdu et al., 1999). Careful evaluation of Akt null flies, by stage dependent heat shock induced expression of a rescue construct to surpass lethality in null animals, revealed that animals were smaller than controls at all stages (Scanga et al., 2000). This supported a role for Akt in growth, phenocopying other members of the insulin/PI3K/PTEN cascade mutants. Mechanistically, Akt was shown to promote cell growth by direct phosphorylation of Tsc2, a negative regulator of mTOR, resulting in impaired interaction with its binding partner Tsc1 and subsequent activation of mTOR (Potter et al., 2002). Interestingly, in flies a study in the Pan laboratory showed that transgenes of Tsc2 with mutated Akt phosphorylation residues could rescue lethality of Tsc2 mutant animals (Dong and Pan, 2004). This suggested that the phosphorylation events mediated by Akt are not required for organismal development and survival in Drosophila. This is in contrast to mammalian findings were Akt phosphorylation residues in Tsc2 are critical for growth and viability (Inoki et al., 2002). One possible explanation was that Akt residues in Tsc1, the binding partner, could compensate functionally. However, mutations of the Akt phosphorylation sites in Tsc1 and 2 were still able to rescue the lethality in Tsc mutant flies (Schleich and Teleman, 2009). Therefore, it is possible that the phosphorylation of Tsc2 serves an

auxiliary role for enhancement of mTOR activation in flies and/or that it directly regulates mTOR in a Tsc2 independent manner. Further characterization of Akt in flies have revealed roles in a number of cellular and developmental processes that include hypoxia signaling, neuronal development, developmental timing and oogenesis via regulation of follicle size (Benmimoun et al., 2012; Dragojlovic-Munther and Martinez-Agosto, 2012; Lavery et al., 2007; Walkiewicz and Stern, 2009).

Structurally, Akt is composed of an N-terminal pleckstrin homology (PH) domain, a central kinase domain and a C-terminal regulatory domain. The PH domain binds PIP3 and allows the recruitment of Akt to the plasma membrane were it promotes Akt dependent signal transduction. Here, Akt is a direct target of PDK1 to promote mTOR activation in flies and mammals (Rintelen et al., 2001). Meanwhile, this activation is antagonized by the phosphatase PTEN (Gao et al., 2000). Phosphorylation of Akt at Threonine-308 in the kinase region and Serine-473 in the regulatory region by PDK1 and mTORC2, respectively, are required for full activation of its kinase activity resulting in its localization at the plasma membrane (Sarbassov et al., 2005; Zhang et al., 2015). Akt activation results in a signaling relay to downstream targets that include GSK3B and the transcription factor FOXO, among others.

3. Amino acids

Amino acid-dependent activation of mTOR is conserved from yeast to mammals, suggesting that it might have been the first mechanism to promote cell growth and the regulation of energy homeostasis in eukaryotes. The hunt to elucidate the proteins responsible for the integration of this extracellular signal led to the discovery and

characterization of the Rag GTPases by two independent groups (Kim et al., 2008; Sancak et al., 2008).

Rag GTPases are members of the Ras subfamily of GTPases. In flies there are two Rag proteins, RagA and RagC. Meanwhile, duplications events in higher eukaryotes resulted in four Rag proteins RagA/B and RagC/D. In mammals and flies heterodimers of Rag proteins, RagA-GTP/RagC-GDP and RagB-GTP/RagD-GDP, execute their function in a nucleotide dependent manner to activate mTOR in response to amino acids (Groenewoud and Zwartkruis, 2013; Jewell et al., 2013). The Sabatini group has shown that Rag GTPase activation in response to amino acids results in the mobilization of mTOR at the lysosomal surface to be activated by Rheb to promote cellular growth. Genetic analysis in flies revealed that loss of RagA or over-expression of dominant negative RagA results in reduction in cell size under basal states. Meanwhile, expression of a constitutive active version of RagA inhibits autophagy and promotes cell growth under starvation further supporting their role in mTOR activation and growth. The physiological relevance of this pathway was shown using RagA-GTP knock-in mice, revealing they develop normally but die after birth as these period is accompanied by starvation and inhibition of mTOR. Knock-in mice fail to down regulate mTOR and induce autophagy for de novo production of glucose and amino acids during the neonatal period (Efeyan et al., 2013).

Amino acid-dependent activation of mTOR occurs and is regulated at the lysosomal surface. Mechanistically, amino acids are sensed in an inside-out mechanism by the vacuolar V-ATPases at the lysosome (Zoncu et al., 2011). In turn, this results in the recruitment of the Ragulator complex (C7orf59, HBXIP, MAPKSP1, ROBLD3 and

c11orf5) to the surface of the lysosomal via a physical interaction with the Rag GTPases upon amino acid stimulation. At this point, the Ragulator serves as the Guanine Exchange Factor (GEF) of RagA, to promote the exchange of GDP for GTP resulting in its activation. In parallel, folliculin (FLCN) a GTP-hydrolysis activating protein (GAP) is recruited to the lysosomal surface under amino acid stimulation to mediate hydrolysis of GTP to the GDP bound state of RagC (Tsun et al., 2013). Activation of the Rag protein heterodimer (RagA-GTP/RagC-GDP) results in the translocation of mTOR to the lysosomal surface to encounter Raptor, which can interact with RagA. Recruitment of mTOR to the lysosomal surface results in its activation by Rheb (Sancak et al., 2010; Sancak et al., 2008). However, and potentially, the biggest question remaining is who is mobilizing mTOR during distinct nutrient states and where is it being translocated in the absence of nutrients and growth factors.

4. Insulin Signaling

The main cellular task of mTOR in cells is to serve as an integration center for upstream activators that include: cytokines, mitogens, nutrients and hormones such as insulin (Dibble and Manning, 2013).

Insulin signaling is initiated upon systemic insulin binding to the tyrosine kinase insulin receptor, InR, in response to a nutrient stimulus at the plasma membrane (Dibble and Manning, 2013; Hietakangas and Cohen, 2009). In flies, eight Drosophila insulin like-peptides (dilp) have been found. Of these dilp 2, 3 and 5 have been postulated to carry insulin growth promoting roles with dilp2 and dilp3 responding to systemic levels of amino acids and sugar, respectively (Kannan and Fridell, 2013; Kim and Neufeld, 2015). Upon insulin binding, cross phosphorylation of the InR recruits the insulin receptor

substrate (IRS), *chico* in flies, to be phosphorylated and activated. IRS activation results in the recruitment of class I PI3 kinase at the plasma membrane. At this point, phosphatidyl-4, 5-biphosphate is phosphorylated by this kinase to synthetize phosphatidyl-3,4,5-biphosphate (PIP3) that will allow the activation of mTOR (Carracedo et al., 2008).

The resulting elevated levels of PIP3, following class I PI3 kinase activation, allow recruitment of pleckstrin homology (PH) domain containing proteins. Among these proteins, phosphoinositide-dependent kinase-1 (PDK1) and Akt are recruited at the plasma membrane were PDK1 activates Akt via phosphorylation (Li and Marshall, 2015). The phosphatase with Tensin Homology (PTEN) protein, a common mutated protein in cancer, antagonizes the action of Class I PI3K via de-phosphorylation of PIP3 to PIP2.

Akt activation results in the downstream activation of mTOR. Akt achieves this task in two ways. First, it directly phosphorylates TSC2, the Rheb GTPase GAP, allowing the disinhibition of Rheb and activation of mTOR. Secondly, it phosphorylates PRAS40 allowing mTOR binding and interaction with its substrates (Brown and Toker, 2015; Dibble and Cantley, 2015). Once Rheb is activated in its GTP bound state it directly activates mTOR. Subsequently, mTOR phosphorylates downstream targets S6 kinase, 4E-BP and Atg1 promoting cell growth, protein synthesis and autophagy inhibition (Shimobayashi and Hall, 2014). In parallel, the stimulus provided by insulin results in the removal of the TSC complex away from the lysosome allowing the activation of mTOR

by Rheb at the lysosomal surface (Menon et al., 2014). The compartment Tsc2 is mobilized in response to growth factors is unknown.

c. Lysosomal positioning and mTOR localization

The reciprocal regulation between autophagy and mTOR under distinct nutrient states is incompletely understood. This prompted the Rubinsztein laboratory to evaluate and monitor the localization of mTOR under distinct nutrient states in HeLa cells (Korolchuk et al., 2011). They decided to use a starvation protocol reflecting physiological conditions by removing amino acids and serum for 5 hours. They observed that lysosomes acquired a perinuclear position upon starvation (amino acid/serum withdrawal) while re-addition of nutrients lead to cytoplasmic dispersal of the same compartment. This phenomenon was correlated with co-localization between lysosomes and phospho-Akt staining upon re-addition of nutrients and absent during starvation. Surprisingly, mTOR was not released from the lysosomal compartments during their experiments, but rather trafficked together with the lysosomes. Further characterization revealed that the dispersal of lysosomes upon nutritional stimulus was dependent on microtubules, kinesins (KIF1B and KIF2) and the Arl8 GTPase. Last, they showed that the movement of the lysosomes was accompanied by changes in the pH of the lysosomes. As lysosomes move inwards upon starvation pH dropped to allow formation of autolysosomes and as they dispersed outwards upon nutrient re-addition pH increased. The group was able to describe a novel mechanism, mimicking physiological conditions, were upon removal of nutrients mTOR is removed from its upstream regulators (InR and Akt) on lysosomes to allow induction

of autophagy. Conversely, nutrient stimulus resulted in mobilization of mTOR on lysosomes to become activated at the plasma membrane.

The findings of the Rubinsztein group are in contrast to models proposed by the Sabatini and Hall groups in which mTOR and Tsc2 localization at the lysosome are responsive to nutrient and growth factor stimulation. Rather than continuously being present at the lysosomal surface and their activity influenced by lysosomal positioning (Groenewoud and Zwartkruis, 2013; Jewell et al., 2013; Menon et al., 2014). These findings provide a physiologic example of the regulation of mTOR localization in response to an environmental stimulus, starvation and re-addition of nutrients. Taking into consideration the accepted localization of Rheb at the lysosome provided by the Sabatini group and the Rubinsztein group findings that mTOR is never released from the lysosomes. It begs the question of why is mTOR activity abolished under starvation if autolysosomal activity is an alternative source of amino acids and sugars that could potentially activate mTOR under their experimental setting. Further characterization to pinpoint the localization of other mTOR activators, the Rag GTPase/Ragulator network and Tsc2 remains to be elucidated. These may shed light onto the regulatory mechanisms governing mTOR signaling under this physiologic experimental setting

IV Rab GTPases and cellular trafficking

a. Overview

Rab family proteins, 33 in Drosophila and 70 in mammals, are small lipidated G proteins that regulate vesicular traffic, defined here as directed vesicle movement between organelles and other cellular compartments in cells (Hutagalung and Novick,

2011; Mizuno-Yamasaki et al., 2012; Zhang et al., 2007). Their function is executed in a nucleotide-dependent manner via recruitment of effector proteins and tethering of molecules to promote fusion and fission at organelle surfaces (Hutagalung and Novick, 2011; Mizuno-Yamasaki et al., 2012). Rab GTPases have been shown to regulate anterograde and retrograde trafficking between ER and Golgi, endosomal to lysosomal maturation and protein secretion among other cellular tasks (Hutagalung and Novick, 2011; Mizuno-Yamasaki et al., 2012). Given the exquisite and specific role of these GTPases in cellular trafficking it is not surprising that their function is required for proper development and signaling in organisms ranging from yeast to mammals. However, most of the research on Rab mediated trafficking has been focused on the secretory and endosome/lysosomal pathway (Hutagalung and Novick, 2011; Mizuno-Yamasaki et al., 2012). A potential role for Rab mediated traffic in other cellular pathways, such as autophagy and insulin/mTOR signaling regulation remains incompletely understood and unexplored.

The role of Rab GTPases in the regulation of autophagy has begun to emerge in recent years in yeast and mammalian cell culture, revealing that a small subset of Rab GTPases regulate this process at different stages. At the beginning steps, Rab 1 is required for IM synthesis and was recently shown to regulate mTOR localization at the Golgi in a Rag-independent fashion (Thomas et al., 2014; Zoppino et al., 2010). In yeast it is required for the formation of autophagosomes as it is found on the same vesicles as Atg9 and is required for the activation of Atg1 (Kakuta et al., 2012; Shirahama-Noda et al., 2013; Wang et al., 2013b). Similarly, Rab32 and Rab33 are required for the

recruitment of Atg proteins and the synthesis of the IM (Hirota and Tanaka, 2009; Itoh et al., 2008). Meanwhile, under growth factor limitation or starvation Rab5 is activated by the class I PI3K subunit p110 to promote activation of class III PI3K kinase Vps34 to promote autophagy induction and PI3P synthesis (Dou et al., 2013; Ravikumar et al., 2008). Rab11 has been associated with autophagy at two distinct steps, first regulating AP formation via sequestration of Atg1 at recycling endosomes and allowing fusion of maturing APs with multivesicular bodies (Chua et al., 2011; Fader et al., 2008; Longatti et al., 2012). At the last step, Rab 7 was shown to be required for fusion of late endosomes/lysosomes with mature APs to allow termination of the process and cargo degradation (Gutierrez et al., 2004; Jager et al., 2004). As most of this work has been performed in yeast and mammalian systems, evaluation in a more complex system than yeast but with less gene redundancy than mammals, such as Drosophila, awaits.

b. Endosome-to-Golgi traffic

Retrograde traffic is used in cells to shuttle proteins and lipids between the Golgi network and the endomembrane system. The pathway is commonly used by signaling receptors to escape lysosomal degradation and as a traffic route step after their internalization as they recycle towards the plasma membrane (Chia et al., 2013; Johannes and Popoff, 2008). Endosome-to-Golgi trafficking has been shown to be important for a plethora of cellular tasks that include receptor signaling, glucose homeostasis, protein secretion, lysosomal biogenesis and function and autophagy among others (Chia et al., 2013; Johannes and Popoff, 2008). The importance of the pathway is exemplified by its hijack by pathogens and bacterial toxins to gain control of the cellular machinery. The pathway starts via

endocytosis at the plasma membrane followed by sorting of proteins at endosomes and their subsequent segregation on distinct endosomal domains before being sent to their final destinations (Chia and Gleeson, 2011). Although in concept the general mechanism for retrograde traffic are well understood from yeast to mammals the complexity due to distinct parallel pathways at the sorting step on endosomal platforms, the parallel pathways regulating a similar traffic route and the proteins regulating the pathway make the field complex and challenging. The proteins regulating retrograde trafficking include: dynamin, AP-1, epsin, GGA, Arl and Rab GTPases, tethering complexes (Retromer, GARP and COG) and SNAREs.

Early studies in yeast demonstrated endosome-to-Golgi trafficking was essential for the Cvt pathway, a selective type of basal autophagy in yeast, but not starvation induced autophagy regulation (Ohashi and Munro, 2010; Reggiori et al., 2003; Ye et al., 2014). This prompted the Munro laboratory to carry a comprehensive analysis of double mutants for regulators of endosome-to-Golgi trafficking in yeast to examine if the lack of retrograde traffic routes as regulators of autophagy was due to functional redundancy. The group combined mutants on lobe B of the conserved oligomeric complex (Cog; composed of Cog5-8), shown to regulate endosome-to-Golgi traffic, with SNAREs (tlg2 and gos1) regulating distinct and independent retrograde traffic routes. Importantly, the single mutants had been shown to have defects in the Cvt pathway but not starvation-induced autophagy. Surprisingly, double mutants between Cog lobe B subunits and each independent SNARE displayed defects under starvation-induced autophagy. The data suggested functional redundancy in retrograde traffic routes from endosome-to-Golgi

under starvation-induced autophagy. Similar observations were obtained when Cog lobe B mutants were combined with sorting nexins (Snx), Atg20 and 24, previously shown to harbor defects in Cvt pathway and endosome-to-Golgi trafficking. In addition, the group showed that this might be a general phenomena for endosome-to-Golgi proteins affecting the Cvt pathway as combination of mutants of a retrograde route regulated by the VFT/Vps51-4 and Ypt6 displayed defects in starvation induced autophagy when combined with Atg20 or Atg24 mutants. Further characterization of different combinations of double mutants revealed that they were required for the traffic of Atg9 to the nascent autophagosome under starvation conditions. This opened the autophagy field to the analysis of proteins regulating retrograde routes and highlights the robustness for these traffic regulators in cells.

Golgi associated retrograde protein (GARP) forms a tether complex that regulates the fusion of retrograde vesicles trafficking from endosomes to the Golgi. The complex is composed of Vps51-54 and it conserved from yeast to mammals. In mammals and yeast the complex can bind the GTPase Rab6 and the SNAREs tlgp1, sytaxin6, syntaxin16 and Vamp4 to carry mediate the transport and docking of vesicles (Liewen et al., 2005; Perez-Victoria and Bonifacino, 2009; Siniossoglou and Pelham, 2001). Mutations of GARP components in C. elegans and mammalian cell culture result in aberrant lysosomal morphology (Luo et al., 2011; Perez-Victoria et al., 2010). In mammals, ablation of the complex results in impaired retrieval of M6PR, TGN6 and Shiga toxin from endosomes towards the Golgi (Chia and Gleeson, 2011). In addition, the Bonifacio group recently showed that the GARP complex is required during autophagy. Loss of Vps52/Ang2

resulted in accumulation of autophagic vesicles, mis-sorting of Cathepsin D hydrolase and impaired degradation of the autophagic cargo p62 (Perez-Victoria et al., 2010). They showed the GARP mediates the retrograde retrieval of M6PR from endosome to the Golgi via physical interactions with the GTPases Arl1 and Rab6 (Perez-Victoria and Bonifacino, 2009; Perez-Victoria et al., 2008; Perez-Victoria et al., 2010). Similar studies in yeast have implicated the GARP complex in the regulation of autophagy and the Cvt pathway (Ohashi and Munro, 2010; Reggiori et al., 2003; Ye et al., 2014).

Retromer is perhaps one of the best-studied coat complexes regulating retrograde traffic from endosomes towards the trans-Golgi network (TGN). Retromer is composed of Vps26/Vps29/Vp35 and has been evolutionarily conserved from yeast to mammals. Functionally, in mammals, retromer forms distinct complexes with the Bar containing-Snx1/2 or –Snx4/5/6 allowing the complex to carry curvature of membranes destined to retrograde traffic towards the Golgi and/or plasma membrane. The complex has been localized at endosomes to regulate retrograde traffic towards the Golgi. Although it has also been shown to regulate endosome to plasma membrane trafficking in cells. Retromer co-localizes with the late endosomal marker Rab7 and regulates the retrograde traffic of cargos that include: M6PR, sortilin and Shiga toxin (Lu and Hong, 2014). In addition, Retromer can recruit Wiskott-Aldrich syndrome and scar homolog protein (WASH) to coordinate actin nucleation events and AP-1 to coordinate its fusion events.

Retromer is essential for organismal development. Mutations in flies cause lethality and defects in the retrograde trafficking of wingless binding protein resulting in defective secretion of wingless (Lu and Hong, 2014). Point mutations in the retromer subunit

Vps35 have also been associated with Autosomal dominant Parkinson's disease. Expression of the point mutation was shown to impair autophagy and have decrease binding of WALS resulting in defective recruitment of the protein at endosomes (Lu and Hong, 2014). However, the contribution of retromer during autophagy is controversial. A separate study by the Dikic group, using USO cell lines, showed that knockdown of retromer subunit Vps29 did not affect autophagy progression and co-localization between Vps29 and LC3/Atg8 under starvation induced autophagy was not detected (Popovic and Dikic, 2014). However, it is entirely possible that retromer contribution to autophagy could be tissue dependent and functionally redundant in mammals as has been shown in yeast (Ohashi and Munro, 2010). Therefore, the role of retrograde traffic during autophagy is incompletely understood in higher eukaryotes and could shed new insight in the regulation of autophagy and disease states such as Parkinson's disease.

c. Lysosomal function

In 1955, de Duve and collaborators described for the first time the lysosome as an organelle harboring hydrolytic enzymes using liver cells (Coutinho et al., 2015). Today we know that lysosomes harbor lipases, hydrolases, nucleases, phosphatases and an array of lysosomal trans membrane proteins essential for its functionality. The synthesis of these enzymes and structural proteins relies exclusively in the biosynthetic role provided by the ER and Golgi network. In parallel, lysosomes are dependent on correct sorting from the Golgi in anterograde and retrograde fashion towards endosomes and plasma membrane.

We have gained great insight on lysosomal function from the studies of lysosomal storage diseases. Characterization of the single genes mutated in this group of diseases has revealed their function in degradation of macromolecules, as they accumulate when the protein is mutated, or in structural role maintaining morphology of the lysosomes (Appelqvist et al., 2013). This has allowed the development of gene therapy and drugs for the treatment of these diseases.

d. Hydrolase Sorting

Hydrolase sorting is a multistep process in cells to ensure their correct processing and delivery to the lysosome. Enzymes are synthesize at the ER and directed towards the Golgi complex with minor post-translational modifications. Once at the Golgi, enzymes are heavily glycosylated as they sequentially move from the cis-Golgi to the medial-Golgi en route to the TGN. The glycosylation event at conserved residues allows correct recognition and permits loading onto hydrolase receptors in the Golgi, while ensuring their protection from degradation upon arrival at the lysosome. Once glycosylated proteins are loaded onto their receptors they are packaged for anterograde at the TGN for sorting towards endosomes. At endosomes, these organelles progress and mature to lysosomes allowing a drop in pH and acidification that promotes unloading of the hydrolase from their receptor. At this point hydrolases are able to cleave their substrates and lysosomal receptors are recycled retrogradely towards the Golgi (Appelqvist et al., 2013; Braulke and Bonifacino, 2009; Zaidi et al., 2008).

Sorting of lysosomal proteins is mediated by the Mannose-6-phosphate receptor (M6PR) and sortilin (Appelqvist et al., 2013; Braulke and Bonifacino, 2009). Two versions of

M6PR exits in mammals: cation-dependent (CD-M6PR) and cation-independent (CI-M6P). Meanwhile, flies contain a single copy called lysosomal enzyme receptor protein (LERP) (Dennes et al., 2005). Both mammalian copies have structural and functional differences in cells.

M6PRs have been localized at the plasma membrane, endosomes and the Golgi. Compartments they decorate and traffic during their cycle of sorting events between the Golgi and the lysosome. M6PRs carry their function via recognition of M6P residues in cargo. Upon cargo recognition, complexes of M6PR-hydrolase are packaged into clathrin-coated vesicles destined to endosomes. The packaging of hydrolases is regulated by recognition of acidic residues in the cytoplasmic tail of M6PRs by the clathrin family of adaptor proteins GGA (Golgi-localized, γ-ear-containing, ADP ribosylation factor binding). In addition, the GTPase Arf1, PIP4 and AP-1 regulate the delivery of these vesicles towards endosomes from the Golgi. Upon fusion with endosomes, vesicles uncoat and the complex remains bound until the pH is acidic enough to promote release of the hydrolases. At this point, the hydrolase resides in the lysosome to be activated. Rab9 and TIP47 mediate the retrieval of the M6PR from endosomes towards the Golgi. However, it should be pointed that the retrieval of the M6PR is a matter of debate and the literature provides extensive examples for alternate ways to retrieve this protein retrogradely to the Golgi from endosomes for further rounds of hydrolase delivery. Dysfunction of M6PR results in impaired delivery of hydrolases to lysosomes, swelling of lysosomes and lysosomal dysfunction in flies and mammals (Coutinho et al., 2012a; Dennes et al., 2005).

Sortilin is a glycoprotein with structural and functional similarity to the M6PR in mammals and Vps10 in yeast (Coutinho et al., 2012b). The protein is localized at endosomes and the Golgi network and utilizes the same machinery as M6PR to deliver lysosomal proteins. In addition, it can associate with the coat complex retromer to mediate retrograde traffic from endosome. Most surprisingly, it was shown that sortilin regulates the sorting of hydrolases Cathepsin D and H (Coutinho et al., 2012b). The dynamics and possible cross talk and parallel regulation of Cathepsin hydrolases by sortilin and M6PR are not understood and remain a matter of speculation.

Cathepsin hydrolases are essential to sustain lysosomal function in higher eukaryotes. Hydrolases are synthesized in pre-pro-form and subsequently cleaved to pro-form in the ER, removing a secretion signal in the N-terminal region. Next, they are trafficked to the Golgi with minimal post-translational modifications to become heavily glycosylated and modified by addition of a mannose-6-phosphate to ensure its loading on M6PR (Zaidi et al., 2008). Once packaged, hydrolase-M6PR complexes are sorted towards endosomes. Mutations in Cathepsin genes result in early lethality, lysosomal and neurodegenerative disorders and associated with the development of with heart disease and cancer (Anderson et al., 2013; Cheng et al., 2014; Reiser et al., 2010). In flies, mutation of Cathepsin D results in adult onset neuronal accumulation of lipofuscin and neurodegeneration (Myllykangas et al., 2005). Similarly, mammalian studies in mice have revealed that removal of Cathepsin D or dual removal of Cathpsin B/L leads to lysosomal and autolysosomal dysfunction (Koike et al., 2000; Shacka et al., 2007; Walls et al., 2007). In addition, these mice show accumulation of autophagic vesicles and

decreased activation of the pro-survival protein Akt. More strikingly, is the fact that loss of a Cathepsin D in human causes Batten's disease, a juvenile lethal neurodegenerative condition (Siintola et al., 2006). This suggests functional conservation across higher eukaryotes for hydrolase function in a tissue specific manner.

Summary

Autophagy is a conserved lysosomal dependent pathway used by cells as an alternative source of nutrients under stress conditions to maintain cellular homeostasis and promote survival. Insulin and amino acids negatively regulate autophagy by promoting mTOR signaling and inhibition of the negative regulator kinase Atg1. In addition vesicular traffic input from an array of cellular organelles (e.g. Golgi, ER, endocytic pathway and mitochondria) is required for the delivery of proteins, enzymes and lipids throughout the distinct steps of the autophagic pathway. Deregulation of autophagy has been shown in disease states ranging from cancer and metabolic diseases to neurodegeneration. Therefore, elucidation of the mechanisms governing autophagy under physiological conditions, in vivo model systems and distinct disease states holds clinical promise in the development of pharmacological agents to modulate autophagy.

The field of autophagy has experienced extensive growth in the last decade but still gaps in knowledge remain unanswered. For example, we have gather extensive information on the role and structure of Atg proteins in the regulation of autophagy. However, how Atg proteins are mobilized from their constitutive compartments under distinct nutritional states and their basal function remain incompletely understood. Similarly, how organelles

(e.g. ER, Golgi, mitochondria, etc.) switch from their constitutive roles to supply autophagy with proteins and lipids upon induction is not fully understood. An additional lack of understanding is found in the regulatory mechanisms responsible for achieving adequate autophagosomal size. It has been established that autophagic vesicle growth is as a direct consequence of exchange and fusion with components of the endocytic pathway (regulated by Rab7 and 11, SNAREs and ESCRT proteins), recruitment of Atg proteins, post-Golgi Sec proteins and the levels of Atg8 (mRNA and protein), among others (Chen and Klionsky, 2011; Fader et al., 2008; Jager et al., 2004; Jin and Klionsky, 2014; Lamb et al., 2013; Mehrpour et al., 2010; Yang and Rosenwald, 2014). Nonetheless, the molecular mechanisms regulating growth of autophagic vesicles and the role traffic regulators serve upon autophagy induction are still elusive. Similarly, retrograde traffic has been shown to be important for autophagy induction and regulation of Atg9 delivery to the PAS in yeast (Ohashi and Munro, 2010). Whether it is required in higher eukaryotes for autophagy regulation and functionally redundant like in yeast remains to be determined.

In the last decade, mTOR was localized to the lysosomal surface under amino acid stimulation, although other cellular compartments have been invoked to harbor the kinase (Betz and Hall, 2013). Yet, the localization and compartments mTOR complex 1 is mobilized in a tissue and stimulus dependent manner are not completely understood. More strikingly, the regulatory mechanisms governing insulin receptor (InR) trafficking are not understood. Therefore, how autophagy and its upstream regulators, mTOR and InR, are reciprocally regulated in a stimulus and tissue specific manner in vivo remains

puzzling. Understanding these mechanisms will increase the number of potential targets sites that can be used for the development of pharmacological manipulations of autophagy in the treatment of diseases.

The field of autophagy has been extensively researched and advanced using yeast and mammalian cell culture model systems. Studies have revealed the pathway to be conserved and require a set of Atg proteins for their regulation. However, evolution has added proteins required for the regulation of autophagy (e.g. insulin receptor, Rubicon, etc.), not found in yeast, in the mammalian repertoire suggesting that multicellular eukaryotes require a more complex autophagic network of proteins to carry its function. A possible explanation for some of the remaining gaps left in the field in higher eukaryotes might be the functional and gene redundancy found in them. Evaluation of autophagy in simpler eukaryotes such as Drosophila can provide the following advantages in the studies of autophagy: 1) reduced gene redundancy, 2) short life cycles, 3) in vivo model system, 4) manipulation of external stimuli to mimic physiologic conditions and 5) powerful genetic manipulations. We decided to test this premise by carrying a reverse genetic screen to uncover novel traffic regulators in fat body cells in Drosophila. The main goal of this thesis is: 1) to uncover novel traffic regulators of the Rab GTPase family required for starvation-induced autophagy in fat body cells, 2) evaluate their role in mTOR signaling regulation and 3) establish their requirement in the reciprocal coordination between autophagy and mTOR signaling during distinct nutrient conditions.

Here we present novel vesicular traffic regulators of the Rab GTPase family required for the regulation of autophagy. We show that a subset of Rab GTPases (Rab2, 5, 7 and 14) is required for the regulation of autophagosomal growth and induction and autolysosomal function. Additionally, we show that Rab5 is required for autophagic vesicle growth and induction, endocytosis and lysosomal maturation. Our characterization of Rab5 shows that it is sufficient and required for autophagy induction. Last, we fully characterized the role of Rab6 during starvation-induced autophagy. We show that loss of Rab6 causes an accumulation of autophagosomes, reduction in cell size and expansion of the lysosomal compartment. Characterization of these phenotypes revealed that they result by parallel defects in mis-sorting of Cathepsin D from lysosomes and defective recycling of InR at the plasma membrane resulting in reduced insulin dependent activation of mTOR signaling. Our findings suggest that loss of Rab6 interferes with the reciprocal regulation between autophagy and mTOR during distinct nutrient conditions by affecting two distinct retrograde traffic routes.

Chapter 2: Materials and methods

Materials and Methods

Screen Design

For our screen we used the flip-out system in the larval fat body of Drosophila (Mauvezin et al., 2014).The system allows us to target UAS-dsRNAi of the gene of interest to clonal cells labeled with GFP leaving the rest of the tissue as an internal control. Using this system we expressed RNAi constructs for the 33 Rab GTPases encoded in the fly genome and monitored the effects on the autophagosomal marker Atg8a. We evaluated our effects under full rich food (Fed) and upon 4 hours of amino acid starvation (STV, starvation). With completion of the screen we selected a few candidates for further characterization.

Fly Strains and Genetic Manipulations

Flies were raised at 25°C on standard cornmeal/molasses/agar media. The following *D. melanogaster* strains were used: $Rab5^{2}$FRT40A and UAS-Rab5-GFP (gift of L. Bilder, University of Berkeley, CA, USA), $Rab6^{D23D}$FRT40A (gift of A. Ephrusi, Developmental Biology Unit, European Molecular Biology Laboratory, Heidelberg, Germany), Rab 14 null and UAS-Rab14RB-mRFP (gift of L.Wu, University of Maryland, MD, USA), UAS-mCherry-VhaM8.9, UAS-GFP-VhaM8.9 (gift of Matias Simmons, University of Freirburg, Freiburg, Germany), UAS-Vha55-EGFP (gift of Julian Dow, University of Glasgow, Glasgow, UK), UAS-LAMP-green fluorescent protein (GFP; gift of Helmut Krämer, University of Texas, Dallas, TX), Tub-UAS-InR-CFP (gift of Hugo Stocker, Institute of Molecular System Biology, Zurich, Switzerland) and Tub-Vps29-mCherry (gift of Jullie Brill, University of Toronto, Toronto, CA). Additional strains were

obtained from the Bloomington Stock Center (Bloomington, IN) and Vienna Stock centers.

Heat shock-induced flippase (hsFLP)/Flippase Recognition Target (FRT)-mediated loss of function clones in the larval fat body were induced in 0–4 hours old embryos by a 1-1 ½ hour heat shock at 37°C and were marked by fat body–specific activation of Upstream Activating Sequence (UAS)-green fluorescent protein (GFP) lines on FRT-linked chromosomes. flip-out clones were generated through spontaneous hsFLP-dependent activation of Act>CD2>GAL4.

Autophagy Induction and Detection

To induce starvation, 25-30 larvae were transferred to fresh food media 72 hours after egg laying for 20–24 hours to avoid crowded conditions. Afterwards they were transferred to 20% sucrose solution for 4 hours before dissection. LysoTracker Red (Invitrogen) staining was performed as described (Juhasz and Neufeld, 2008).

Re-Feeding Experiments

25-30 larvae were transferred to fresh food media, 72 hours after egg laying, for 16–24 hours to avoid crowded conditions. Afterwards they were transferred to 20% sucrose solution for 4 hours before dissection followed by transfer of 10-15 larvae to regular laboratory cornmeal food mixed with 1mL of water plus a fine granulated layer of yeast pellets covering the food for a duration of 6-7hrs.

Immunohistochemistry

For imaging and analysis of fluorescent tagged proteins, 10–12 larvae per genotype were dissected and inverted in PBS and fixed overnight at 4°C in 4% paraformaldehyde/PBS. The next day, samples were washed extensively in PBS + 0.1% Triton X-100 (PBST), and counterstained with DAPI. A single section or lobe of fat body from each carcass was dissected and mounted in Vecta Shield.

Samples to be used for immunohistochemistry were fixed and washed as above. Subsequently, they were blocked in PBST + 4% normal goat serum for 3hrs at room temperature and then incubated overnight in blocking solution containing the primary antibody of interest. The following antibodies and concentrations were used: Cathepsin D (1:300) (gift of Dr. Dennes, Universitaets-Klinikum-Muenster, Muester, Germany) and CP1/Cath L (1:2:500) (gift of Dr. Dolph, Darthmouth college, NH, USA).

Confocal images were captured on a Zeiss LSM710 confocal microscope equipped with a ×40 (W) objective lens (APO DIC III numerical aperture 1.2) and acquired using Zeiss software Zen 2010. Laser lines used in this study were 405, 488 and 561 nm. Red/green/blue (RGB) and grayscale images were further processed with ImageJ (National Institutes of Health, USA) or Photoshop CS3. Live images of LysoTracker Red–stained samples were obtained on a Zeiss Axioscope-2 microscope equipped with a Nikon DXM1200 digital camera (Melville, NY), using a 40× Plan-Neofluar 0.75 NA objective lens and Nikon ACT-1 software. Images were further processed and assembled into figures using Adobe Photoshop CS (San Jose, CA) and ImageJ.

Quantification of punctae was carried using ImageJ Software and Adobe Photoshop CS3. Each clone was surrounded with a line to save area. Next, for clonal analysis of mCherry-Atg8 punctae, nuclei marked with DAPI were used to create masks on mCherry channels to remove nuclei from red and blue channels before quantifications. Punctae structures were marked by adjusting threshold levels to select desired punctae per clone analyzed. Data was analyzed using the analyze particle function on ImageJ. N values on mosaic analysis indicates a clone of a given genotype, at least 7 fat body lobes of each genotype were used to image all clonal cells per experiment.

For InR-CFP measurements, a line was drawn in a given cell from plasma membrane to plasma membrane avoiding the nuclei. Subsequently, cells were analyzed using the Box-plot function of ImageJ. The final signal values were obtained by averaging both plasma membrane highest peaks subtracted from the 2 highest peaks in the cytoplasm. 2 cells per fat body were used for analysis; n indicated a single cell in the data set.

Complete set of N values for all the desired statistics were evaluated using student t-test in Microsoft Excel.

Texas Red tracer endocytic assay

10 larvae per genotype were bisected and inverted in PBS. Next larvae were transferred to a 1.5-mL tube containing 80 μg/ml TR-Avidin (Invitrogen) in M3 insect medium (Sigma-Aldrich) containing 5% fbs, 1× insect medium supplement (Sigma-Aldrich) and penicillin/streptomycin antibiotics (Invitrogen). Carcasses were incubated for 30 min at room temperature with gentle agitation followed by two rinses and three washes for 5

min with ice-cold PBS + 0.5% BSA at 4°C. Afterwards, larvae were fixed, washed, counterstained with DAPI and mounted in Vecta Shield reagent.

Western Blot Analysis

For general Western blots, fat bodies were dissected in PBS and lysed directly in SDS sample buffer. Extracts were boiled 3 min, separated by polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore, Billerica MA). The following antibodies were used: rabbit anti-phospho-T398 dS6K 1:250 (Cell Signaling Technology, Beverly, MA), mouse anti-β-tubulin E7 1:250 (Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit anti-phospho-S505 Akt 1:1000 (Cell Signaling Technology, Beverlyl, MA). Signals were visualized using Super Signal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL) with BioMax Light (Kodak, Rochester NY) or HyBlot CL autoradiography film (Denville Scientific, Metuchen NJ) and quantitated using Adobe Photoshop software. 5 larvae were used per sample and at least three replicates evaluated for quantifications.

Chapter 3: Screen to identify novel vesicular traffic regulators of autophagy

A need to uncover novel vesicular trafficking regulators

Autophagy a housekeeping catabolic mechanism used by cells to maintain organelle and protein quality control and combat cellular stress. The pathway continuously receives vesicular input from a plethora of organelles (e.g. ER, Golgi, etc.) to deliver regulatory proteins, enzymes and lipids. Similarly, the mechanisms responsible for expansion and maturation of autophagosomes rely on vesicle donor events and the proper recruitment of Atg proteins. However, it is not completely understood how traffic routes shift from their constitutive roles to fulfill these tasks upon autophagy induction. Furthermore, the traffic requirements and regulators required for the recycling of autolysosomes and the complex process of ALR remains unexplored.

mTOR, amino acids and insulin signaling negatively regulate autophagy. The localization of mTOR has been shown to impact its activation and the regulation of autophagy (Betz and Hall, 2013). The Rubinsztein laboratory used a physiological starvation protocol to establish that lysosomal positioning and mTOR localization at this compartment affected its activation and autophagy (Korolchuk et al., 2011). In contrast, the Sabatini laboratory has extensively characterized the regulatory mechanisms responsible for localizing mTOR at the lysosomal surface in response to amino acid stimulus but has not examined autophagy under their experimental setting (Betz and Hall, 2013; Groenewoud and Zwartkruis, 2013; Shimobayashi and Hall, 2014). Last, the Yu group has localized mTOR at autolysosomes for ALR and tubule formation under prolonged starvation (Yu et al., 2010). Additional complexity to the regulation of mTOR signaling and localization is added by the fact that mTOR has been localized to different organelles in cells (Betz and

Hall, 2013). A recent study provided further insight into the role traffic in mTOR activation and localization as it showed Rab1 is required for mTOR mobilization to the Golgi in response to amino acids in a Rag independent manner (Thomas et al., 2014). Therefore, mTOR localization is a mechanism employed by cells to adjust to environmental cues and availability of growth factors and nutrients. However, the dynamics of mTOR localization during distinct nutrient states and the regulators carrying this task are not completely understood, have not been examined in vivo and remain to be uncovered.

Our understanding of the regulatory mechanisms governing autophagy has increased exponentially over the last decade. However, the majority of research evaluating the role of vesicular movement in the regulation of autophagy has been performed in yeast and mammals. Limitations on both systems might prevent full understanding of the pathway. Mammals have gene redundancy while yeast cannot fully model the complexity observed in mammals in the regulation of cellular processes. This prompted us to carry out a reverse genetic screen using Drosophila. The fruit fly has less gene redundancy for family of proteins compared to mammals and it is a multicellular organism amenable to in vivo studies. Our motivation to perform this screen was: 1) to uncover novel traffic regulators of the Rab GTPase family required for starvation-induced autophagy in fat body cells, 2) evaluate their role in mTOR signaling regulation and 3) establish their requirement in the reciprocal coordination between autophagy and mTOR signaling during distinct nutrient conditions.

Results

In an attempt to uncover novel regulators of autophagy we performed a comprehensive screen using the flip-out system in the larval fat body of Drosophila to knock down the 33 Rab GTPases encoded in the fly genome (Mauvezin et al., 2014). Using mCherry-Atg8a as a reporter to label autophagic vesicles (AV) we found a spectrum of Rab GTPases affecting the size and number of AV under fed and amino acid starvation conditions (Table 1). The significant number of Rab GTPases that we obtained as candidates suggest that there might be little redundancy in flies for Rab GTPases in the regulation of autophagy. Here we characterize a subset sharing a common phenotype.

Subset of Rab proteins is required for autophagic vesicle induction and growth

From our candidate genes we were interested in a set of Rab proteins that reduced the size of AV upon amino acid starvation in fat body cells of Drosophila larvae. We observed that when Rab 2, 5, 7 or 14 were knocked down the percentage of the cytoplasm occupied by AV and their size was dramatically reduced as compared to control neighbor cells (Fig. 4, A-F). From these four Rab GTPases we decided to focus our attention on Rab2, 5 and 14 as a role for Rab7 in autophagy and lysosomal and autolysosomal function has been well characterized (Gutierrez et al., 2004; Jager et al., 2004). Next, we wanted to determine if our phenotypes for these proteins was a direct or indirect effect by evaluating if they co-localized with the AV marker, Atg8. To this end we took advantage of the Rab GTPase YFP library for our Rab's of interest (Zhang et al., 2007).

Table. 1 Rab screen results

Name	Fat body expression	Autophagic Vesicle Number	Cell Size	Co-localization with Atg8
Rab1	High level expression	Decreased	Decreased	Yes
Rab2	Low expression	Decreased	No change	Yes
Rab3	No expression	No change	No change	No
Rab4	Moderate expression	No change	No change	Partial
Rab5	High level expression	Decreased	No change	No
Rab6	Moderate expression	Increased	No change	Yes
Rab7	Very high expression	Decreased	No change	Yes
Rab8	Moderate expression	No change	No change	
Rab9	Low expression	Increased	Decreased	Yes
Rab10	Moderate expression	No change	No change	
Rab11	Very high expression	Increased	Decreased	Yes
Rab14	Moderate expression	Decrease	No change	Yes
Rab18	Moderate expression	Increased	No change	
Rab19	Moderate expression	Decreased	No change	Yes
Rab21	Low expression	No change	No change	
Rab23	No expression	No change	No change	
Rab26	No expression	No change	No change	
Rab27	NA	No change	No change	
Rab30	Low expression	No change	No change	
Rab32	Very high expression	No change	No change	Yes
Rab35	Low expression	Decreased	No change	
Rab39	Moderate expression	Decrease	Decreased	
Rab40	Low expression	No change	No change	

RabX1	Low expression	No change	No change	Yes
RabX2	NA	No change	No change	
RabX4	Low expression	No change	No change	
RabX5	No expression	No change	No change	
RabX6	Low expression	Decreased	No change	Yes
Rab9D	NA	No change	No change	
Rab9Db	NA	No change	No change	
Rab9E	NA	No change	No change	
Rab9Fa	NA	No change	No change	
Rab9Fb	NA		Decreased	Yes

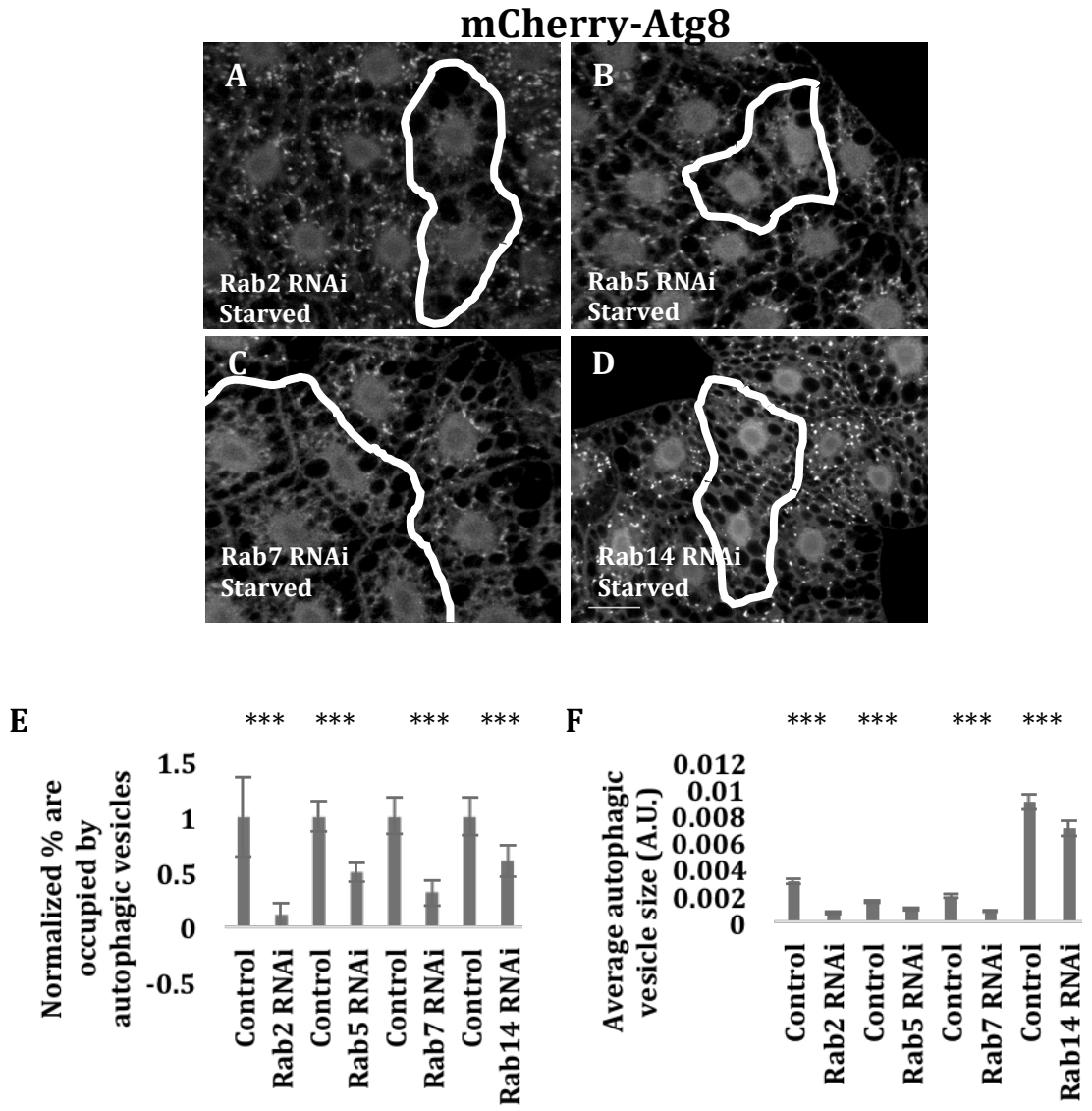


Figure 4. Rab2, 5, 7 and 14 are required for autophagic vesicle growth

A-F) Representative image of larval fat body containing a *Rab2* (A), *Rab5* (B), *Rab7* (C) and *Rab14* (D) RNAi expressing cell clone (outlined in white), showing reduced size and percentage area occupied of mCherry-Atg8a-marked autophagic vesicles relative to surrounding control cells under starvation conditions. Relative area occupied and mean average size of mCherry-Atg8a punctate per cell are indicated in (E) and (F), respectively. Scale bar, 25µm. n=20 clones per genotype and condition. *p<0.05, ***p<0.01, Student's t-test. Error bars indicate s.e.m. Genotypes: 1-4) *hs-flp; UAS-Dicer/+; r4-mCherry-Atg8a, Act<CD2<Gal4, UAS-GFP /UAS-Rab-dsRNA*

We found that Rab2, 7 and 14 co-localized with Atg8 under starvation conditions (Fig. 5, A-C). Collectively, our data suggest that these subset of proteins is directly required for production and growth of AVs.

Rab2 and Rab14 are required for autolysosomal formation and function

Autophagy induction results in formation of an isolation membrane that expands and grows to form an AV/autophagosome that fuses with the lysosome to form the degradative unit of autophagy, the autolysosome (Singh and Cuervo, 2011). Therefore, we wanted to determine if these Rab subset of proteins affected autolysosome and lysosomal function. We began our characterization with Rab2 and Rab14. First, we evaluated two different P-elements transposons insertions commercially available (Rab2-EY and Rab2-KG, hereafter) that are located upstream of the 5'-UTR of the Rab2 gene. Homozygotes of each allele showed severe motor dysfunction phenotypes in two distinct assays (Table2) and pupal lethality for homozygous larvae that managed to crawl along the vials. Heterozygotes containing both P-elements alleles and/or each P-element insertion crossed to a deficiency of the same chromosomal region showed similar phenotypes. Therefore, we concluded that these two insertions are at least hypomorphic alleles of Rab2. Using these hypomorphic alleles we evaluated whether Rab2 affected the formation of autolysosomes using the acidic dye Lysotracker red. Our data shows that Rab2 hypomorphs have a severe impairment in the formation and acidification of autolysosomes under starvation, as we observed a decreased in punctae formation when compared to control larvae after 4 hours amino acid starvation (Fig. 6, A-E).

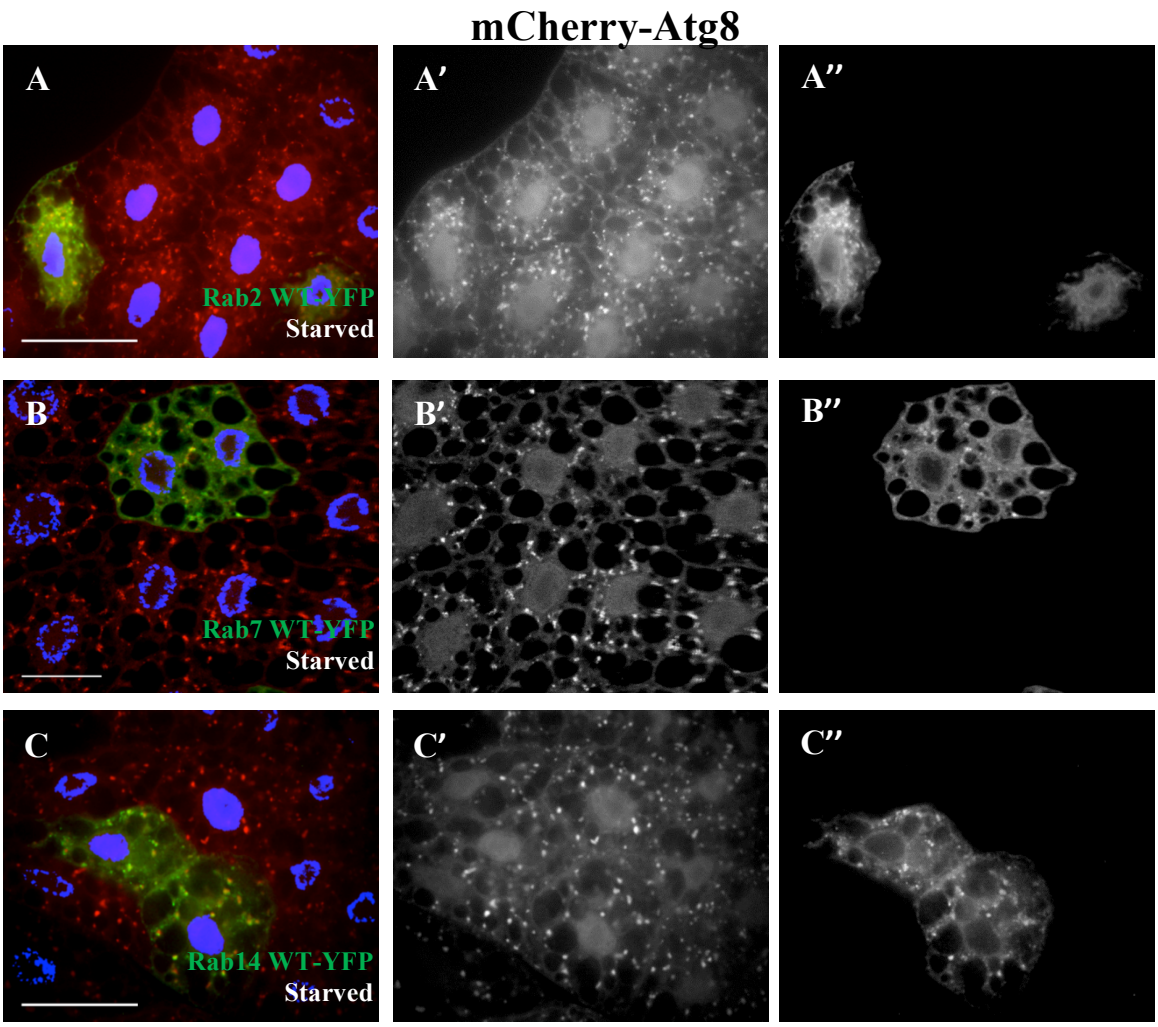


Figure 5. Rab 2, Rab7 and Raab14 co-localizes autophagic vesicle size under starvation

A-C) Representative image of larval fat body containing a cell clone marked by expression of Rab2-YFP (A), Rab7-GFP (B) and Rab14 (C), showing punctate formation under starvation of mCherry-Atg8a-marked autophagic vesicles co-localizing with Rab proteins under starvation conditions. A', B', C') and A", B", C") depict red and green channels, respectively for better visualization. Scale bar, 25µm. Genotypes: A) *hs-flp; +/+; r4-mCherry-Atg8a, Act<CD2<Gal4 /UAS-Rab2-WT-YFP. B) hs-flp; UAS-Rab7-GFP/+; r4-mCherry-Atg8a, Act<CD2<Gal4 /+. C) hs-flp; UAS-Rab14-WT-YFP; r4-mCherry-Atg8a, Act<CD2<Gal4 /+.*

Table 2. Rab2 Hypomorphs have prolonged inversion times

Genotype	Mean	SD	Variance	N	P value
yw	0.23 sec	0.049	0.0024	10	
Rab2-EY	1 min 14 sec	0.60	0.36	10	$P < 0.05$
yw	0.34 sec	0.11	0.012	8	
Rab2-KG	2 min 55 sec	1.03	1.06	8	$p< 0.05$

Table 3. Rab2 Hypomorphs have decreased peristalsis of the body wall

Genotype	Mean	SD	Variance	N	P value
yw	42.3	1.88	3.43	6	
Rab2-EY	15.7	4.48	20.04	6	$P< 0.05$
Rab2-KG	20.2	10.03	100.8	5	$P< 0.05$

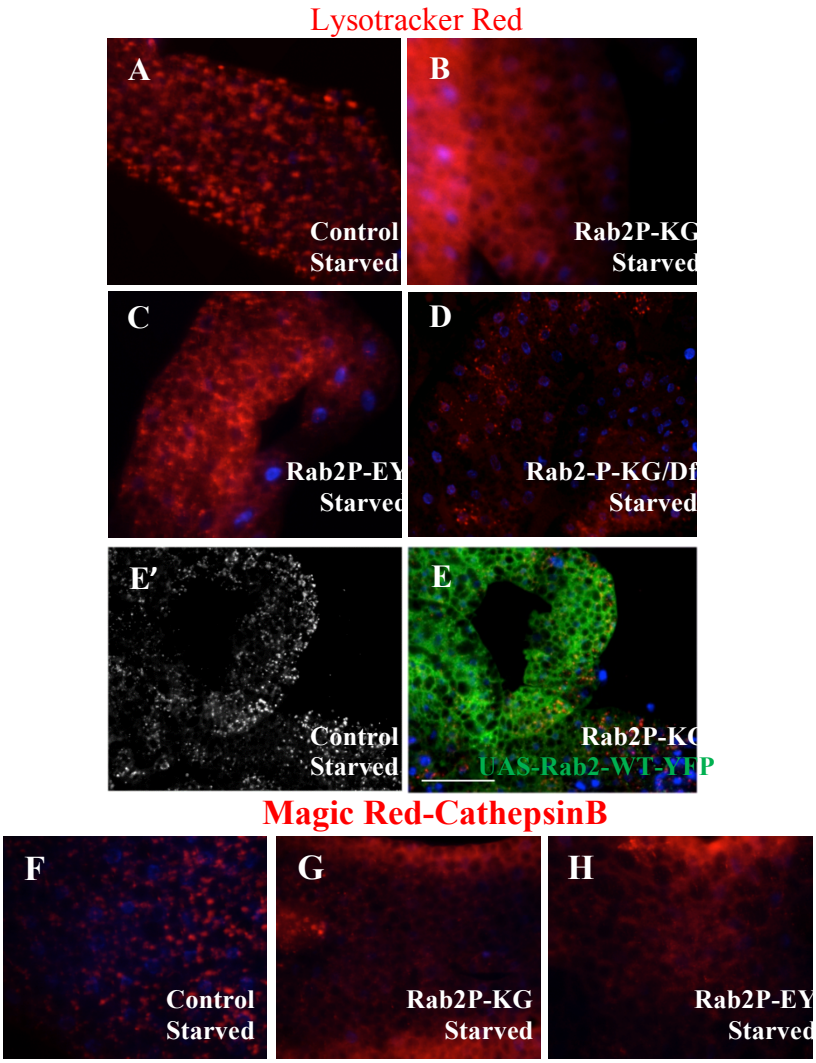


Figure 6. Rab2 hypomorphs have decreased acidification and autolysosomal size
A-D) Representative image of Rab2-KG (B), Rab2-EY (C) and Rab2-KG/Df (D) fat body tissue showing decreased Lysotracker Red marked autolysosomes as compared to control tissue (A) under starvation conditions. 5) Representative image of fat body over-expression of Rab2-YFP in a Rab2-KG homozygous animal showing Lysotracker Red marked autolysosomes similar to control tissue (A) under starvation conditions. F-H) Representative image of Rab2-KG (G) and Rab2-EY (H) hypomorph animals showing decreased cleavage and punctate formation of the Cathepsin B substrate (MagicRed) as compared to control tissue (F) under starvation conditions. Scale bar, 25µm. Genotypes: A) *yw B) yw; Rab2-KG/Rab2-KG. C) yw; Rab2-EY/Rab2-EY. D) yw/+; Rab2-KG/Df. E) yw; Rab2-KG/Rab2-KG; r4-Gal4, UAS-Rab2-WT-YFP. F) yw. G) yw; Rab2-KG/Rab2-KG. H) yw; Rab2-EY/Rab2-EY*

This was also observed when the Rab2-KG allele was “in trans” to a deficiency, suggesting that the phenotype we observed in homozygous animals is not due to a second mutation in the chromosome harboring the P-element insertion but rather due to reduced protein levels of Rab2 (Fig.6, D). Importantly, we could rescue this defect by over-expression of Rab2 in fat body tissue using the r4-Gal4 driver while retaining the motor dysfunction (Fig. 6, E). Similarly, we used an available null allele for Rab14 (Garg and Wu, 2014) to examine autolysosome formation. We observed a defect in the formation of Lysotracker Red punctae under starvation, as compared to control (Fig. 7, A and B) Acidification of lysosomal compartments is a requirement to ensure activation of lysosomal hydrolases to allow proper lysosomal function and prevent aggregation of damage organelles and proteins (Mauvezin et al., 2015). We showed that Rab2 and Rab14 loss of function results in reduced staining of the acidic dye lysotracker. Therefore, we decided to evaluate lysosomal and autolysosomal function. To test hydrolase activity we used a commercially available cell permeable reagent substrate, MagicRed, in whole fat body tissue of larvae. The reagent is a substrate for lysosomal hydrolases, in our case Cathepsin B, which upon cleavage becomes fluorescent and can be detected using a standard fluorescent microscope. Our data shows that loss of Rab2 function results in reduced staining, indicating reduced cleavage of the substrate as a result of reduced hydrolase activity (Fig. 6, F-H). To examine a role for Rab14 in lysosomal function, we used null tissue to monitor the levels of the autophagic substrate Ref(2)p/p62 via western blotting. Ref(2)p is an adaptor protein that has binding domains

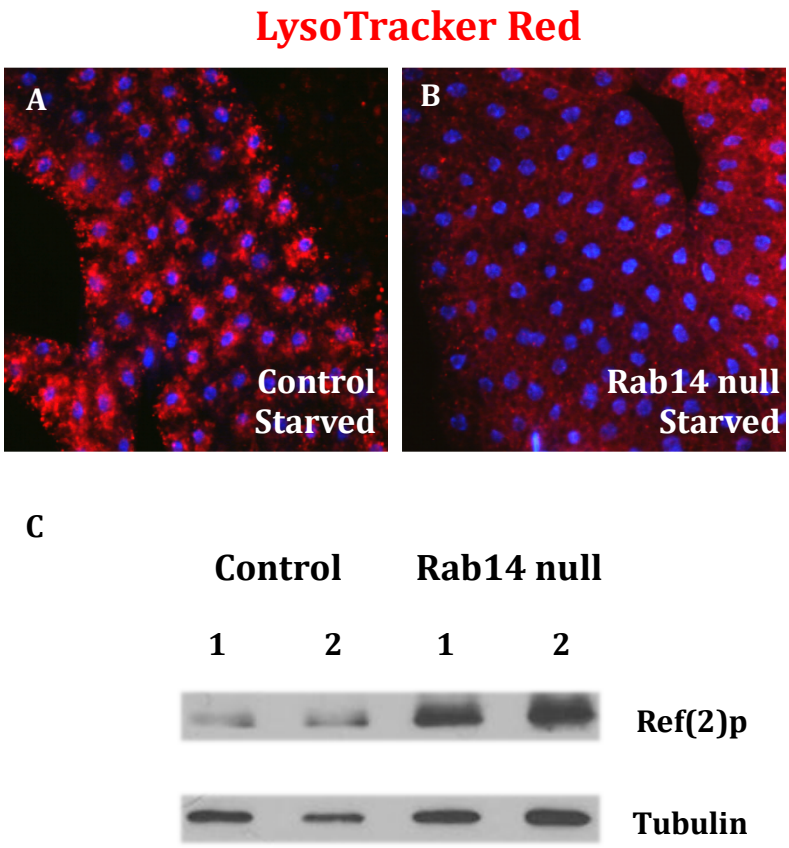


Figure 7. Rab14 is required for autolysosome formation and autophagic substrate degradation

A, B) Representative image of Rab14 null (B) animals showing decreased accumulation of Lysotraker Red-marked autolysosomes as compared to control tissue (A) under starvation conditions. C) Rab14 null animals fat bodies have increased levels of the autophagic substrate Ref(2)p under basal states, as compared to control tissue. Fat body extracts from larvae were used to monitor levels of Ref(2)p. Scale bar, 25µm. Genotypes: A) *yw. B) Rab14-/-. C) As A, B)*

for Atg8 and ubiquitin and can serve as a carrier molecule of ubiquitinated cargo to the nascent autophagosome. As such, Ref(2)p levels are routinely assayed in the evaluation of autophagy (Pircs et al., 2012). Our results show that loss of Rab14 increased the levels of p62 under basal conditions as compared to control tissue, suggesting reduced function of autophagy (Fig. 7, C).

The similarity of phenotypes between these four Rab GTPases suggests that they might work in parallel or sequentially to ensure proper autophagic vesicle production and growth. Therefore, we decided to evaluate whether they could decorate the same vesicles at a given time looking for co-localization among Rab2, 5, 7 and 14. We observed that Rab14 partially co-localized with the early endosomal marker Rab5 under fed conditions (Fig. 8, A). Meanwhile, it displayed extensive co-localization with the late endosomal markers, Rab7 and LAMP (Fig. 8, A- C). Surprisingly, we also observed extensive co-localization between Rab2 and Rab14 (Fig. 8, D). Altogether, our data supports a direct role for Rab2 and Rab14 in the formation and growth of autophagic vesicles as these markers localized at autophagic vesicles and the endocytic pathway.

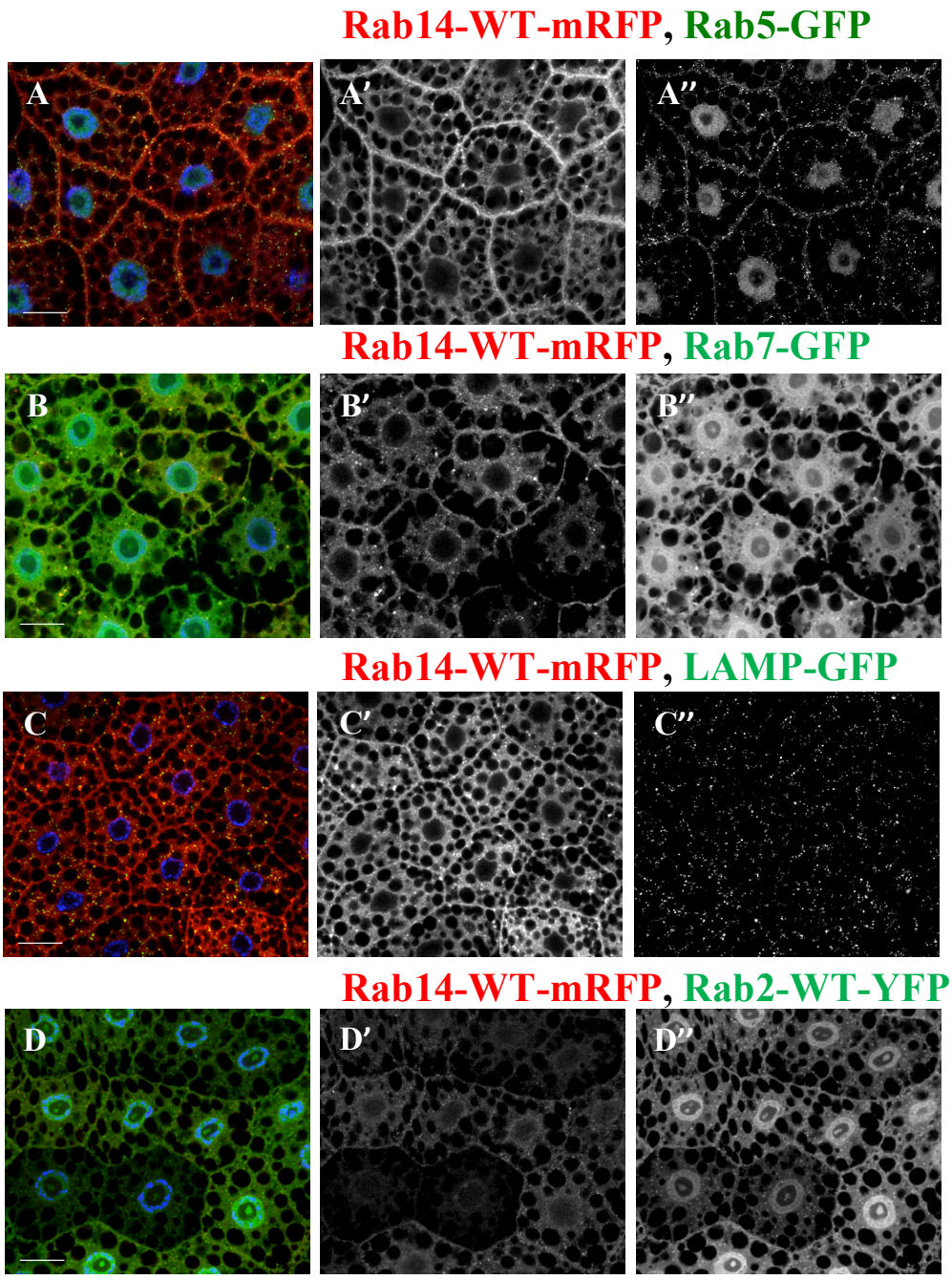


Figure 8. Co-localization between Rab2, Rab5, Rab7 and Rab14.

A, B) Co-localization between Rab5-GFP and Rab14-RFP (A), Rab7-GFP and Rab14-RFP (B), LAMP-GFP and Rab14-RFP (C) and Rab2-YFP and Rab14-RFP (D) expressed throughout the larval fat body under basal states. A', B', C', D') red and A", B", C", D") green channels are depicted for better visualization. Scale bar, 25µm. Genotypes: A) r4-*Gal4 UAS-Rab5-GFP/UAS-Rab14-RFP. B) Cg-Gal4 UAS-Rab7-GFP/+; UAS-Rab14-RFP/+. C) Cg-Gal4 UAS-LAMP-GFP/+; UAS-Rab14-RFP/+. D)* r4-*Gal4 UAS-Rab2-YFP/UAS-Rab14-RFP*

Rab5 is required for autophagic vesicle formation and endocytosis

Rab5 has been previously shown to be required for autophagy induction in mammalian cell culture (Ravikumar et al., 2008). Here we show that the function is conserved in flies, as knock down of Rab5 resulted in a decrease in the percentage of area occupied by Atg8 in cells (Fig. 4, B and D). In addition, we show that Rab5 has a separate function in the growth regulation of autophagic vesicles (Fig. 4, F). We further validated our results by using an available Rab5 null allele (Morrison et al., 2008) for mosaic analysis and a Rab5 dominant negative construct (Rab5-S43N, GDP-locked) to determine their effect on autophagic vesicle production. Rab5 null clones or Rab5-S43N over-expressing clones showed a dramatic reduction in the production and size of autophagic vesicles that were formed under starvation, compared to control cells (Fig. 9, A and B). Next, we wanted to determine if Rab5 is sufficient to induce autophagy in an active GTP bound state. To test this we over-expressed a constitutive active form of Rab5 (Rab5-Q61L, GTP-locked) in flip-out clones. We observed over-expression of this construct resulted in a cell autonomous accumulation of autophagic vesicles under basal states while control neighbor cells did not (Fig. 3F, 3-4). Importantly, we observed that these autophagic vesicles were capable of acidification, as activation of Rab5 resulted in mobilization of the V0-subunit VhaM8.9 from the plasma membrane to the cytoplasm where it co-localized with Atg8a (Fig. 3F, 3-4). This data suggests that activation of Rab5 results in the formation of autolysosomes. Altogether, our results indicate that Rab5 is sufficient to induce autophagic vesicle production and their growth in the fat body of Drosophila.

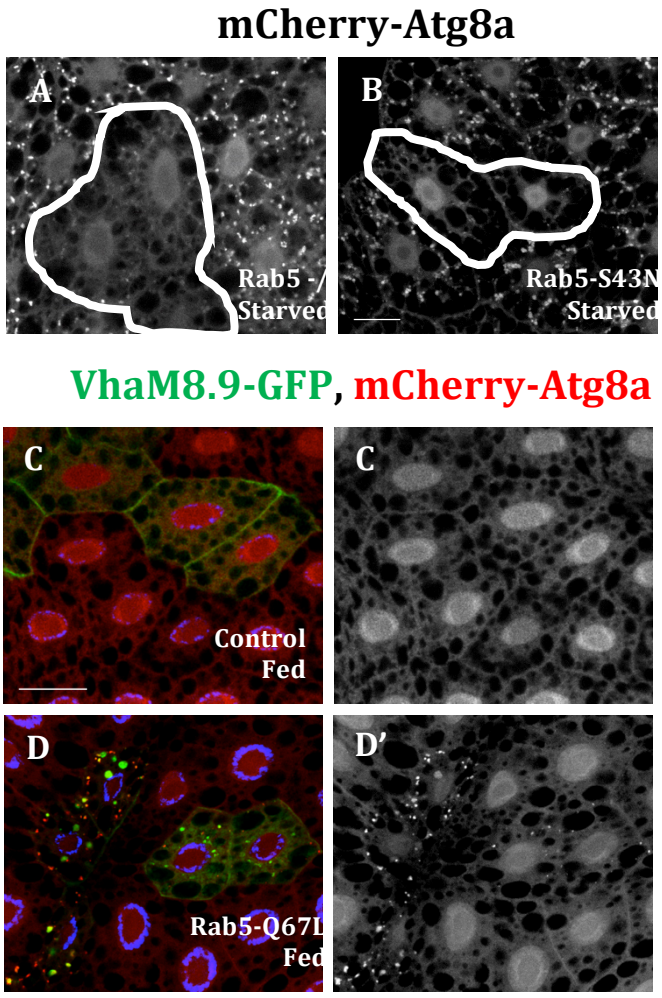
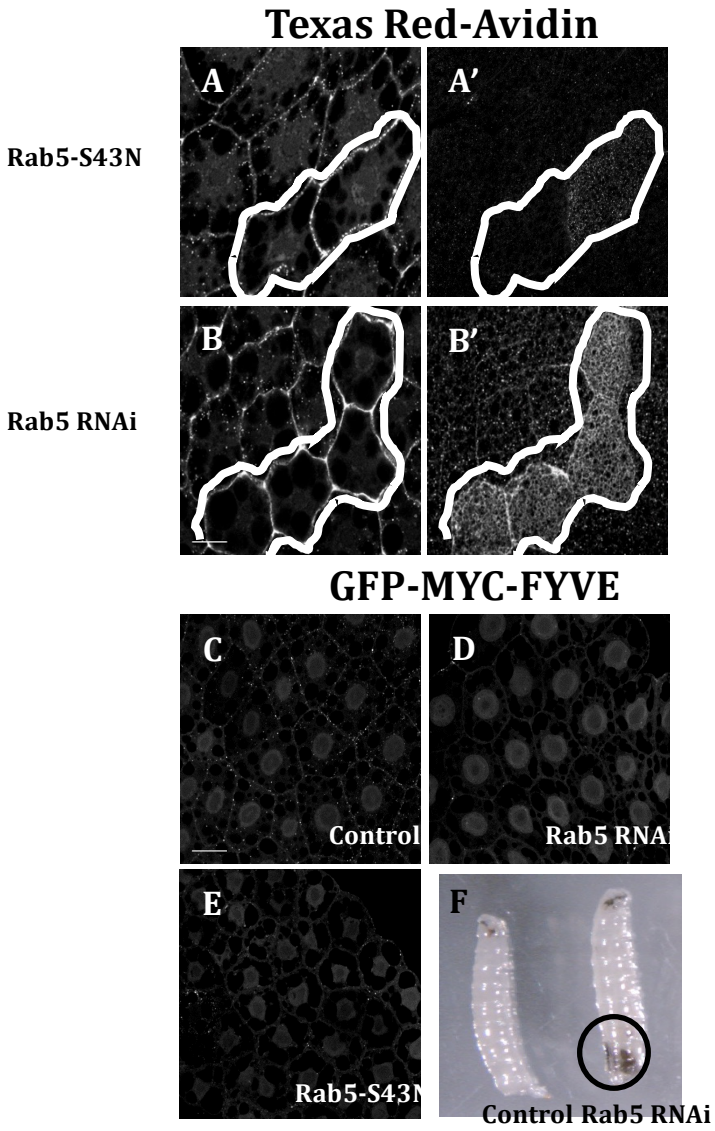


Figure 9. Rab5 is required and sufficient for autophagy induction

A, B) Representative image of larval fat body containing a Rab5 null cell (A) or expression of a Rab5 dominant negative (B) clone (outlined in white), showing decreased accumulation of mCherry-Atg8a-marked autophagic vesicles relative to surrounding control cells under starvation conditions. C-D) Representative image of larval fat body containing a *Rab5* constitutively active (D) expressing cell clone marked by expression of VhaM8.9-GFP showing increased accumulation of mCherry-Atg8a-marked autophagic vesicles relative to surrounding control cells under fed conditions. C', D') depict red channels for easier visualization. Scale bar, 25µm. Genotypes: A) *hs-flp; Rab5*^{2}*, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/+. B) hs-flp; UAS-Dicer/UAS-Rab5-S43N; r4-mCherry-Atg8a, Act<CD2<Gal4, UAS-GFP /+. C) hs-flp; UAS-VhaM8.9-GFP/+; r4-mCherry-Atg8a, Act<CD2<Gal4/+. D) hs-flp; UAS-VhaM8.9-GFP/+; r4-mCherry-Atg8a, Act<CD2<Gal4/UAS-Rab5-Q61L.*

Rab5 is a classic early endosomal marker with a conserved role from yeast to mammals in the regulation of endocytosis (Wucherpfennig et al., 2003). Therefore, we wanted to validate our tools by examining if Rab5 regulated endocytosis in the larval fat body. For this, we evaluated the uptake of Texas Red Avidin (TR-Avidin) in clonal cells over-expressing Rab5-S43N and clonal cells were Rab5 was knockdown. We observed a decreased in the uptake of the tracer as less punctae where observed when Rab5 function was reduced while enrichment occurred at the plasma membrane (Fig. 10, A and B). Importantly, the absence of tracer was not due to turnover since we observed the tracer in these clonal cells enriched distally to the nucleus plane at the cell surface (Fig. 10, A' and B'). Next, we wanted to determine if the role for Rab5 in the regulation of autophagy could be by regulating the activity of Vps34, a protein required for autophagic vesicle production and class III phosphoinositide-3-phosphate (PI3P) synthesis from yeast to mammals (Ravikumar et al., 2008; Wucherpfennig et al., 2003). We over-expressed an established class III PI3P reporter (GFP-MYC-FYVE) in control, Rab5 knockdown and Rab5-S43N expressing tissue. We found that tissue where Rab5 function was reduced there was a decrease in the punctae of this reporter as compared to control tissue (Fig. 3G, 3-5). Altogether, our data suggests that Rab5 might regulate autophagy induction and autophagic vesicle growth by regulating the production of PI3P lipids.



.

Figure 10. Rab5 is required for endocytosis and class III PIP3 synthesis.

A, B) Representative image of larval fat body containing a *Rab5 dominant negative* (A) and *Rab5* RNAi (B) expressing cell clone (outlined in white), showing reduced TR-Avidin uptake relative to control surrounding cells under fed conditions. Distal (A', B') and nuclear (A, B) focal planes are shown. C-E) Depletion of Rab 5 (D) or expression of a Rab5 dominant negative (E) throughout the larval fat body results in decreased punctate of the FYVE-MYC-GFP PIP3 reporter compared to control tissue (C). F) Depletion of Rab 5 throughout the larval fat body results in increased formation of melanotic masses as compared to control tissue. Scale bar, 25µm. Genotypes: A) *hs-flp; UAS-Rab5-S43N/+; Act<CD2<Gal4, UAS-GFP /+. B) hs-flp; +/+; Act<CD2<Gal4, UAS-GFP /UAS-Rab5-dsRNA. C) Cg-Gal4, UAS-FYVE-MYC-GFP/+; +/+. D) Cg-Gal4, UAS-FYVE-MYC-GFP/UAS-Rab5-S43N; +/+. E) Cg-Gal4, UAS-FYVE-MYC-GFP/+; UAS-Rab5-dsRNA/+. F) As in C) and D)*

Vps34 forms a molecular complex with Atg6/Vps15/Atg14 to regulate autophagy. Atg6 was recently shown to regulate endocytosis and autophagy in flies, mutants showed impaired function of both processes and the formation of melanotic tumors (Shravage et al., 2013). In the course of our experiments we observed similar findings when Rab5 was knockdown throughout the larval fat body (Fig. 10, F). Taken together, our data suggests that Rab5, like Atg6, might regulate similar pathways by regulating the function of the Vps34 kinase to produce PI3P lipid required for autophagy and endocytosis.

Rab5 is essential for lysosomal and autolysosomal function

Rab5 has been proposed to be required for endocytosis, endocytic pathway maturation and lysosome biogenesis (Hutagalung and Novick, 2011). However, direct functional evidence for a role of Rab5 in the function of lysosomes is lacking in the literature. Therefore, we decided to determine if Rab5 was required for lysosomal and autolysosomal function. First, we evaluated if expression of a dominant negative version of Rab5 affected formation of autolysosomes using Lysotracker Red. We observed that over-expression of dominant negative Rab5 was sufficient to reduce cell autonomously the staining of Lysotracker, as compared to control cells (Fig. 11, A and A'). Acidification is a pre-requisite for the activation of lysosomal hydrolases (Braulke and Bonifacino, 2009; Guo et al., 2014; Zaidi et al., 2008). Given that we observed reduced acidic labeling we wanted to evaluate whether loss of Rab5 could be affecting the processing of lysosomal hydrolases from pro-enzyme (immature enzyme) to mature form, in turn impairing lysosomal degradative capacity. For this we obtained fat body tissue from control and dominant negative Rab5 expressing flies to examine via western blotting the processing of Cathepsin L. Our results show that under fed and starvation

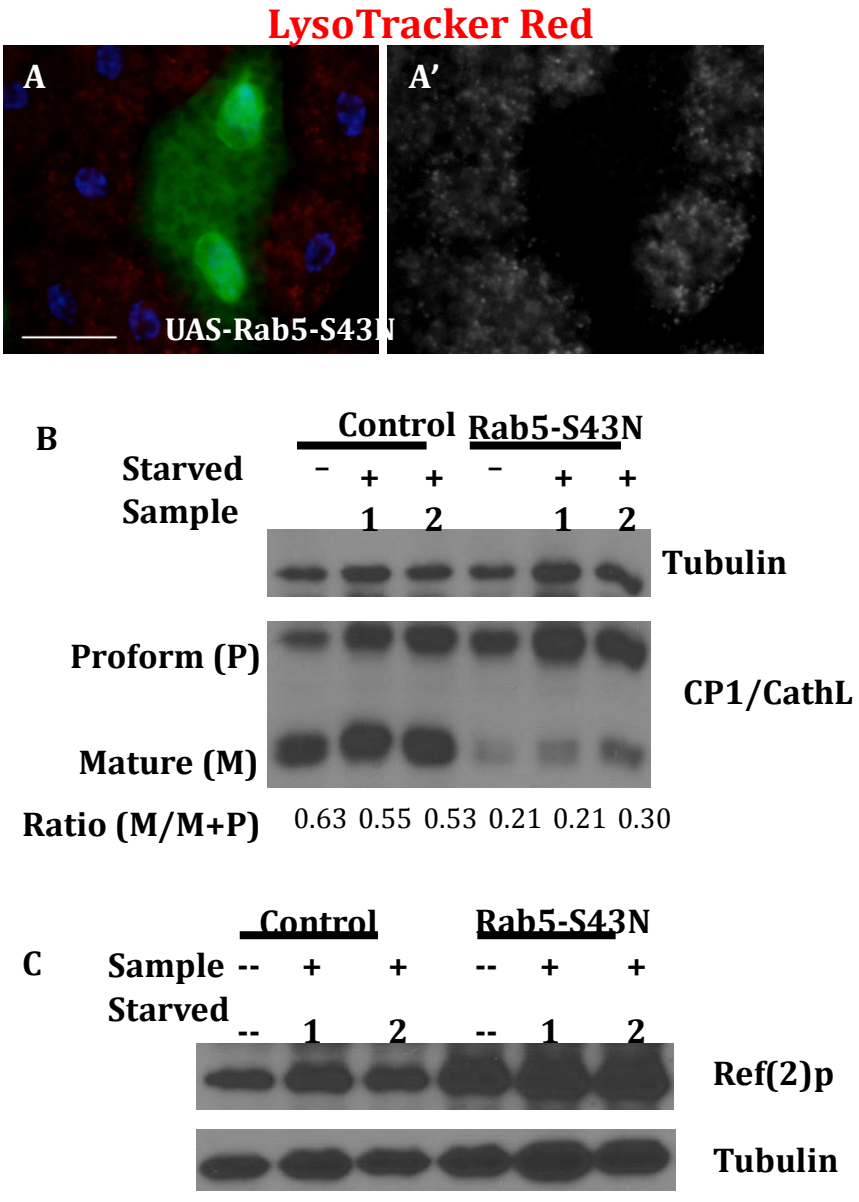


Figure 11. Rab5 loss results in impaired lysosomal and autolysosomal function.

A) Representative image of larval fat body containing a *Rab5 dominant negative* expressing cell clone (marked in green), showing reduced Lysotracker Red punctate formation relative to control surrounding cells under Starvation conditions. A') depicts red channel for better visualization. B) Rab5 dominant negative expression in decreased conversion of mature Cathepsin L from pro-form species as compared to control. Fat body extracts from larvae at the indicated time points and nutritional states were used to monitor levels of the Cathepsin L hydrolase. C) Rab5 dominant negative expression increased the levels of the autophagic substrate Ref(2)p as compared to control. Fat body extracts from larvae at the indicated time points and nutritional states were used to monitor levels of Ref(2)p. Scale bar, 25µm. Genotypes: A) *hs-flp; UAS-Rab5-S43N/+; Act<CD2<Gal4, UAS-GFP /+. B-C) Control: r4-Gal4/+. Rab5-S43N: r4-Gal4/UAS-Rab5-S43N.*

conditions control larvae are able to process Cathepsin L from a pro-enzyme form to mature species. However, expression of the dominant negative Rab5 impaired the processing of Cathepsin L resulting in higher levels of pro-enzyme and reduced levels of the mature form (Fig. 11, B). Next, we evaluated whether the defect in acidification and hydrolase processing affecting degradation of the autophagic substrate Ref(2)p. We obtained fat body tissue from control and Rab5 knockdown flies to monitor via western blotting the levels of Ref(2)p. Knockdown of Rab5 resulted in accumulation of Ref(2)p under basal and amino acid starvation conditions, as compared to control tissue (Fig. 11, C). Collectively, our data suggests that Rab5 function is essential for lysosomal and autolysosomal function to prevent the accumulation of proteins and autophagic cargo.

Suppression of autophagic vesicle induction and growth upon Rab5 loss is not due to sustained mTOR activation

Autophagy is negatively regulated by the serine/threonine kinase mTOR. Meanwhile, Rag GTPases and insulin signaling promote parallel activation of mTOR to promote cell growth and inhibit autophagy (Shimobayashi and Hall, 2014). We have shown that Rab5 loss results in reduced autophagic vesicle number and reduced growth (Fig.4 and 9). This raises the possibility that Rab5 may negatively regulate mTOR and in turn, positively autophagy. This is further supported by our observations that Rab5 null clones or expression of a dominant negative Rab5 increases cell size area (Fig. 10 and Fig. 12, A and B). Therefore, we examined if the phenotypes we obtained for Rab5 loss are the result of enhanced or sustained mTOR activation and in turn inhibition of autophagy. To test this we decided to over-express a dominant negative RagA GTPase (RagA-T121N,

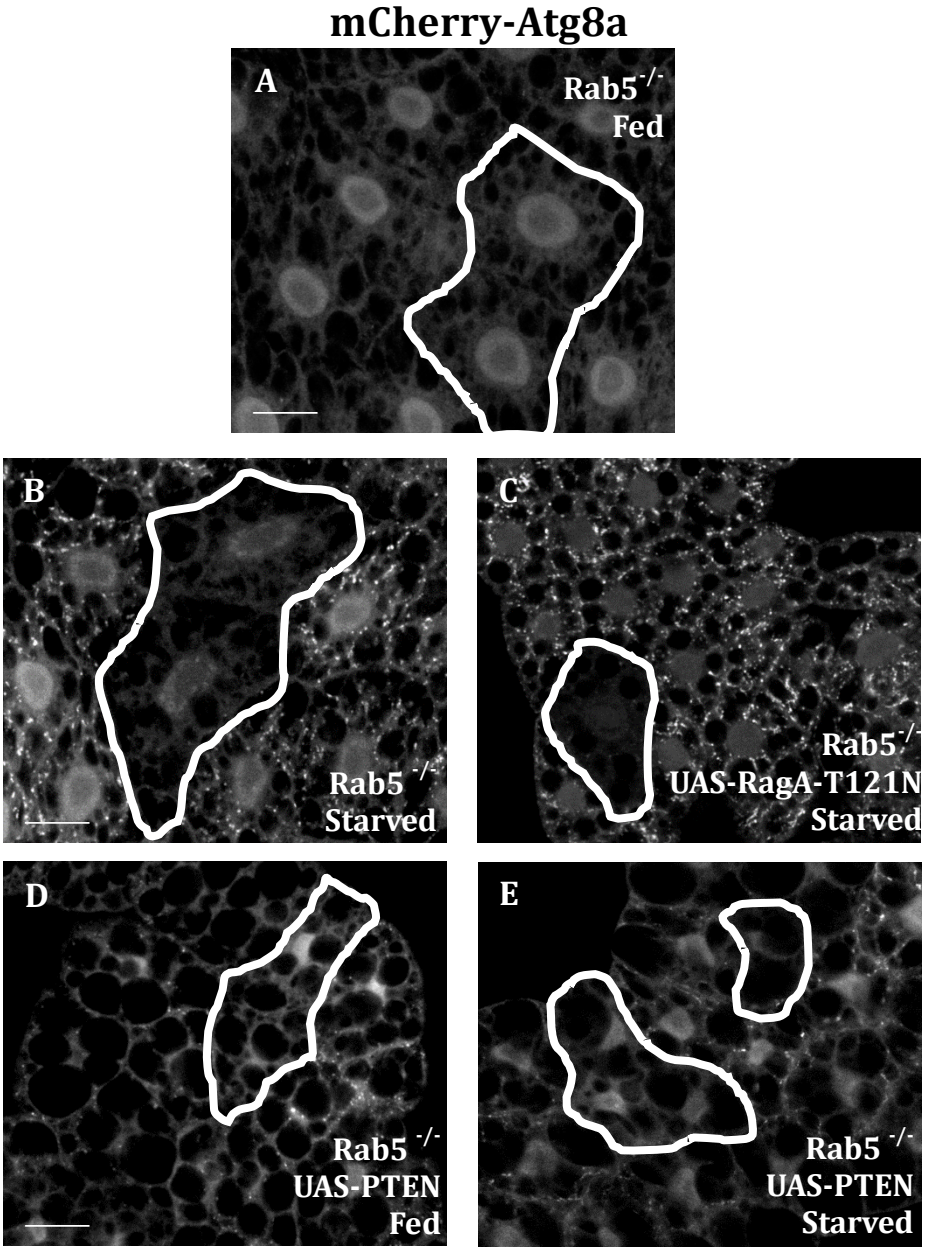


Figure 12. Inhibition of mTOR signaling is not sufficient to promote autophagic vesicle growth in Rab5 null cells.

A-E) Rab5 mutant cell clones (outlined in white) were induced in control background (A and B) or in the presence of fat body-specific expression of $RagA^{T121N}$ (C) or PTEN (D and E), and observed under fed or 4 hour starvation conditions. Genotypes: A, B) *hs-flp; $Rab5^{2}$, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/+*. C) *hs-flp; $Rab5^{2}$, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/UAS-RagA-T121N*. D, E) *hs-flp; $Rab5^{2}$, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/UAS-PTEN*

GDP locked) or PTEN in entire fat bodies to down-regulate amino acid and insulin signaling dependent activation of mTOR, respectively. In parallel, we generated Rab5 null clones while we starved larvae to remove systemic nutrient dependent inputs for mTOR activation. Our results show that over-expression of RagA-T121N was not sufficient to induce autophagic vesicles and restore to normal the cell size when Rab5 is lost in cells, as compared to wild-type neighbor cells (Fig. 12, C). Similarly, over-expression of PTEN was not able to restore autophagic vesicle induction and size upon Rab5 loss under fed and starvation conditions (Fig. 12, D and E). In sum, our results suggest that down-regulation of mTOR signaling and autophagy induction via over-expression of PTEN is not sufficient to restore autophagic vesicle production when Rab5 is lost, supporting a direct role for Rab5 in the formation of autophagic vesicles. Additionally, these data positions Rab5 parallel or downstream to RagA and PTEN in the regulation of the autophagic pathway.

Summary

Autophagy has been exhaustively studied using yeast and mammalian cell culture. Studies have shown great conservation in function of the core machinery required for autophagy regulation. However, apart from the core machinery of Atg proteins the yeast system does not contain additional regulators found in mammals. Meanwhile, mammals have gene redundancy and an expanded autophagy network repertoire of proteins, as compared to yeast, which can obscure the discovery of potential regulators of autophagy. Therefore, evaluation of a multicellular model system with less gene redundancy such as Drosophila might aid in the identification of novel regulator of autophagy.

The Rab GTPase subfamily has gathered much attention in the field of autophagy due to their role in vesicle trafficking and many potential regulation sites, from formation of the isolation membrane to completion of the autolysosome and its recycling via ALR. Interestingly, from 70 Rab's encoded in the human genome only 3-6 (Rab1, 5, 7, 9, 11 and 33) have been directly shown or speculated to be important for the formation of the isolation membrane or simply autophagy induction. To date none has been shown to regulate ALR or mTOR/insulin receptor localization, directly, under distinct nutrient states. It seems paradoxical that from all the different organelles invoked to regulate isolation membrane formation and its expansion more Rab GTPases haven’t been discovered considering the numerous protein and lipids that need to be mobilized upon autophagy induction. We hypothesized that this is mainly due to gene redundancy found in mammals. Therefore we decided to test this and perform a reverse genetic screen by

knocking down all the 33 Rab GTPases found in the fly genome while we monitored the effect on autophagic vesicles.

Our screen confirmed our hypothesis, as we were able to detect novel GTPases at the completion of our screen. Importantly, we obtained established Rab GTPases regulating autophagy in other model systems that included Rab5, Rab7, Rab9 and Rab11 (Fader et al., 2008; Gutierrez et al., 2004; Jager et al., 2004; Ravikumar et al., 2008). In the course of our studies Rab1 was shown to be required for autophagy and has been well characterized in yeast and mammals (Ao et al., 2014; Wang et al., 2013b; Zoppino et al., 2010). Interestingly, the majority of the Rab GTPases we uncovered as candidates showed a similar phenotype, reduction in autophagic vesicles number, block of autophagy and/or decrease in size of autophagic vesicles. It suggests that in fat body cells the main role of Rab GTPases is the positive regulation of autophagy and growth of autophagic vesicles. Characterization of their intracellular localization and effect on the core machinery should shed information on the mechanisms of autophagosome growth.

Here, we briefly characterized four of our candidates (Rab2, 5, 7 and Rab14). Examination of the literature revealed these four GTPases acted in a Rab cascade to regulate phagolysosome formation in C. elegans (Guo et al., 2010). Interestingly, autolysosomes and phagolysosomes in C. elegans use similar regulatory proteins to form their degradative unit (Huang et al., 2013; Kovacs and Zhang, 2010; Yang and Zhang, 2014). We showed that Rab2, 5 and 14 are required for formation and growth of autophagic vesicles and autolysosomal function. In addition, that this Rab subset is

needed for lysosomal function as they are required for the degradation of the autophagic substrate Ref(2)p. Further characterization of Rab5 revealed that, like mammals it is required for PI3P production. Since PI3P is mainly produced by the class III PI3P kinase Vps34, this presents a potential way Rab5 could regulate autophagosomal growth (Juhasz et al., 2008). A similar mechanistic role has been shown in mammals where Rab5 was shown to regulate and bind Vps34 (Ravikumar et al., 2008). However, a role for Rab5 in the regulation of autophagic vesicle growth in mammals was not discussed in this study, highlighting a novel role for Rab5 in flies.

Our data and screen results validate Drosophila as a powerful model system in the study of vesicular trafficking in autophagy regulation. Furthermore, our screen data suggest that Rab GTPases main role in fat body cells is the induction and regulation of autophagic vesicle growth.

Chapter 4: Rab6 dual regulation of the autophagic pathway and mTOR signaling in larval fat body cells.

Introduction

Autophagy is a catabolic housekeeping mechanism employed by cells to maintain energetic balance under stress conditions, such as starvation. The pathway starts by the formation of an isolation membrane that expands forming a double membrane autophagosome. Subsequent fusion with the lysosome results in the formation of an autolysosome and ensures delivery of lysosomal hydrolases to promote degradation of internalized cargo. The degradation of cargo serves as an alternative source of nutrients under nutrient limitation conditions.

Rab family proteins, 33 in Drosophila and 70 in mammals, are small lipidated G proteins regulating vesicular trafficking (Ao et al., 2014; Zhang et al., 2007). Their function is executed in a nucleotide-dependent manner via recruitment of effector proteins and tethering of molecules to promote fusion and fission at organelle surfaces (Ao et al., 2014). Rab6, a member of this family of proteins, has been characterized from yeast to mammals with established roles regulating secretion, endosome-to-Golgi, intra Golgi, and Golgi-to-ER traffic (Liu and Storrie, 2012). In Drosophila, Rab6 has been shown to regulate oocyte and eye development via regulation of Golgi-to-plasma membrane receptor targeting (Coutelis and Ephrussi, 2007; Januschke et al., 2007; Shetty et al., 1998; Tong et al., 2011). Nonetheless, most of the research on Rab GTPase mediated trafficking has been focused on the secretory and endosome/lysosome pathways (Ao et al., 2014). A potential role for Rab6 and other Rab GTPases in other cellular

pathways in higher eukaryotes, such as autophagy and insulin/mTOR signaling regulation remains incompletely understood and unexplored.

Canonical insulin signaling (CIS) and amino acid signaling (AAS) promote cellular growth via activation of the serine /threonine kinase mTOR and inhibition of autophagy. CIS is activated upon binding of insulin to the receptor at the plasma membrane surface resulting in subsequent downstream activation of class I PI3K, Akt and Rheb to activate mTOR (Shimobayashi and Hall, 2014). In parallel to this cascade, amino acids enter cells via transporters and activate the Rag/Ragulator complex resulting in mTOR translocation, from and unknown organelle, towards the lysosomal surface to be activated by Rheb (Sancak et al. 2010, Zoncu et al. 2011 and Wang et al. 2015). Activation of mTOR results in activation of S6 kinase and suppression of 4EBP promoting growth and protein synthesis (Shimobayashi and Hall, 2014). Interestingly, it has been shown that CIS and AAS culminate in activation of mTOR at the lysosomal surface, positioning the lysosome as the main nutrient sensing organelle in cells (Menon et al., 2014; Tong et al., 2011). However, how CIS, Rab proteins and autophagy are reciprocally regulated during various nutrients states in vivo remains unexplored. Furthermore, how nutrient states and Rab GTPases regulate the localization of mTOR and its upstream activators remains incompletely understood.

The role of Rab GTPases in the regulation of autophagy has begun to emerge in recent years in yeast and mammalian cell culture, revealing that a small subset of Rab GTPases regulates this process at different stages (Ao et al., 2014; Zhang et al., 2007). At the beginning steps, Rab 1 and 32 are required for IM synthesis while Rab5 is required to

promote activation of class III PI3K leading to autophagy induction (Hirota and Tanaka, 2009; Ravikumar et al., 2008; Zoppino et al., 2010). Rab11 has been associated with autophagy at two distinct steps, first regulating AP formation and secondly for fusion of maturing APs with multivesicular bodies (Fader et al., 2008; Longatti et al., 2012). At the last step, Rab 7 was shown to be required for fusion of late endosomes/lysosomes with mature APs to allow termination of the processes and cargo degradation (Gutierrez et al., 2004; Jager et al., 2004). As most of this work has been performed in yeast and mammalian systems, we decided to study the role Rab GTPases may play in autophagy regulation using as a model system the fruit fly Drosophila melanogaster. The model system allowed us to reduce the redundancy of family proteins found in mammalian cell lines as we uncovered novel and established regulators of autophagy in our screen results (Ayala CI and Neufeld TP, *unpublished*).

Here we describe the characterization of Rab6 as a novel regulator of autophagy and insulin/mTOR signaling in the larval fat body of *D. melanogaster*. We show that loss of Rab6 causes an accumulation of autophagosomes, reduction in cell size and expansion of the lysosomal compartment. Characterization of these phenotypes revealed that they result primarily from mis-sorting of Cathepsin D from lysosomes, rendering them with a reduced degradative capacity upon autophagosome-lysosome fusion. Interestingly, these defects can be rescued by inactivation of the mTOR inhibitor PTEN and over-expression of Rheb, but not by re-feeding of exogenous nutrients nor constitutive activation of amino acid signaling, indicating an additional novel role for Rab6 in regulation of the mTOR signaling axis. Evaluation of the insulin receptor (InR) following nutrient re-feeding after

starvation revealed that InR recycling to the plasma membrane is compromised and alternatively mis-routed to the lysosome upon Rab6 loss. Our findings suggest that loss of Rab6 interferes with the reciprocal regulation between autophagy and mTOR during distinct nutrient conditions by affecting two distinct traffic routes.

Results

Rab6 loss results in accumulation of autolysosomes

In an attempt to uncover novel regulators of autophagy we decided to screen Rab GTPases, the masters regulators of intracellular traffic. We exploited the UAS/Gal4 bipartite system to express RNAi transgenes, for 31 of the 33 Rab GTPases encoded in the Drosophila genome, using the well described reporter Atg8a as a marker to monitor autophagosomes and autophagy (Mauvezin et al., 2014). Among the novel candidates genes uncovered (Ayala CI and Neufeld TP, unpublished) we decided to fully characterize the role of Rab6 during autophagy, as a role in higher eukaryotes in the regulation of autophagy and the insulin/mTOR signaling was unknown. First, we expressed an RNAi against Rab6 in flip-out clones (Neufeld, 2008) and observed that it resulted in an accumulation of autophagic vesicles during fed states as compared to control cells (Fig. 13, A and B). Upon a 4-hour amino acid starvation we observed that some clones had enlarged autophagic vesicles relative to control cells (data not shown). Next, we decided to confirm our RNAi was targeting Rab6. For this we expressed Rab6-YFP in fat body tissue in parallel with Rab6 RNAi and observed that it decreased total protein when western blots where probed with GFP antibody (Fig. 13, C). Last, we

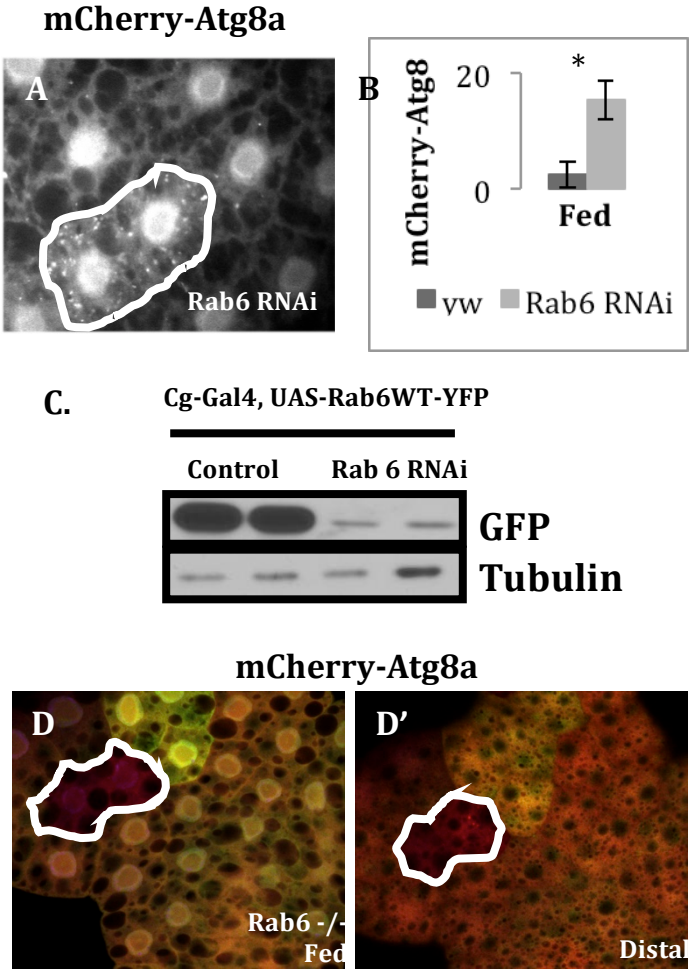


Figure 13. Knockdown of Rab6 leads to accumulation of autolysosomes.

A) Representative image of larval fat body containing a *Rab6* RNAi expressing cell clone (outlined in white), showing increased accumulation of mCherry-Atg8a-marked autophagic vesicles relative to surrounding control cells under fed conditions. Mean number of mCherry-Atg8a punctate per cell is indicated in B. C) Rab6-YFP is reduced in extracts of Rab6-depleted fat body tissue under basal and conditions as compared to control fat body tissue. YFP was detected via western blot using a GFP antibody. D) Representative images of larval fat body containing a *Rab6* null cell clone (outlined in white), showing absence of mCherry-Atg8a-marked autophagic vesicles similar to surrounding control cells under fed conditions in early L3 instar larvae. Nuclear (D) and distal (D') focal planes are shown. Scale bar, 25µm. n=20 clones per genotype and condition. *p<0.05, ***p<0.01, Student's t-test. Error bars indicate s.e.m. Genotypes: A) *hs-flp; UAS-Dicer/+; r4-mCherry-Atg8a, Act<CD2<Gal4, UAS-GFP /UAS-Rab6-dsRNA. C) Control: Cg-Gal4, UAS-Rab6-WT-YFP/+; +/+. Rab6 RNAi: Cg-Gal4, UAS-Rab6-WT-YFP/+; UAS_Rab6-dsRNA/+*. D) *hs-flp; Rab6^{D23D}, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/+*.

validated our phenotypes using an available null allele, Rab6 D23D, for mosaic analysis and monitoring of autophagy (Coutelis and Ephrussi, 2007; Januschke et al., 2007; Purcell and Artavanis-Tsakonas, 1999). We observed that clonal loss of Rab6 resulted in a cell-autonomous accumulation of autophagic vesicles, using Atg8a as a marker, at the nuclear plane (Fig. 14, A and B) and distal to the nucleus in late L3 larvae under basal states (Fig. 14, A'). Interestingly, this phenomenon was not observed in early L3 larvae suggesting a time dependency and threshold for the accumulation of autophagic vesicles (Fig. 13, D). Additionally and in accordance with our knock down results, we observed at least a 1.5x fold increase in autophagosomal size under amino acid starvation in ~30% of the Rab6 null clonal cells analyzed (Fig. 14, C; 50/171 clonal cells).

Accumulation of autophagic vesicles may arise as a result of a block in fusion between autophagosomes and lysosome in route to form an autolysosome (Itakura et al., 2012; Takats et al., 2013). Therefore, we wanted to test if the autophagic vesicles that accumulated when Rab6 is lost where mature autophagosomes or autolysosomes. To test this, we co-expressed LAMP-GFP (lysosomal marker) and mCherry-Atg8a (autophagosomal marker) in control and Rab6 RNAi expressing larvae. Our results show that loss of Rab6 does not impair autolysosome formation, as observed in control larvae (Fig. 14, E-G). Similarly, we could not observe a defect between the co-localization of mCherry-Atg8a and the late endosomal marker Rab7 when Rab6 was knocked down in flip-out clones (Fig. 15, A and B). Together our results show that loss of Rab 6 results in the accumulation of autolysosomes.

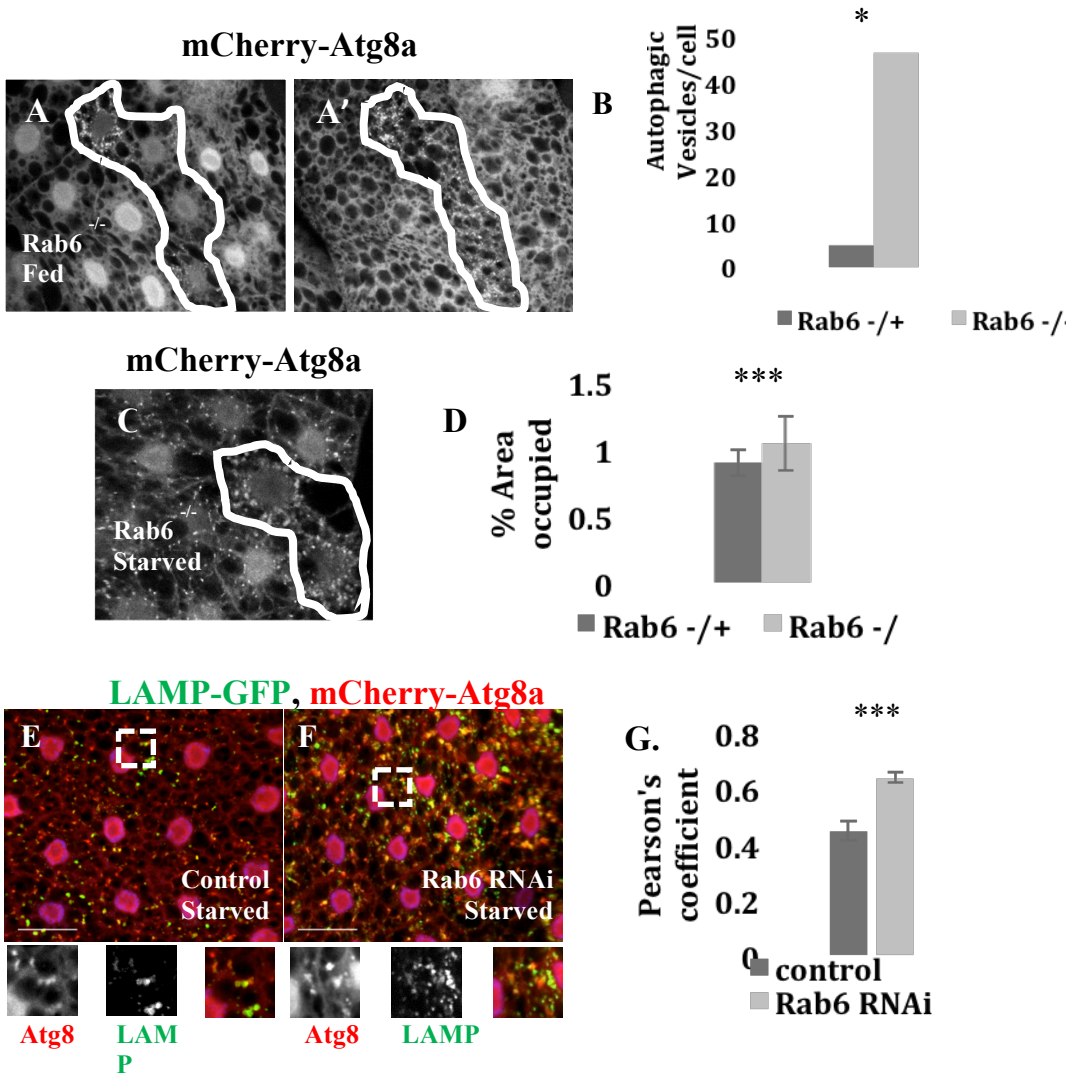


Figure 14. Loss of Rab6 leads to accumulation of autolysosomes.

A, B) Representative images of larval fat body containing a *Rab6* null cell clone (outlined in white), showing increased accumulation of mCherry-Atg8a-marked autophagic vesicles relative to surrounding control cells under fed conditions. Distal (A) and nuclear (A') focal planes are shown. Mean number of mCherry-Atg8a punctae per cell is indicated in B. C-D) Formation of autophagic vesicles is observed in response to 4hr starvation in both *Rab6* null cell clones and surrounding control cells, Relative area of mCherry-Atg8a punctae is quantified in D. E-G) mCherry-Atg8a punctae co-localize with LAMP-GFP in both control (E) and Rab6 depleted cells (F) under starvation conditions. Co-localization coefficient of these markers is shown in (G), n=8. Scale bar, 25µm. B) n=60 and D) n=171; n= clone per genotype and condition. *p<0.05, ***p<0.01, Student's t-test. Error bars indicate s.e.m. Genotypes: A-B) *hs-flp; Rab6^{D23D}, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/+*. E) Cg-*Gal4 UAS-LAMP-GFP, UAS-mCherry-Atg8a/+*. F) *Cg-Gal4 UAS-LAMP-GFP, UAS-mCherry-Atg8a/UAS-Rab6-dsRNA*

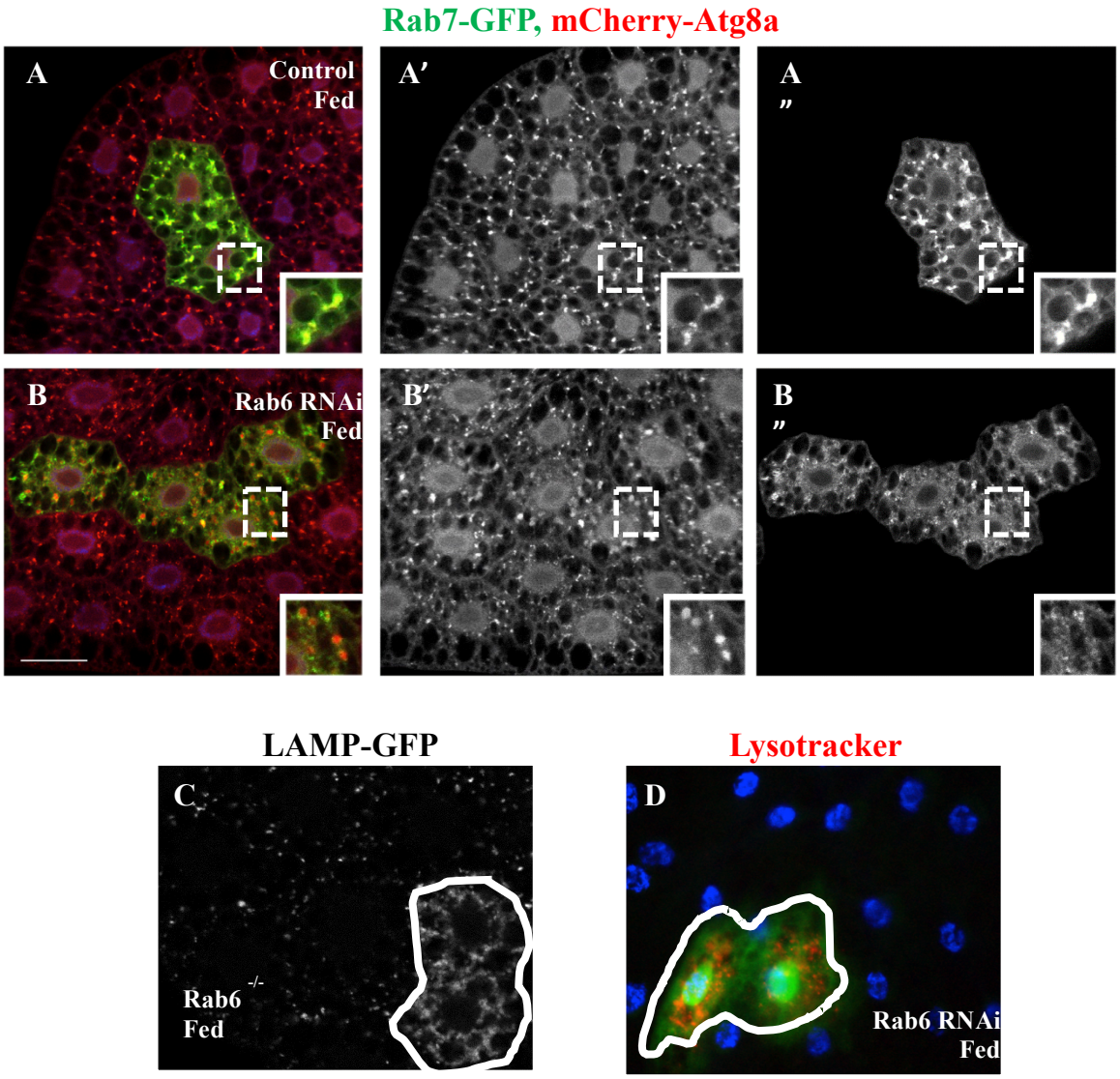


Figure 15. Loss of Rab6 results in expansion of the lysosomal compartment and formation of autolysosomes.

A, B) Representative image of larval fat body containing a *Rab6* RNAi (B) or control (A) expressing cell clone marked by expression of Rab7-GFP, showing punctate formation under starvation of mCherry-Atg8a-marked autophagic vesicles co-localizing with Rab7-GFP under both conditions. A'-B') and A"-B") depict red and green channels, respectively for better visualization. C) Representative image of larval fat body containing a *Rab6* null cell clone (outlined in white), showing expansion of LAMP-GFP labeled lysosomes relative to surrounding control cells under fed conditions. D) Representative image of larval fat body containing a *Rab6* RNAi expressing cell clone (marked by GFP), showing increased accumulation of Lysotracker Red autolysosomes relative to surrounding control cells under fed conditions. Scale bar, 25µm. Genotypes: A*) hs-flp; UAS-Rab7-GFP/+; r4-mCherry-Atg8a, Act<CD2<Gal4, /+. B) hs-flp; UAS-Rab7-GFP/+; r4-mCherry-Atg8a, Act<CD2<Gal4, /+. C) hs-flp; Rab6^{D23D}, FRT40A /UAS-ds-Red, FRT40A, fb-Gal4, UAS-LAMP-GFP; +/+ D) hs-flp; +/+; Act<CD2<Gal4, UAS-GFP/UAS-Rab6-dsRNA*

Lysosomal function is reduced in the absence of Rab6

An increase in auto-lysosomal number could be the result of an imbalance between autophagic vesicle production and/or a defect in their degradation or turnover (Shacka and Roth, 2007; Walls et al., 2007). We decided to test if any of the two or a combination could explain our observations when Rab6 was lost in cells. First, we evaluated the lysosomal compartment using LAMP-GFP as a marker in Rab6 null clones and Rab6 RNAi expressing fat bodies. We observed an expansion of the lysosomal compartment as compared to control tissue when Rab6 was knocked down and in analysis of Rab6 null clones (Fig. 15, A and Fig 16, A-C). Next, we determined if acidification was occurring in the absence of Rab6. We expressed Rab6 RNAi clonally and stained for lysotracker (a dye labeling acidified compartments) under fed and starvation conditions. We detected an accumulation of lysotracker punctae under fed conditions and normal punctae formation under starvation when Rab6 was knocked down, as compared to control (Fig. 4C, 4 and data not shown). In addition, we monitored the co-localization of V-ATPases subunits, Vha55 (V1 complex subunit) and VhaM8.9 (V0 accessory subunit) with autophagic vesicles labeled with mCherry-Atg8 in control and Rab6 RNAi clonal cells. We observed normal co-localization when Rab6 was knocked down compared to control cells (Fig. 17, A-D). However, an obvious enlargement of these autolysosomes was observed in the absence of Rab6.

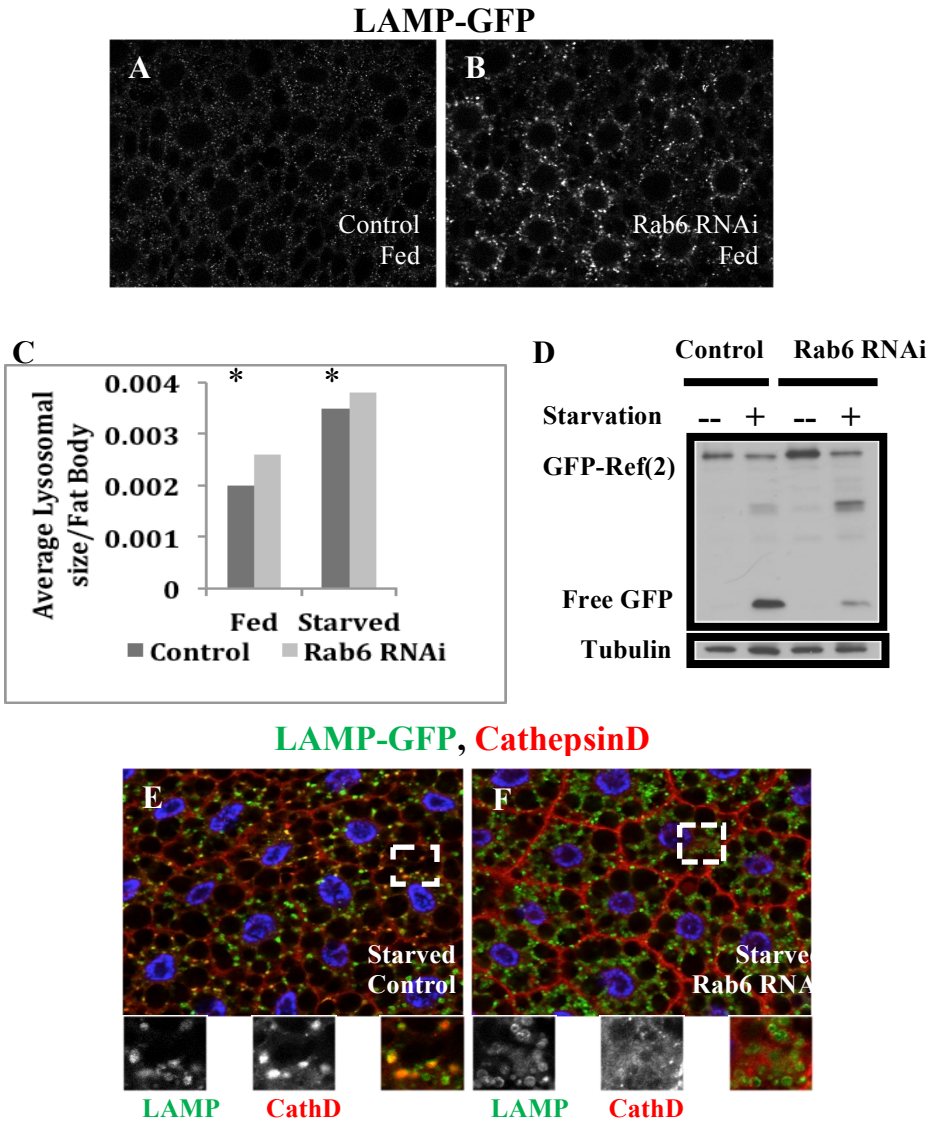


Figure 16. Rab6 loss results in expansion of the lysosomal compartment and reduced lysosomal function.

A-C) Depletion of Rab 6 throughout the larval fat body results in expansion of the LAMP-GFP-marked lysosomal compartment (B) compared to control tissue (A); data are quantified in (C). n=10 larvae per genotype; *p<0.05, ***p<0.01, Student's t-test. Error bars indicate s.e.m. D) Rab6 depletion results in higher basal level of GFP-Ref(2)p under fed conditions and reduced degradation under starvation conditions, as indicated by generation of free GFP. Fat body extracts from fed and starved larvae expressing UAS-GFP-Ref(2)p was used to detect GFP-Ref(2)p and/or free GFP via western blot using a GFP antibody. E and F) Cathepsin D co-localizes with the lysosomal marker LAMP-GFP in control fat body cells but is mis-targeted to the plasma membrane upon Rab6 depletion under starvation conditions. Scale bar, 25µm. Genotypes: A, E) *Cg-Gal4 UAS-LAMP-GFP /+*. B, F) *Cg-Gal4 UAS-LAMP-GFP /UAS-Rab6-dsRNA*.

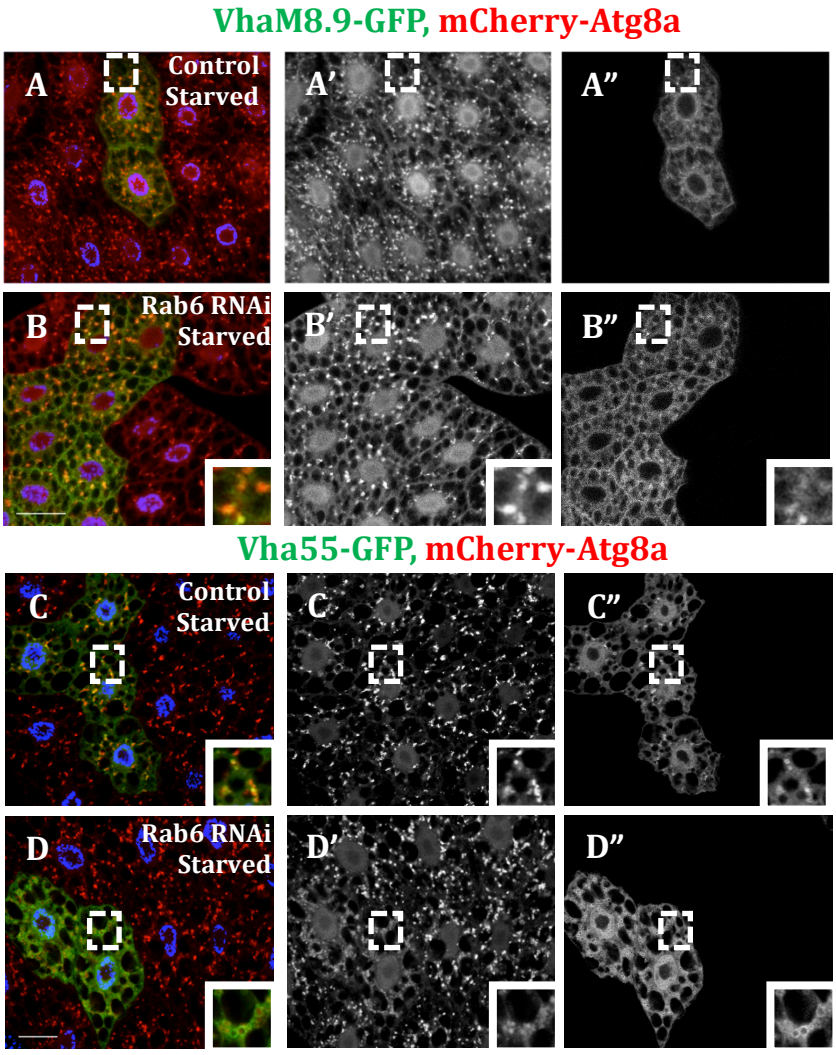


Figure 17. Rab6 is not required for the recruitment of Vacuolar-ATPases at autophagic vesicles.

A, B) Representative image of larval fat body containing a *Rab6* RNAi (B) or control (A) expressing cell clone marked by expression of Vha8.9-GFP, showing punctate formation under starvation of mCherry-Atg8a-marked autophagic vesicles co-localizing with Rab7-GFP under both conditions. A'-B') and A"-B") depict red and green channels, respectively for better visualization. C-D) Representative image of larval fat body containing a *Rab6* RNAi (D) or control (C) expressing cell clone marked by expression of Vha55-GFP, showing punctate formation under starvation of mCherry-Atg8a-marked autophagic vesicles co-localizing with Rab7-GFP under both conditions. C', D') and C", D") depict red and green channels, respectively for better visualization. Scale bar, 25µm. Genotypes: A) *hs-flp; UAS-Vha55-GFP/+; r4-mCherry-Atg8a, Act<CD2<Gal4, /+. B) hs-flp; UAS-Vha55-GFP/+; r4-mCherry-Atg8a, Act<CD2<Gal4, /UAS-Rab6-dsRNA. C) hs-flp; UAS-Vha55-GFP/+; r4-mCherry-Atg8a, Act<CD2<Gal4, /+. D) hs-flp; UAS-Vha55-GFP/+; r4-mCherry-Atg8a, Act<CD2<Gal4/UAS-Rab6-dsRNA*

Last, we decided to test autolysosomal degradation capacity by monitoring the levels and turnover of the autophagic substrate Ref(2)p (Mauvezin et al., 2014; Pircs et al., 2012). To this end, we employed the use of the autophagic substrate Ref2p/p62 tagged to GFP. This allowed us to probe with a GFP antibody to detect full length protein (Ref2p+GFP) and free GFP production under fed and starvation conditions (Mauvezin et al., 2014). Samples from control and fat body tissue where Rab6 was knocked down under fed and starvation conditions were used. Rab6 knock down resulted in higher basal levels of full length Ref(2)p, as compared to control. Under starvation conditions we observed defects in degradation of the full-length protein and diminished production of free-GFP species (Fig. 16, D). We also noted higher levels of intermediary bands (Ref2p-GFP degradation products) when Rab6 was knocked down. Our results suggest that loss of Rab6 results in expansion of the lysosomal compartment and impairs lysosomal and autolysosomal degradation capacity.

In yeast and mammalian cell culture, Rab6 has been shown to regulate hydrolase delivery to the lysosome. This regulation is indirect by regulating retrograde retrieval of the hydrolase receptor (M6PR and Vps10, in mammals and yeast respectively) from late endosomes/lysosomes to the Golgi (Bonifacino and Hierro, 2011; Conibear and Stevens, 2000; Liewen et al., 2005; Perez-Victoria and Bonifacino, 2009; Siniossoglou and Pelham, 2001). Therefore, we decided to evaluate if the lysosomal hydrolases Cathepsin D and L where delivered to lysosomes when Rab6 was knocked down. First, we tested if Cathepsin D/L localized to the lysosome by performing endogenous staining of the protein. In parallel, we examined the specificity of our antibodies by knocked down of

each respective hydrolase followed by staining. Our results show that Cathepsin D and L co-localize with LAMP-GFP and their respective knockdown results in disappearance of the staining (Fig. 18, A-C), suggesting that our antibodies have specificity. Next, we monitor the hydrolases when Rab6 was knockdown and in Rab6 null cells. We generated null clones of Rab6 in fat bodies of starved animals and examined if Cathepsin D was co-localizing in autophagic vesicles. We observed that staining of Cathepsin D disappeared from Rab6 null cells that had an increased in autophagic vesicle size as compared to control (Fig. 18, D). Additionally, we stained fat bodies of animals expressing LAMP-GFP to monitor hydrolase delivery at the lysosome. Our results show that knockdown of Rab6 in fat bodies under starvation have decreased staining of Cathepsin D at lysosomes compared to control tissue (Fig. 17, E and F). We obtained similar findings when we stained for Cathepsin L (data not shown). Altogether, our data suggests that loss of Rab6 affects the sorting of lysosomal hydrolases to the lysosome in turn affecting lysosomal and autolysosomal function.

Next, we set out to determine the localization of Rab6 in fat body cells. We co-expressed an YFP- Rab6 in fat bodies of larvae and observed that it co-localized with a Golgi marker (RFP-Golgi) and lysosomal marker (LAMP-HRP), (Fig. 19, A and B). Additionally, we examined if Rab6 could co-localize with autophagic vesicles, monitoring mCherry-Atg8a, and observed that both co-localize under starvation (Fig. 19, C). Altogether, our data suggests that Rab6 decorates the compartments involved in the sorting of hydrolases, the Golgi, autophagic vesicles and late endosomes supporting a regulatory role for Rab6 in hydrolase sorting.

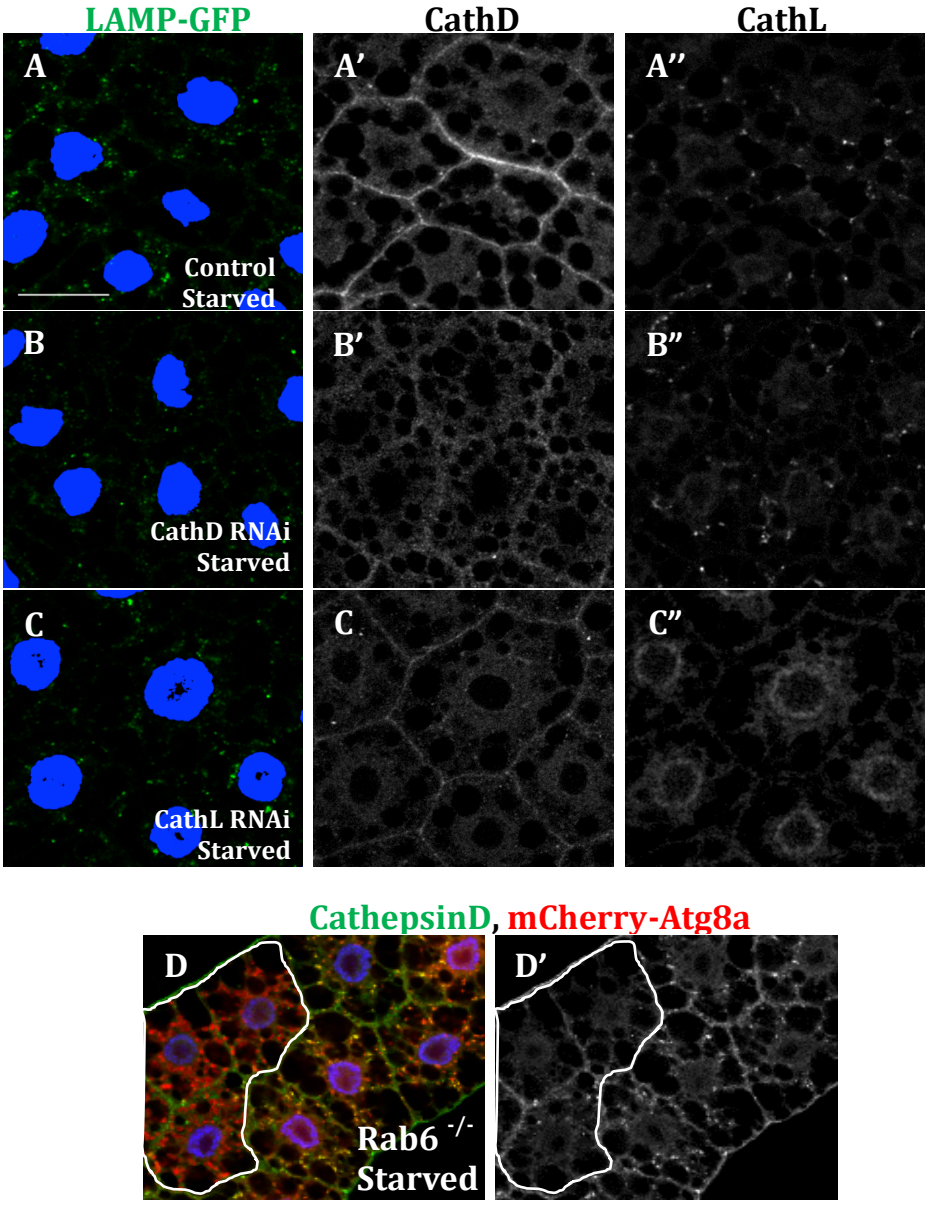


Figure 18. Cathepsin D and L localize at lysosomes under starvation.

A-C) Depletion of Cathepsin D (B) and Cathepsin L (C) throughout the larval fat body results in expansion in reductions of Cathepsin D (B') and Cathepsin L (C") staining co-localizing with LAMP-GFP lysosomal compartment compared to control tissue (A). D) Clones of Rab6 null fat body cells (encircled in white) show reduced Cathepsin D staining on autophagic vesicles relative to surrounding control cells upon 4-hr starvation. D') depicts green channel (Cath D) for better visualization. Scale bar, 25µm. Genotypes: A) *Cg-Gal4 UAS-LAMP-GFP/+. B) Cg-Gal4 UAS-LAMP-GFP/UAS-CathD-dsRNA. C) Cg-Gal4 UAS-LAMP-GFP/UAS-CathL-dsRNA. D) hs-flp; Rab6^{D23D}, FRT40A /UAS-2xeGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/+.*

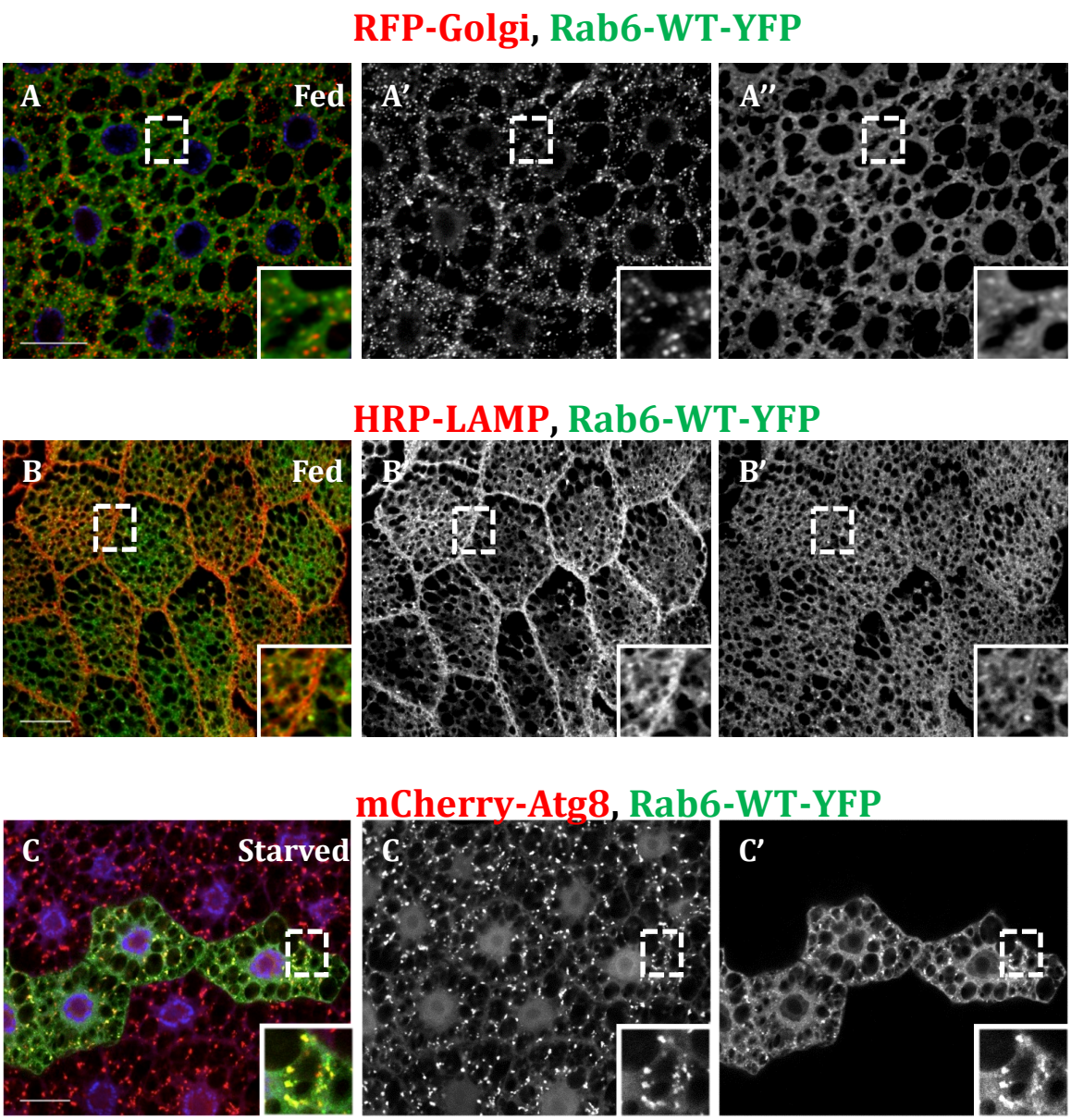


Figure 19. Rab6 subcellular localization in fat body cells

A) Co-localization between Rab6-YFP and Golgi-RFP expressed throughout the larval fat body. A', A") depict red and green channels, respectively for better visualization. B) Rab6-YFP and HRP-LAMP were expressed throughout the larval fat body and immune-stained the HRP antigen (B'). B', B") depict red and green channels, respectively for better visualization. C) Representative image of larval fat body containing expressing cell clone marked by expression of Rab6-YFP, showing punctate formation under starvation of mCherry-Atg8a-marked autophagic vesicles co-localizing with Rab6-YFP. C', C") depict red and green channels, respectively for better visualization. Scale bar, 25µm. Genotypes: A) *Cg-Gal4 UAS-Rab6-WT-YFP/UAS-Golgi-RFP. B) Cg-Gal4 UAS-Rab6-WT-YFP/UAS-HRP-LAMP. C) hs-flp; UAS-Rab6-WT-YFP/+; r4-mCherry-Atg8a, Act<CD2<Gal4, /+.*

Rab6 loss results in impaired turnover of autophagic vesicles and defective nutrient sensing.

Impaired lysosomal function results in accumulation of autophagic vesicles (Mauvezin et al., 2015; Takats et al., 2013; Takats et al., 2014). We have shown that loss of Rab6 results in reduced degradation of lysosomes and autolysosomes and accumulation of autophagic vesicles (Fig. 16). Therefore, we wanted to test if these autophagic vesicles could turnover when Rab6 was lost. We tested this by taking fed larvae, subjecting them to starvation and then transferring them back to rich food to allow cells to clear autophagic vesicles intrinsically. Experimentally, we generated Rab6 null clones while monitoring autophagic vesicles, using mCherry-Atg8, under starvation and after we transferred larvae into rich food for 7 hours following starvation. Our results show that under starvation Rab6 null cells have a non-statistically significant higher size of autophagic vesicles compared to control cells (Fig. 20, A and C). However, when larvae were transferred to rich food conditions Rab6 null cells failed to turnover autophagic vesicles as their size remained bigger than control neighbor cells and similar in size to starvation conditions (Fig. 20, A-C). Our results suggest that defective degradation observed upon Rab6 loss translates into a failure of autophagic vesicle turnover.

Nutrient stimulation is a potent activator of mTOR. To confirm that our experimental design was having the desired effect (e.g. enhancing mTOR activity upon nutrient stimulus conditions) we decided to monitor the phosphorylation of S6 kinase (S6K), a downstream target of mTOR. We obtained fat body tissue from control and Rab6 knockdown flies under fed, starvation and nutrient re-addition conditions and subject

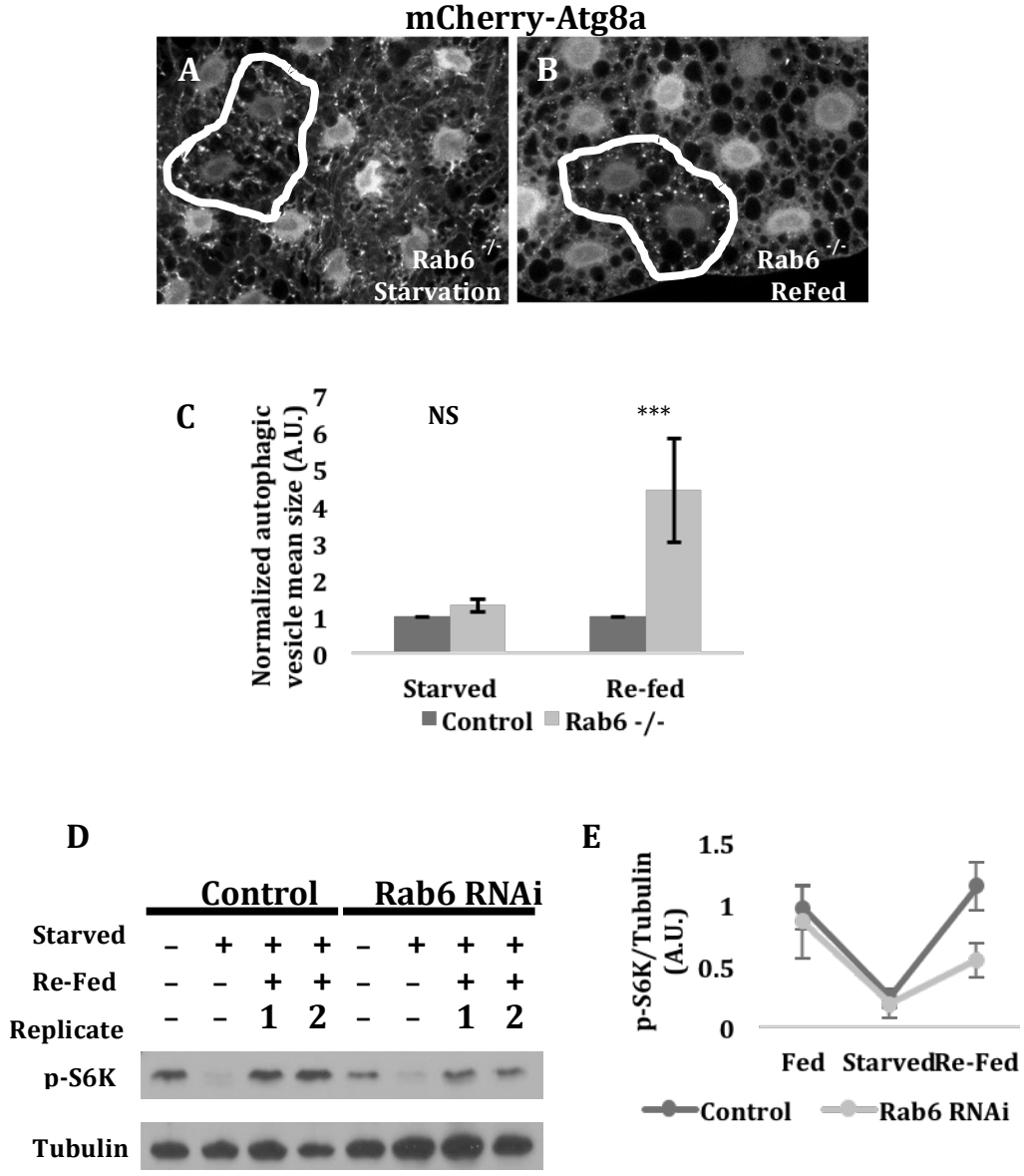


Figure 20. Nutrient sensing and autophagic clearance are compromised in the absence of Rab6.

A-C) Clones of Rab6 null fat body cells (encircled in white) show impaired clearance of autophagic vesicles relative to surrounding control cells upon transition from 4-hr starvation conditions (A) to 7-hr refeeding on full rich food (B). Scale bar, 25µm. Autophagic vesicle size is quantified in (C). A.U. arbitrary units. n=13, n=clone per condition. NS, not significant; ***p<0.01, Student's t-test. Error bars indicate s.e.m. D and E) Rab6 depletion results in decreased activation of mTOR upon nutrient re-addition. Fat body extracts from larvae at the indicated time points and nutritional states were used to monitor phosphorylation of S6 kinase. Quantification of data represented in D (E). Genotypes: A, B) *hs-flp; Rab6^{D23D}, FRT40A /UAS-2xeGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/+. D, E) control, Cg-Gal4/+. Rab6 RNAi: Cg-Gal4/UAS-Rab6-dsRNA.*

them to western blot analysis. We observed phosphorylation of S6K under fed conditions in control animals. Phosphorylation was rapidly lost upon starvation and regained after nutrient re-addition in control animals (Fig. 20, D and E). When Rab6 was knocked down we observed a consistent moderate to slight decrease in the phosphorylation status of S6K. No obvious differences were noted under starvation conditions when compared to control tissue in the same time point. Surprisingly, when Rab6 knockdown tissue was examined for the nutrient re-addition group we observed a more consistent decreased phosphorylation of S6K than fed conditions (Fig. 20, D and E). Our data suggests that is required Rab6 for the activation of S6K by mTOR.

Rab6 loss results in decreased canonical insulin signaling

Amino acid and insulin signaling are upstream activators of mTOR (Shimobayashi and Hall, 2014). We decided to generate Rab6 null clones while independently over-expressing Rheb or a constitutive active form of RagA (RagA-GTP) in the entire fat body of larvae to activate mTOR directly and to mimic amino acid dependent mTOR activation, respectively. As a readout for these experiments we decided to use two well-established phenotypes used for perturbations of mTOR signaling: 1) cell size area measurements and 2) monitoring of autophagic vesicles, using mCherry-Atg8a. We found that Rab6 null clones have a slight reduction in cells size under fed and starvation conditions that could not be rescued by over-expression of RagA-QL but slightly improved by Rheb over-expression when compared to Rab6 null cells (Fig. 21, A-E). In addition, we observed that Rheb over-expression, but not RagA-QL, was able to reduce the percentage area occupied by autophagic vesicles observed in Rab6 null cells under

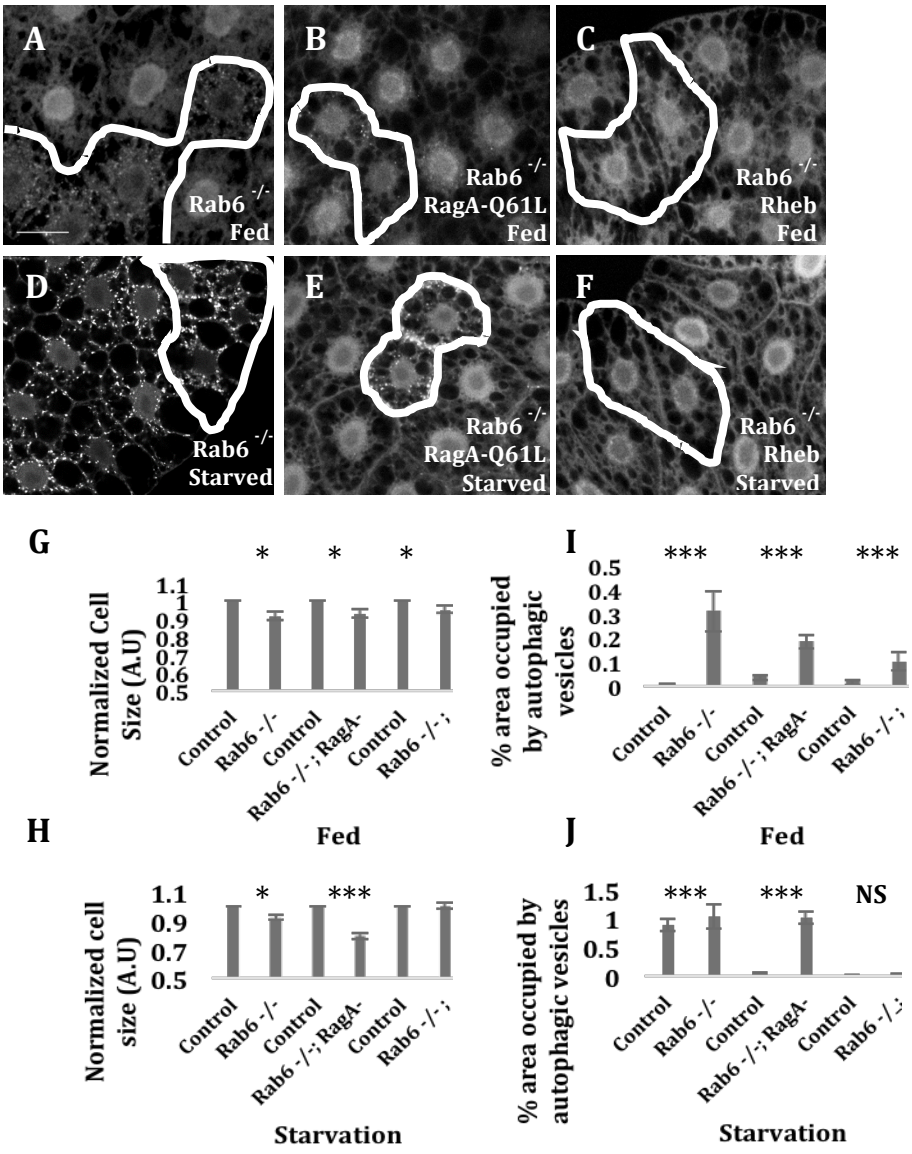


Figure 21. Rab6 mutant phenotypes are rescued by Rheb over-expression, but not by constitutive activation of RagA.

A-F) Rab6 mutant cell clones (outlined in white) were induced in control background (A, D) or in the presence of fat body-specific expression of $RagA^{Q61L}$ (B, E) or Rheb (C, F), and observed under fed or 4-hr starvation conditions. Autophagic vesicles are marked by mCherry-Atg8a. Scale bar, 25µm. G-J) Quantification of cell size (G, H) and autophagic vesicle area (I, J) under fed and 4-hr starvation conditions, for genotypes indicated in A-F). A) n=60, B) n=171, C) n=77, D) n=56, E) n=71 and F) n=77; n= clones per genotype. NS, not significant; *p<0.05, ***p<0.01, Student's t-test. Error bars indicate s.e.m. Genotypes: A, D) *hs-flp; $Rab6^{D23D}$, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/+*. B, E) *hs-flp; $Rab6^{D23D}$, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/UAS-RagA-Q61L*. C, F) *hs-flp; $Rab6^{D23D}$, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/UAS-Rheb-AV4*.

starvation conditions (Fig. 21, I and J). In contrast, we could not observed reduction in the percentage of autophagic vesicle under basal conditions when Rheb or RagA-QL was over-expressed as compared to Rab6 null cells (Fig. 21, G and H). Interestingly, constitutive activation of RagA-QL resulted in enhancement of the Rab6 null phenotypes measured. Our results suggest that loss of Rab6 interferes with Rheb mediated activation of mTOR.

Canonical insulin signaling (CIS) is activated upon binding of insulin to its receptor when nutrients are abundant, resulting in downstream activation of effectors proteins Akt, Rheb and mTOR (Dazert and Hall, 2011; Lavery et al., 2007; Shimobayashi and Hall, 2014). Therefore, we wanted to test if loss of Rab6 loss could affect the phosphorylation of Akt (p-Akt). We tested this by using fat body tissue of control larvae and Rab6 knockdown for western blotting during fed, starved and nutrient re-feeding conditions. In control tissue we observed phosphorylation of Akt under fed conditions, as expected. Starvation resulted in decreased phosphorylation while subsequent placement of larvae in rich food resulted in increased phosphorylation of Akt, as compared to starvation conditions in control fat body tissue. Meanwhile, Rab6 knockdown resulted in decrease phosphorylation of Akt during basal states. No difference was noted during starvation, between Rab6 knock down and control tissue. However, when larvae lacking Rab6 in fat body tissue where placed in rich food following starvation we observed decreased phosphorylation of Akt, as compared to control (Fig. 22, A and B). Our results show that Rab6 is required for nutrient dependent activation of Akt.

The phosphatase and tensin homolog (PTEN) is a negative regulator of CIS opposing activation of Akt and the enhanced PI3P synthesis resulting from insulin binding to its receptor (Gao et al., 2000). Therefore, we decided to determine if we could rescue the Rab6 associated phenotypes by parallel removal of PTEN in Rab6 null clones. We generated Rab6 and PTEN null clones to examine changes in cell size area and percentage area occupied by autophagic vesicles, monitoring mCherry-Atg8a. As described above Rab6 null clones have a slight decrease in cell size under fed and starvation conditions, while PTEN null clones have an increase in cell size (Fig. 22, C, D and F and Fig. 23, A, B and D). Meanwhile, double knockout clones of PTEN and Rab6 showed an increase in cell size under both nutritional conditions (Fig. 22, E and F and Fig. 23, C and D). Additionally, we evaluated the percentage area occupied by autophagic vesicles and observed that double knockout clones had a higher percentage than PTEN null clones, but lower than Rab6 null clones (Fig. 22, C-E and G and Fig. 23, A-C and E). Altogether, tour data suggests that activation of Akt and CIS via removal of PTEN rescues the Rab6 associated phenotypes. Therefore, this positions Rab6 parallel or upstream to PTEN in CIS regulation. Collectively, our data shows that Rab6 genetically interacts with members of the insulin/mTOR cascade and is required for its activation.

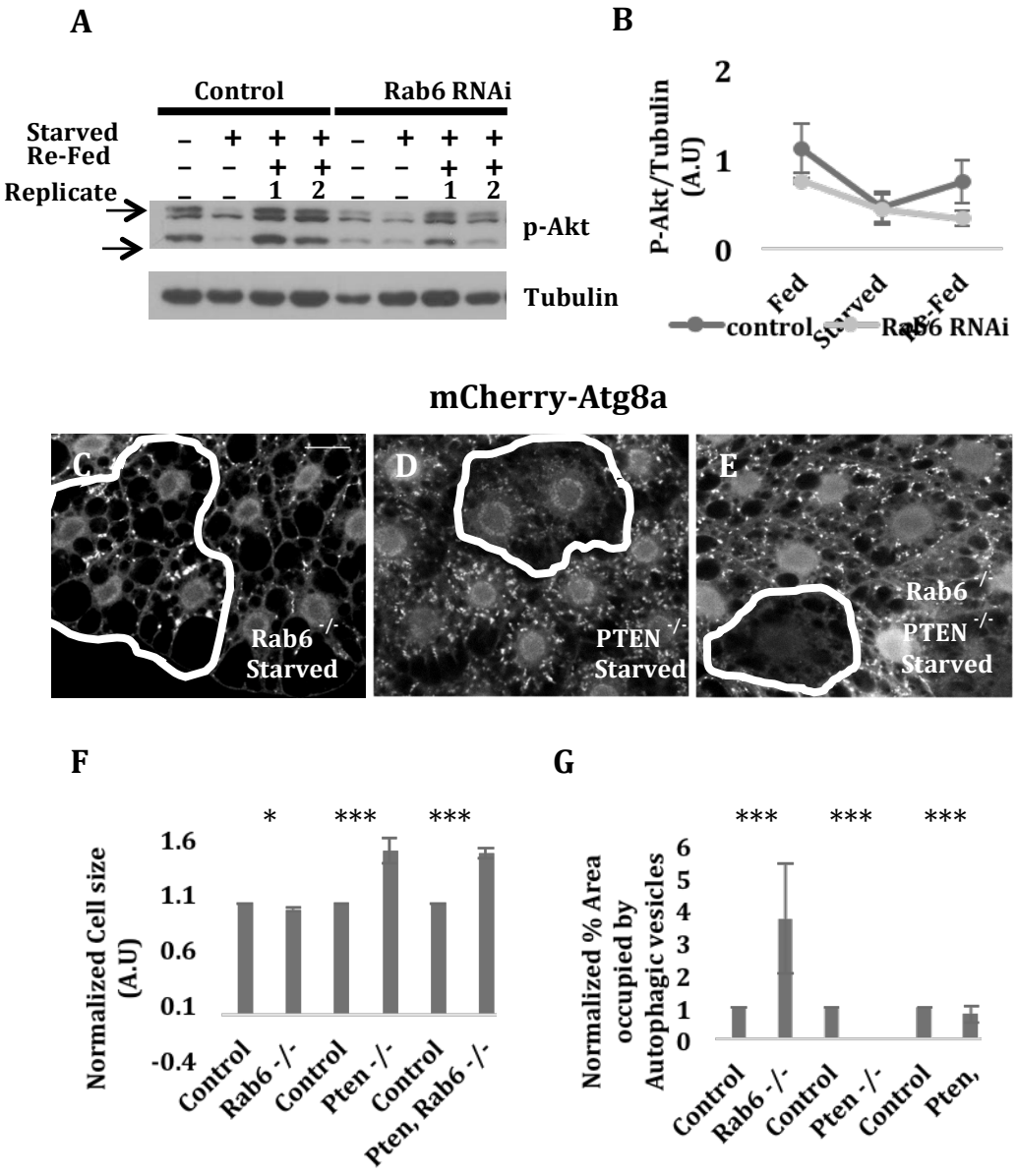


Figure 22. Loss of Rab6 is rescued by parallel removal of PTEN.

A, B) Akt phosphorylation (p-Akt; arrows 2 isoforms) is reduced in extracts of Rab6-depleted fat body tissue under basal and conditions and in response to re-feeding. C-G) Accumulation of mCherry-Atg8a marked autophagic vesicles in response to 4-hr starvation in surrounding control cells and in Rab6$^{-/-}$ (C), PTEN$^{-/-}$ (D), and Rab6$^{-/-}$, PTEN$^{-/-}$ (E) mutant clones (null clones are outlined in white). Cell size and percent area occupied by mCherry-Atg8a (each normalized to surrounding control cells) are indicated for the genotypes shown in in C-E. Scale bar, 25 µm. Genotypes: C) *hs-flp; Rab6^{D23D}, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/+. D) hs-flp; PTEN^{Dj189}, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/+. E) hs-flp; Rab6^{D23D} PTEN^{Dj189}, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/+. C)* n=171, *D)* n=15, *E)* n=65; n= clones per genotype. *p<0.05, ***p<0.01, Student's t-test. Error bars indicate s.e.m.

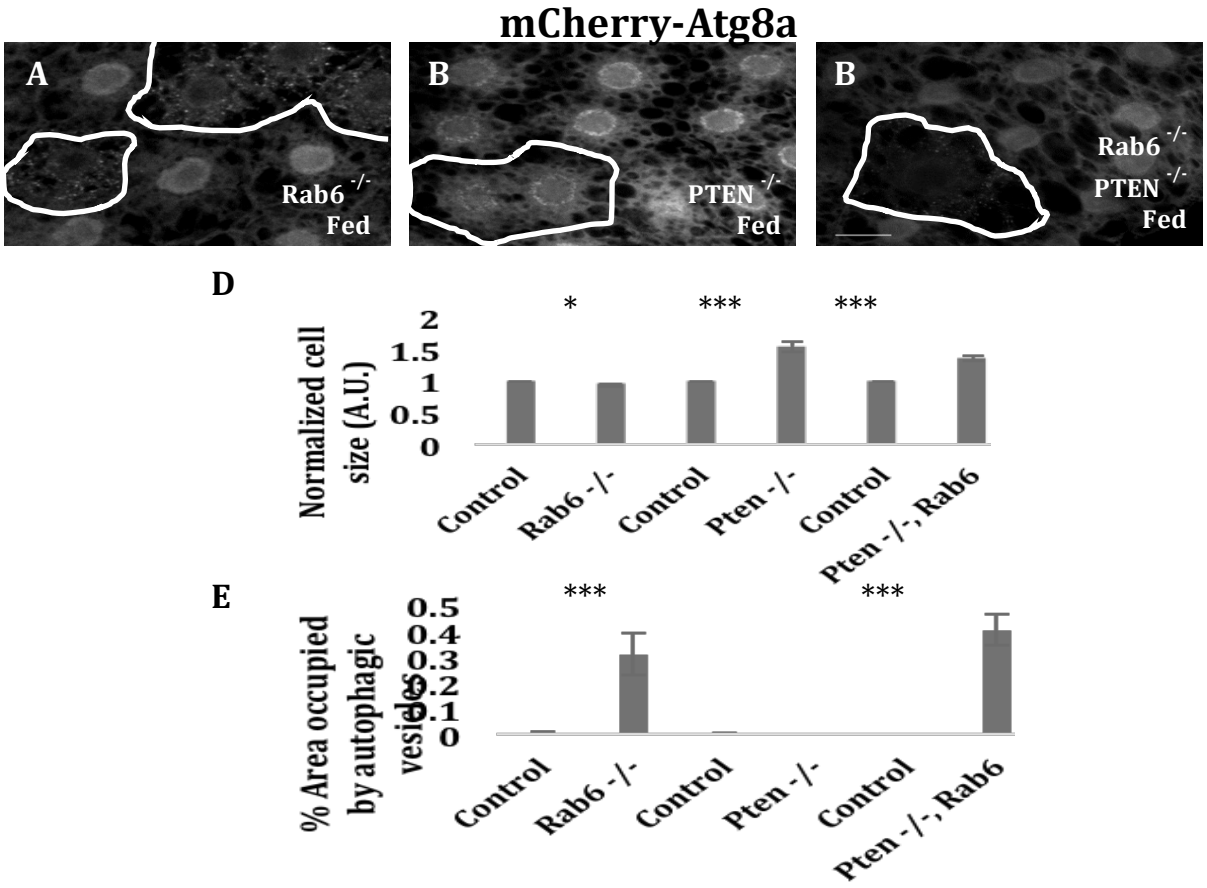


Figure 23. Cell Size reduction in Rab6 null clones is rescued by parallel removal of PTEN under basal states

A-C) Evaluation of mCherry-Atg8a marked autophagic vesicles under basal states in surrounding control cells and in Rab6$^{-/-}$ (A), PTEN$^{-/-}$ (B), and Rab6$^{-/-}$ PTEN$^{-/-}$ (C) mutant clones (null clones are outlined in white). D, E) Cell size and percent area occupied by mCherry-Atg8a (each normalized to surrounding control cells) are indicated for the genotypes shown in in A-C. Scale bar, 25 µm. Genotypes: A) *hs-flp; Rab6^{D23D}, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/+. B) hs-flp; PTEN^{Dj189}, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/+.* C*) hs-flp; Rab6^{D23D} PTEN^{Dj189}, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/+. A)* n=60, B*)* n=21, C*)* n=89; n=clones per genotype. *p<0.05, ***p<0.01, Student's t-test. Error bars indicate s.e.m.

Rab6 loss results in internalization and mis-localization of the insulin receptor towards lysosomes.

Canonical insulin signaling (CIS) is initiated via contact of insulin with the insulin tyrosine kinase receptor (InR) at the plasma membrane. Its presence and continuous shuttling upon internalization onto the endocytic pathway towards the plasma membrane is a basic requirement for CIS. This prompted us to evaluate the localization of the InR in the fat body of Drosophila under fed, starved and nutrient re-addition conditions when Rab6 was knocked down. We co-expressed fluorescent-tagged InR-CFP and LAMP-GFP to determine the localization of both markers in control and Rab6 knocked down fat bodies under distinct nutrient states. We observed consistent localization of the InR at the plasma membrane during all nutrient conditions tested in control larvae and enhancement of the signal after nutrient re-addition in rich food (Fig. 24, A-C and G). When Rab6 knockdown tissue was evaluated we observed no difference in signal intensity at the plasma membrane during fed conditions when compared to control tissue (Fig. 24, C, F and G). Upon starvation we observed moderate InR punctae accumulate in the cytosol and a decrease in the signal intensity at the plasma membrane (Fig. 24, E and F). Interestingly, nutrient re-addition following starvation resulted in enhancement of InR cytosolic punctae and loss of InR plasma membrane labeling in tissue lacking Rab6 when compared to control tissue (Fig. 24, A-F and G). In addition, we were able to determine that the cytosolic InR punctate that form when Rab6 was knocked down co-localize with the lysosomal marker LAMP-GFP (Fig. 24, A-F).

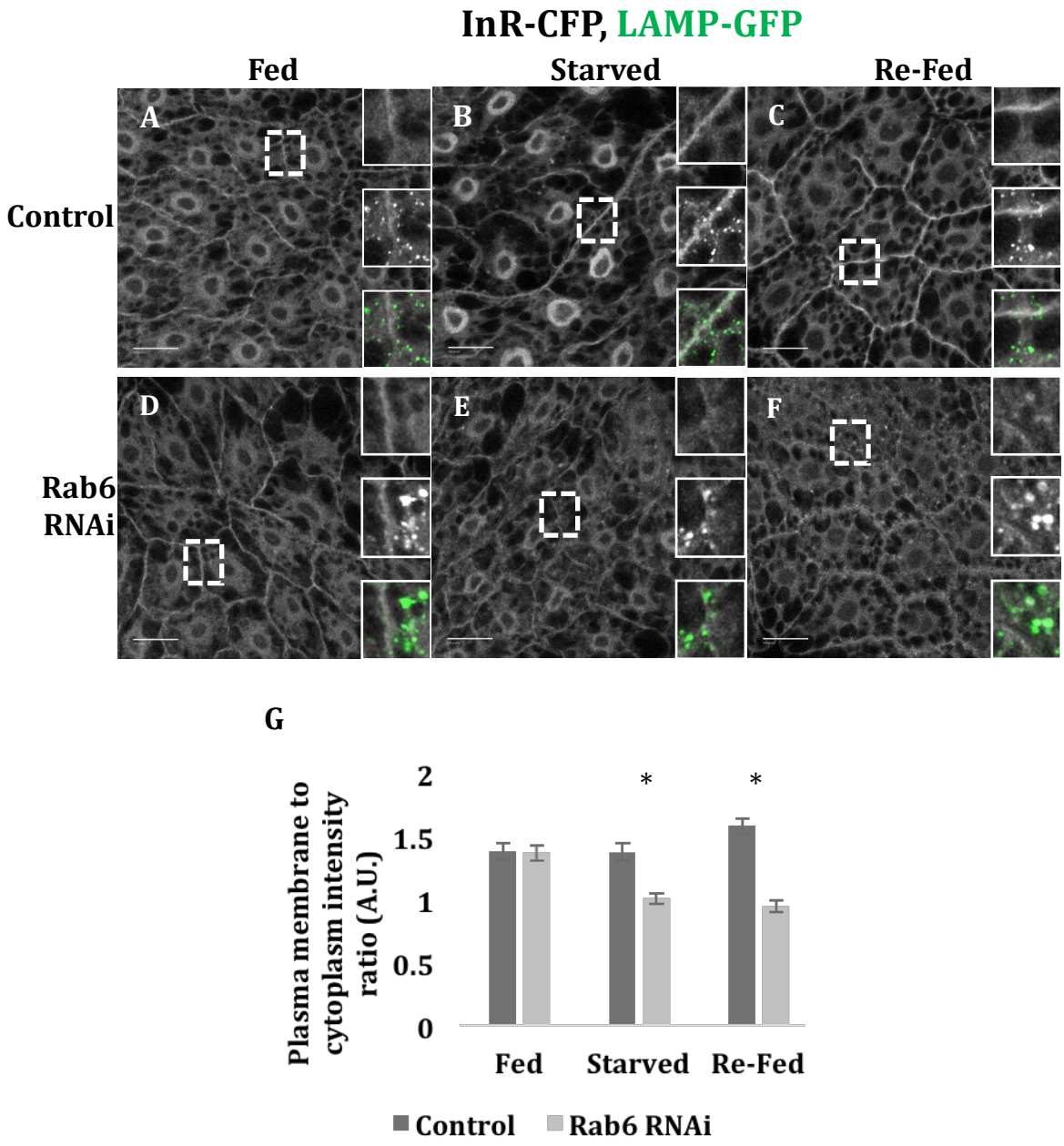


Figure 24. Rab6 depletion results in mis-localization of the insulin receptor.

Representative images of InR-CFP are shown in gray scale for control (A-C) and Rab6-depleted (D-F) fat body cells under fed, 4-hr starved and 7-hr re-fed conditions as indicated. Insets show increased magnification of InR-CFP (top), LAMP-GFP (middle) and merge (bottom; LAMP-GFP in green). Quantified ratio of plasma membrane to cytoplasmic InR-CFP signal is shown in (G). n=19 larvae per condition and genotype. *$p<0.05$, Student's t-test. Error bars indicate s.e.m. Scale bar, 25µm. Genotypes: A-C) Cg-Gal4, UAS-GFP-LAMP/+; UAS-InR-CFP/+. D-F) Cg-Gal4, UAS-GFP-LAMP/+; UAS-Rab6-dsRNA/UAS-InR-CFP

In addition, we monitored the effects on InR trafficking using our null allele. We observed a nutrient-independent effect on Rab6 null clones as we could detect internalization of the receptor and decreased plasma membrane signal under all nutrient states (Fig. 25, A-C). Also, we observed co-localization between InR and mCherry-Atg8a in Rab6 null clones. Taken together, our results suggest that Rab6 affects the retrieval of the InR from the endocytic pathway to avoid its degradation at autolysosomes and lysosomes. Furthermore, these findings provide an additional mechanistic explanation for the autophagic vesicles that accumulate in Rab6 null clones, as the InR is not at the plasma membrane to transduce a nutrient signal to activate mTOR.

Our findings evaluating the InR prompted us to evaluate other stages and/or markers of the endocytic pathway. We have already established that upon Rab6 loss the LE/lysosomal compartment is enlarged when we monitor LAMP-GFP as a marker (Fig. 15 and 16). Therefore, we decided to examine early endosomes, LE and the recycling endosomes compartments using Rab5, VhaM8.9 and the recycling endosome cargo human Transferrin-GFP (hTf-GFP), respectively. Our results show that Rab5 is dispersed throughout the cytosol while VhaM8.9 is localized at the plasma membrane and cytosol in control tissue (Fig. 26, A). Both are in agreement with previous observations in other tissues and the larval fat body for these markers (Juhasz et al., 2008). In contrast, in tissue where Rab6 was knockdown we observed an expansion in the size of early endosomes and increased internalization and expansion of VhaM8.9 compartments (Fig. 26, B). When hTf-GFP was evaluated we observed that it localized at the plasma membrane and cytoplasm in control tissue (Fig. 26, C). In contrast, when Rab6 was knockdown we

observed internalization of this recycling endosome cargo and loss of its labeling at the plasma membrane (Fig. 26, D). Our collective observations, phenotypes and the expansion we observed in the endomembrane system suggest Rab6 is required for the maintenance of endocytic pathway homeostasis and the recycling of membrane bound proteins from the endomembrane system towards the plasma membrane.

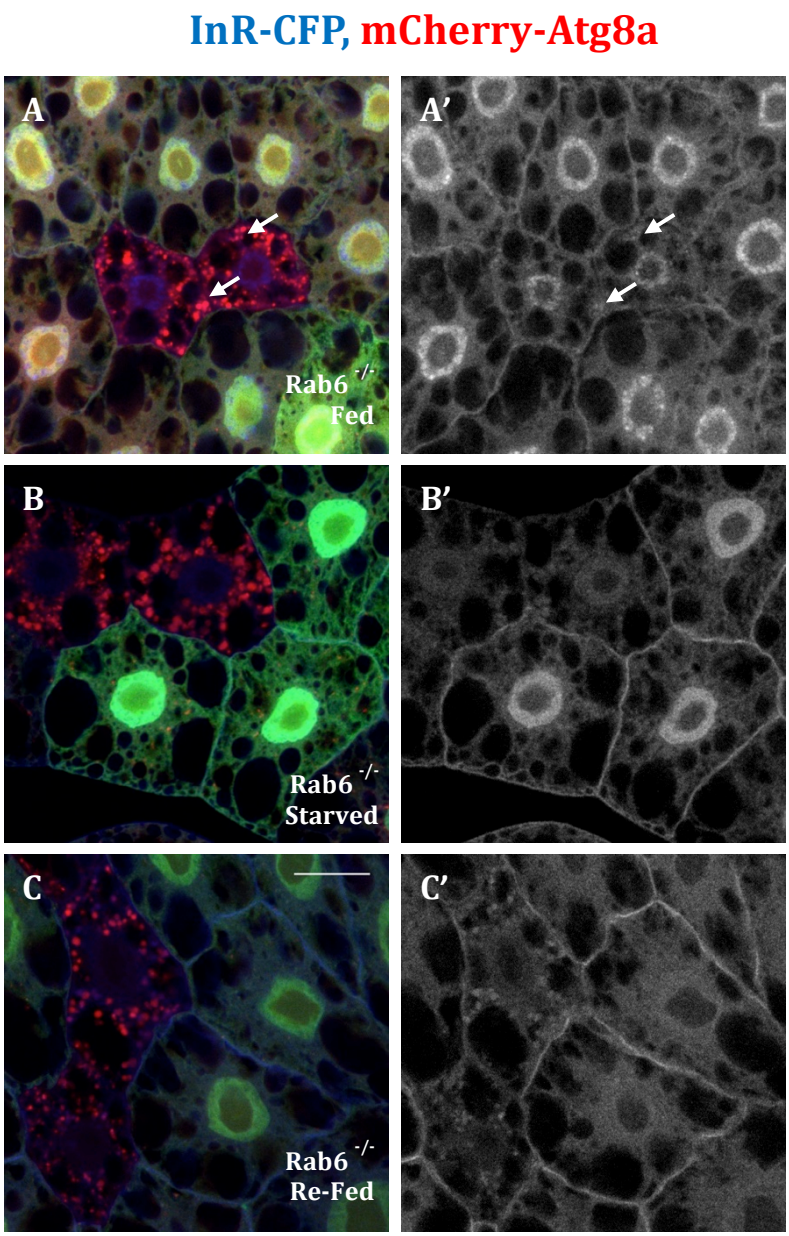


Figure 25. Loss of Rab6 results in internalization of the insulin receptor independent of nutrient status.

A-C) Evaluation of mCherry-Atg8a marked autophagic vesicles and InR-CFP under basal states, starvation and full nutrient re-feeding in surrounding control and in Rab6$^{-/}$ (marked by the absence of GFP) cells. A'-C') is depicted CFP channel for better visualization. Scale bar, 25 µm. Genotypes: A-C) *hs-flp; Rab6^{D23D}, FRT40A /UAS-2x-eGFP, FRT40A, fb-Gal4; UAS-mCherry-Atg8a/UAS-InR-CFP.*

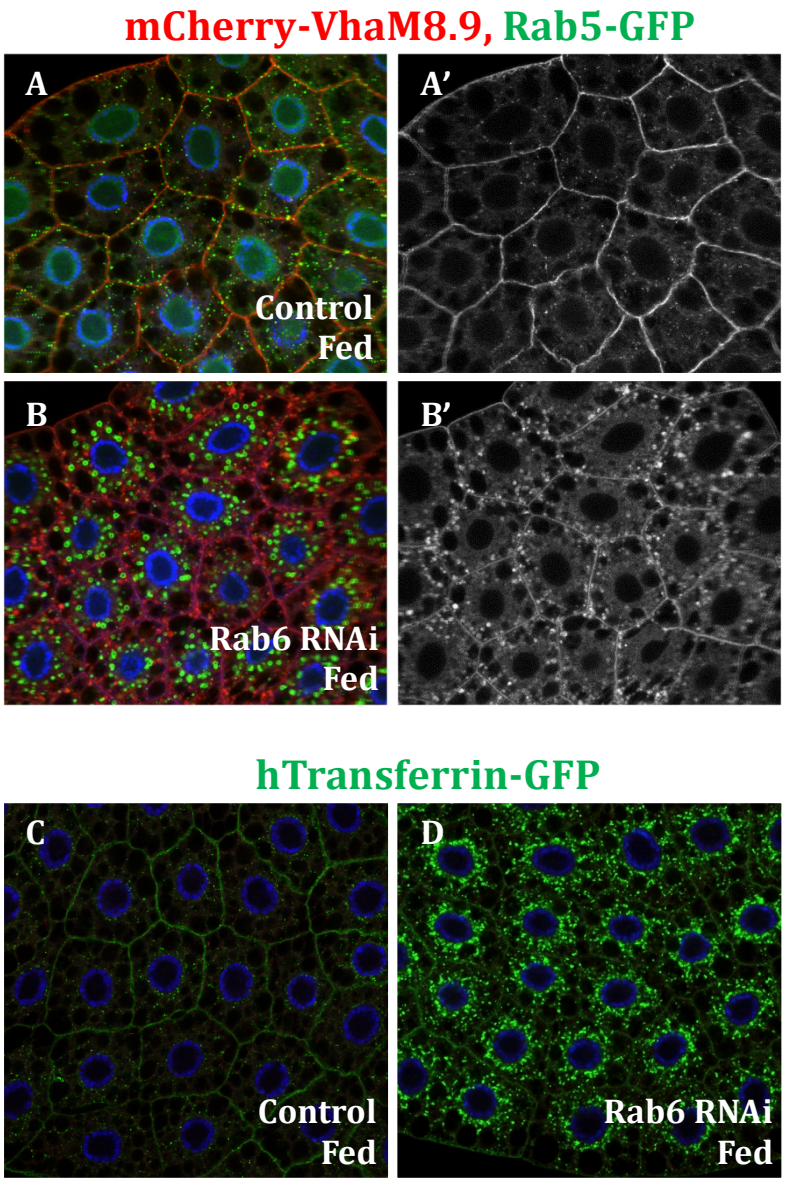


Figure 26. Depletion of Rab6 results in mis-localization of plasma membrane proteins.

A, B) Depletion of Rab 6 throughout the larval fat body results in expansion of the Rab5-GFP-marked early endosomal and VhaM8.9-mCherry late endosomal compartments and loss of plasma membrane localization of VhaM8.9 (B) compared to control tissue (A). A', B') depict red channel (VhaM8.9) for better visualization. C-D) Depletion of Rab 6 throughout the larval fat body results in expansion of the hTransferrin-GFP-marked recycling endosomal compartment (D) compared to control tissue (C); Scale bar, 25µm. Genotypes: A) Cg-Gal4, *UAS-VhaM8.9-mCherry, UAS-Rab5-GFP/+; +. B)* Cg-Gal4, *UAS-VhaM8.9-mCherry, UAS-Rab5-GFP/+; UAS-Rab6-dsRNA/+. C) Cg-Gal4/+; UAS-hTf-GFP/+. D) Cg-Gal4/+; UAS-hTf-GFP/UAS-Rab6-dsRNA*

Chapter 5: Discussion

Discussion

Rab GTPase dependent regulation of autophagy and mTOR signaling

A need to uncover novel Rab GTPases regulating vesicular tracking events during autophagy prompted us to carry a forward genetic screen using Drosophila as a model system. We knocked down all the 33 Rab GTPases encoded in the fly genome using the flip-out system developed in our laboratory. We were able to target UAS-RNAi expression of the gene of interest in clonal cells leaving the rest of the tissue as an internal control. Using these tools we used as readout, mCherry-Atg8a a marker of autophagic vesicles. Upon completion of the screen we were surprised with the number of Rab GTPases affecting the size and number of Atg8a and the cell size of cells. Of great important to us, we uncover new (e.g. Rab2, 6 and 14) and established (e.g. Rab1, 5, 7, 9 and 11) Rab proteins regulating autophagy.

Rab2 and Rab14 are required for autophagosomal growth

Rab2 has been localized to pre-Golgi intermediary sites, segregation stations for anterograde and retrograde traffic of vesicles, to regulate ER-to-Golgi traffic in mammalian cell culture (Tisdale, 1999). In C. elegans, Rab2 mutants (unc-108) display dense core vesicle (DCVs) maturation defects and show motor dysfunction (Edwards et al., 2009; Sumakovic et al., 2009). This is due to failure of retention of factors required for maturation at the Golgi-endosome interface. In addition, Rab2 mutants in worms are defective in apoptotic body removal and maturation of the phagolysosome system (Guo et al., 2010; Mangahas et al., 2008). Similarly, in flies Rab2 has been shown to be

required in hemocytes to allow phagosome maturation (Garg and Wu, 2014). However a role for Rab2 in the regulation of autophagy in Drosophila is remains unexplored.

Interestingly, Rab2 was one of our candidate genes. We find that hypomorphic alleles of Rab2 in flies display pupal lethality and severe motor dysfunction in two distinct assays. This suggests functional conservation of Rab2 function in the nervous system between flies and worms. In addition, we present Rab2 as a novel regulator of autophagy required for the biogenesis and growth of autophagosomes. Evaluation of lysotracker staining using knock down or hypomorphic alleles of Rab2 revealed impaired formation of autolysosomes. Importantly, we could rescue this defect by over-expressing Rab2 in fat bodies of one of our hypomorphic alleles. Evaluation of Cathepsin B activity using a substrate dye revealed reduced staining in Rab2 hypomorphs. Taken together, our data for Rab2, suggest that it is required for induction and growth of autophagic vesicles and the acidification and function of autolysosomes.

Rab14 has been localized to endosomes and the Golgi in mammalian cell studies and extensively associated in the cellular response to pathogens (Proikas-Cezanne et al., 2006). Rab14 localizes and distributes with Rab5 and can regulate endocytic processes in mammalian cells and the kidney (Su et al., 2013). Functionally, it has been associated to maturation of phagolysosomes in response to infection and the regulation of Glut4 recycling to the plasma membrane in adipocytes, among other functions (Garg and Wu, 2014; Kyei et al., 2006; Okai et al., 2015; Reed et al., 2013). In “Drosophila” Rab14 null animals are not lethal suggesting two possibilities: 1) a non-essential gene or 2)

functional redundancy with a different GTPase. Regardless, Rab14 was shown to be important for lysosomal trafficking and phagolysosome maturation in hemocytes (Garg and Wu, 2014). Here we have examined the phenotypes associated with loss of Rab14 and found that loss of Rab14 results in a reduction of size and area covered by autophagic vesicles. In addition, we observed impaired degradation of the autophagic substrate Ref(2) under basal states.

Interestingly, in C. elegans Rab2 and Rab14 have been shown to regulate phagolysosome maturation. In turn, this is required for the removal of apoptotic bodies during nematode development (Guo et al., 2010). Mechanistically, the study showed that Rab2 and Rab14 were sequentially required for the recruitment of Rab7 at the maturing phagosome, whereas Rab5 initiated the cascade by modulating the formation of PIP3 (Guo et al., 2010). Loss of these set of Rab GTPases resulted in impaired degradation and acidification of phagosomes and accumulation of apoptotic bodies. This was fascinating as we recovered the same set of proteins (Rab2, 5, 7 and 14) in our screen with the same autophagy phenotype. Similarly to findings in C. elegans studies, we show that loss of Rab2 and Rab14 affect size and number of autophagic vesicles and have a reduction in lysotracker staining. Taking into consideration the similar phenotypes in the nematode and our data it is tempting to propose a similar sequential mechanism for these GTPases in the regulation of autophagy. More so, considering recent findings showing the similarities between phagolysosome and autolysosome formation, as cells remove apoptotic bodies using components of the autophagic machinery and autophagy (Wang et al., 2013a). In addition, a recent proteomic analysis in flies uncovered that Rab2 can bind

the HOPS subunits Vps39 and Vps41 further supporting a role for Rab2 in lysosomal fusion and/or Rab7 recruitment to the endomembrane system via the HOPS complex (Gillingham et al., 2014). Elucidation of the mechanism and potential regulation of Atg proteins by this set of Rab GTPases will shed light on how they regulate autophagosomal growth.

Rab5 function in endocytosis and autophagy is conserved in flies

Rab5 is a well-characterized GTPase regulating endocytosis and its function is conserved from yeast to mammals. Here we present evidence, using knock down and dominant negative expression of Rab5, that Rab5 is required for endocytosis of the tracer TR-Avidin fat body cells of Drosophila larvae. This validated our tools for further characterization of this protein and suggests Rab5 role in endocytosis is conserved in all tissues examined to date in flies.

Rab5 was one of the first Rab GTPases linked to the autophagic pathway. Using COS-7 cells it was shown to inhibit autophagy by reducing the number of autophagosomes. Characterization of the phenotypes revealed that it is required for the binding of the class III Vps34 complex (Vps15-Atg14-Vps34-Atg6/Beclin) to promote its function and synthesis of PIP3 (Ravikumar et al., 2008). This study also showed that over-expression of Rab5 was sufficient to recues the rough eye phenotype in a Drosophila model of Huntington's disease. Recent evaluation of Vps15 in flies was showed a conserved function in the regulation of autophagy under distinct stress conditions, including starvation (Anding and Baehrecke, 2015). Similarly, Atg6 mutant in flies and mammals

have been shown to be required for endocytosis, Vps34 dependent PIP3 synthesis and autophagy induction (Funderburk et al., 2010; Lorincz et al., 2014; Shravage et al., 2013). In yeast Vps21, the Rab5 orthologue, was shown to localize at the PAS and be required for selective and non-selective autophagy (Chen et al., 2014). However, a conserved role for Rab5 function in the regulation of autophagy in flies remained unexplored.

Here, we present the characterization of Rab5 in the regulation of autophagy in the larval fat body of flies. We found that loss of Rab5 impairs the formation and growth of autophagosomes. This suggests that Rab5 is important in the inductive and expansion/maturation step of autophagy. In addition, we show that Rab5 is required for the degradation of the autophagic substrate Ref(2)p, autolysosome formation and PIP3 synthesis. Importantly, we found that over-expression of active Rab5 (Rab5-Q61L) is sufficient to induce autophagy without stressors under basal states. This is in contrast to findings showing Vps34 over-expression is not sufficient to induce autophagy in flies under basal states (Juhasz et al., 2008), suggesting that activation of Rab5 is a pre-requisite for autophagy induction and activation of Vps34 complex in fly fat body cells. Taken together, our data points to a conserved role for Rab5 in the induction of autophagy by regulating production of PIP3 and point to a novel finding in the growth of autophagosomes.

Mathematical modeling and evaluation of lysosomal populations was shown to support a requirement for Rab5 in the biogenesis of lysosomes (Hirota et al., 2007; Zeigerer et al., 2012). In flies genetic analysis between Rab5 and proteins required for endocytic

maturation, such as Hrs, Rab5 effectors and ESCRT proteins, revealed that upstream removal of proteins regulating early endosomes affected the size of downstream compartments and the accumulation of signal transduction receptors (Vaccari et al., 2009). This suggested that lysosomal compartment degradation was compromised and lysosomes dysfunctional. However, direct evidence provided by lysosomal assays and the evaluation of the lysosomal compartment is absent in the field. Here, we show that expression of a dominant negative construct of Rab5 (GDP-locked) impairs the staining of lysotracker dye and increases the level of the autophagic substrate Ref(2)p. Most strikingly was the observation that dominant negative expression of Rab5 impaired the processing of the Cathepsin L from pro-enzyme to mature form. Importantly, the fact that we could detect the enzyme suggested that Rab5 is not required for synthesis and sorting of the hydrolase. In addition, we observed loss of lysotracker staining in the absence of Rab5 suggesting it might be required for acidification of the endocytic pathway. This is completely possible considering that components of the vacuolar ATPases reside at the plasma membrane, where endocytosis occurs.

Rab5 and its effectors Rabenosyn and Rabex have been shown to be tumor suppressor in flies (Morrison et al., 2008; Thomas and Strutt, 2014). In the larval fat body, we observed that cell autonomously removal of Rab5 using a null allele or expression of a Rab5 dominant negative construct resulted in an increase in cell size while expression of a constitutive active Rab5 reduced it. Given that mTOR and its upstream activators inhibit autophagy we considered if the reduction of autophagosomes we observed when Rab5 was lost was the result of enhanced mTOR activation. However, over-expression of

dominant negative RagA (GDP-locked) or PTEN could not rescue the autophagic vesicle defects observed when Rab5 was lost suggesting that Rab5 may cause an increase in cell size independently of mTOR or that it acts downstream or parallel to the proteins evaluated. Interestingly, analysis of the growth phenotype upon Rab5 loss in other tissues has revealed the growth to be a combinatorial effect of enhanced JNK and Ras signaling driving tumorigenesis (Takino et al., 2014). However, evaluation of Rab5 in S2 Drosophila cells revealed that constitutive active Rab5-QL or inhibition of its function via knockdown or dominant negative expression inhibited S6 kinase phosphorylation in an amino acid dependent manner (Li et al., 2010). We should emphasize that our laboratory has shown that loss or dysfunction of Vps34, the kinase responsible for PIP3 synthesis, did not show defects in mTOR activation in line with our finding for Rab5 (Juhasz et al., 2008). Studies in mammalian cell studies have shed conflicting results in the activation of mTOR upon Rab5 loss (Flinn et al., 2010; Ravikumar et al., 2008). The differences observed could be due to cell type differences, the use of constructs to evaluate true function and/or the nutrient conditions used. Further evaluation of selective nutrient food feeding using live model systems (e.g. flies, worms, mice) will aid in the understanding of a potential role for Rab5 in the regulation of mTOR signaling.

We propose that Rab5 is required for two distinct steps in the regulation of autophagy in the larval fat body. First, it is required for the production of autophagosomes via the production of class III PI3P similar to mammalian studies. Secondly, regulating maturation of the endomembrane from endosome to lysosome to ensure its degradation capacity and function.

Rab6 dually regulates the autophagic pathway

Rab6 is a classic trans-Golgi marker with established roles in the regulation of protein secretion and retrograde endosome-to-Golgi traffic (Luo and Gallwitz, 2003). However, a role in coordinating the reciprocal regulation between TOR signaling and autophagy during distinct nutrient states remains an unexplored topic. Here we have characterized the role of Rab6 as a novel GTPase required to maintain a balance between autophagy and canonical insulin signaling in the larval fat body of flies.

Rab6 in yeast (Ypt6) has an established role in the regulation of CPY (a lysosomal hydrolase) sorting and the processing of the lysosomal enzyme APE1 by regulating endosome-to-Golgi traffic (Bensen et al., 2001). Ypt6 mediates the recruitment of the GARP tether complex at the Golgi to ensure retrieval of the lysosomal hydrolase receptor, Vps10 (Bonifacino and Hierro, 2011; Conibear and Stevens, 2000; Liewen et al., 2005; Perez-Victoria and Bonifacino, 2009; Siniossoglou and Pelham, 2001). Impaired lysosomal hydrolase receptor sorting has been shown to translate in degradation defects and changes in lysosomal morphology in Cathepsin D and GARP mutants (Perez-Victoria and Bonifacino, 2009; Perez-Victoria et al., 2008; Perez-Victoria et al., 2010). Consistent with these findings, we observed expansion of the lysosomal compartment and reduced degradation of the autophagic substrate Ref(2)p when Rab6 was lost. Similar to our findings, loss of the GARP complex, Ypt6 and Ric1/Rgp1 in yeast (Rab6 Guanine exchange factor) have been shown to have defects in the degradation of GFP-Atg8 and liberation of free GFP (Ohashi and Munro, 2010; Ye et al., 2014). This supports a role for

Rab6 in the regulation of endosome-to-Golgi retrograde trafficking, to ensure hydrolase sorting, in flies.

Our observations regarding the sorting of Cathepsin D could be explained by failure to retrieve the M6PR in endosome-to-Golgi (retrograde) or a Golgi-to-endosome (anterograde) traffic route. We favor a retrograde route for Rab6 with our collective data for the following reasons: 1) We have observed that proteins that must traffic through the Golgi, V-ATPases, to their final target are able to reach the autolysosome suggesting that traffic out of the Golgi-to-LE/Lys is not impaired; 2) acidification occurs in the absence of Rab6 suggesting that the vacuolar proton pump complex assembles and functions as would be expected if anterograde traffic from Golgi-to-LE/Lys is not impaired; and 3) based on the incomplete penetrance of our phenotype using the null allele. If Rab6 has a role in retrograde traffic it would be expected that the phenotype be dependent on the rate at which a given cell needs to continuously recycle the hydrolase receptor to maintain lysosomal function according to demand by the cell. As a result, a cell with more demand will be depleted of hydrolases faster than a cell with normal or moderate demand. This is in contrast with an anterograde scenario where the delivery of hydrolases will be independent of demand and developmental time to show a phenotype, as the delivery will be impaired from the start rather than with continuous

It has been reported in the literature that Ypt6 mutants in yeast are hypersensitive to rapamycin. Recently, a yeast screen for rapamycin insensitivity revealed ypt1 (Rab1 orthologue in yeast) regulated the amino-acid re-stimulation response after deprivation of

amino acids by mobilizing mTOR to the Golgi (Thomas et al., 2014). The screen also showed that ypt6 and ypt7 (Rab7 orthologue) were hypersensitive to rapamycin. Interestingly, a GTPase screen in S2 cells showed that knockdown of Rab6 reduced the basal phosphorylation state of S6 kinase. This suggests that Rab6 might regulate mTOR activity in yeast and flies through an unknown mechanism. During our re-feeding experiments we observed that Rab6 loss resulted in decreased phosphorylation of S6 kinase and Akt during re-feeding stimulation. This suggested that Rab6 was affecting mTOR activation in an Akt dependent manner. In addition, we found that over-expression of Rheb and parallel removal of PTEN could rescue the defects associated with Rab6 loss but not over-expression of constitutive active RagA (RagA-CA). Rescue by Rheb supported a role for Rab6 in the regulation of mTOR. However, the observation that RagA-CA did not rescue but enhanced our phenotypes was surprising. Taken together, our data provides the first evidence for Rab6 playing a role in the insulin dependent activation of mTOR.

Currently, the mechanism responsible for trafficking the insulin receptor is unknown. Moreover, how upstream activators of mTOR and autophagy reciprocally regulate each other under different nutrients states is un-explored. Here, we show that loss of Rab6 results in a progressive internalization of the insulin receptor towards the lysosomal compartment. Meanwhile, control cells display a continuous labeling and localization of the InR at the plasma membrane regardless of nutrient conditions. Using our null allele, we observed internalization of the receptor, towards autophagic vesicles (AV), independently of nutrient availability. The data suggests that Rab6 is required for the

regulation of retrograde traffic of the InR after it is internalized from the plasma membrane. Interestingly, we observed a similar finding monitoring the human transferring receptor and a V-ATPase subunit that localizes at the plasma membrane, suggesting Rab6 might be regulating general retrograde traffic of membrane proteins. However, our findings for InR suggest it traffics from endosomes to the Golgi where Rab6 localizes before it recycles to the plasma membrane. Similarly, it has been shown that the glucose transporter 4 (GLU4) internalizes onto early endosomes were it is sorted to the Golgi before being retrieved to the plasma membrane (Brewer et al., 2014). Interestingly, Rab6 and the retromer subunit Vps35 are synthetic lethal and show severe growth defects, suggesting they regulate a common pathway (Luo and Gallwitz, 2003). In mammals, Rab6 has been shown to regulate retromer-Rab11 vesicle docking at the Golgi before delivery to the plasma membrane (Miserey-Lenkei et al., 2007). This suggests the possibility that Rab6 might coordinate a similar retrograde mechanism for recycling endosomal vesicles, carrying membrane bound proteins, docking at the Golgi en route to the plasma membrane in fat body cells. This would ensure InR localization at the plasma membrane to inhibit autophagy induction.

Accumulation of AVs can be the result of an imbalance between production and degradation, a phenomenon termed autophagic stress and first described in Cathepsin D null mice (Koike et al., 2000; Shacka et al., 2007; Walls et al., 2007). In addition, accumulation of AVs can be the result of defective fusion between lysosome and AVs, as has been shown for syntaxin17 and HOPS mutants in flies (Takats et al., 2013; Takats et al., 2014). We began our characterization of Rab6 with the observation that its loss

resulted in the accumulation of AVs under fed states and we ruled out defects between lysosomes and AVs fusion upon Rab6 loss. Our characterization revealed that one of the two reasons AVs accumulate is the failure to properly sort lysosomal hydrolases to lysosomes and/or AVs. This prompted us to evaluate the turnover of AVs in Rab6 null clones in a re-feeding assay. We observed that following starvation placement of larvae in rich food promoted the turnover of AVs in control cells but not in Rab6 null clones. Our data suggest, that similar to Cathepsin D null animal, Rab6 null cells are under autophagic stress as a result of impaired degradation at lysosomes and an over-imposed production of AVs due to internalization of InR. This imbalance can explain why upon parallel removal of PTEN or Rheb over-expression we can still observe AVs. Because even though we are inhibiting autophagy by activating mTOR, when Rab6 is lost, the degradation capacity is still reduced resulting in impaired turnover and accumulation of AVs.

Conclusion

We set out to carry a forward genetic screen to uncover novel traffic regulators of the autophagic pathway and mTOR signaling. One of the motivations to carry this screen was the premise that mammalian cell culture studies had failed to uncover more traffic regulator in the Rab GTPase family due to gene redundancy. Our screen results confirm this as we obtained new Rab GTPase candidates with uncharacterized function in autophagy regulation. Furthermore, our results highlight the strength and advantage of our model system in the study of family of proteins in the regulation of autophagy. In

sum, the screen results indicate that the major role of Rab GTPases is the regulation of autophagic vesicle induction and growth, as a third of our studied proteins when knocked down caused a reduction in number and/or size of autophagic vesicles.

Collectively, we have discovered and characterized a subset of our candidate genes in the regulation of autophagy (Fig. 27). We show that Rab2 and 14 are required for the growth and induction of autophagic vesicles and autolysosomal function. Examination of their role in LE/Lys fusion with autophagic vesicles and traffic of Atg proteins will shed light on their mechanistic role. In addition, we show that Rab5 is required for autophagic vesicle growth and induction by regulating the synthesis of PI3P lipids. Furthermore, we show that it is required for the maturation of the endomembrane system and endocytosis. This ensures lysosomal and autolysosomal function. Whether Rab5 regulates the Atg machinery and/or mTOR regulators in fat body cells will help in the elucidation of how this Rab GTPase coordinates cell growth and autophagy and the growth of autophagosomes. Examination of Rab5 dependent regulation of additional potential growth promoting pathways, such as Ras and Hippo signaling, would aid in establishing whether this GTPase regulates cell growth independently of mTOR.

Last, we show that Rab6 is required for the sorting of lysosomal hydrolases and the insulin receptor upon its internalization at the endomembrane system to avoid its degradation. However, we were not able to establish if the hydrolase sorting was a direct defect of anterograde delivery vs. retrograde recycling. Examination of the fly lysosomal hydrolase receptor (LERP) in parallel to markers of the Golgi, endosomal compartments

and Rab6 could help in further pinpointing the traffic route regulated by Rab6. However, the fact that Rab6 has a conserved role in the retrograde regulation of lysosomal hydrolase receptor in yeast and mammals (Vps10 and M6Pr, respectively) and in light of our findings supports a similar role for Rab6 in retrograde traffic regulation in flies. Similarly, examination focusing on the insulin receptor and the Golgi and endomembrane system will aid in establishing if the InR is recycled retrogradely Golgi dependently vs. independently and whether Rab6 might play a direct or indirect role in this routes. We propose a model where Rab6 regulates general retrograde traffic from endosome-to-Golgi to maintain the delicate balance between AV production and turnover in fat body cells (Fig. 27).

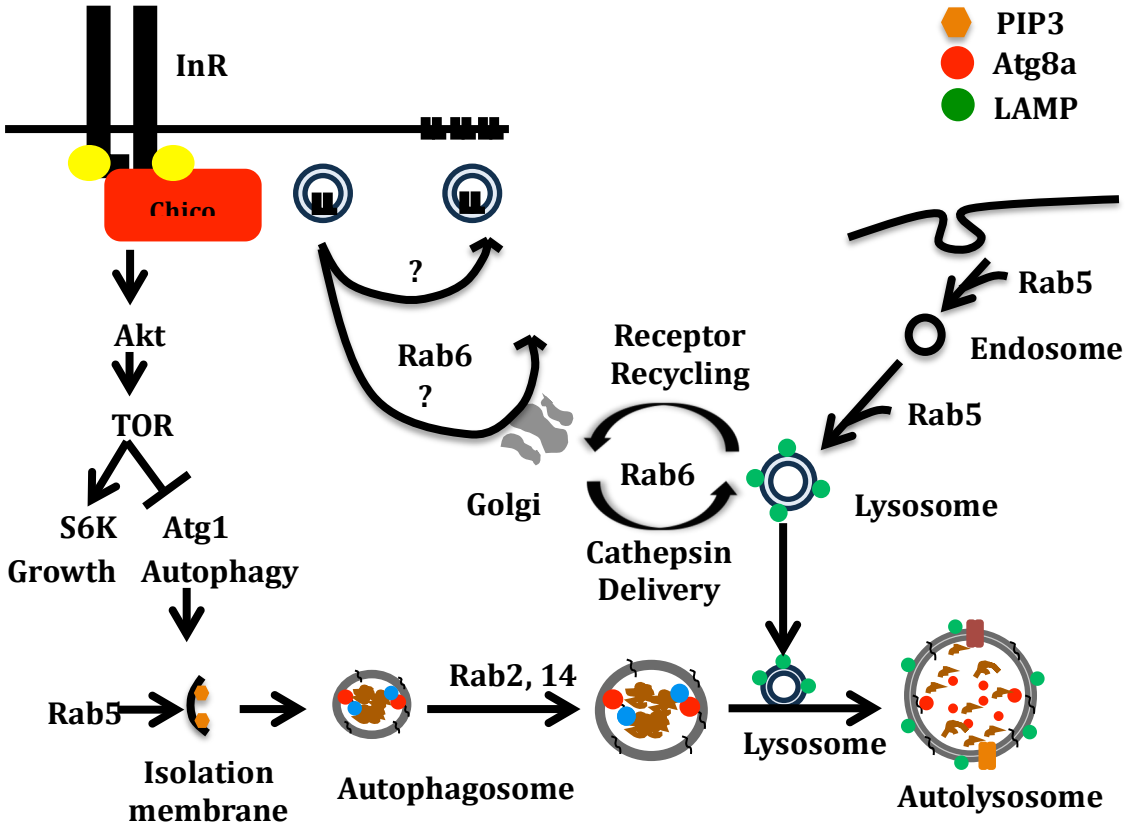


Figure 27. Rab GTPase dependent regulation of autophagy and mTOR signaling in fat body cells in *Drosophila melanogaster*.

This thesis has established that the main role of Rab GTPases in fat body cells of flies is the induction and growth of autophagic vesicles. We characterized a subset of these growth regulators revealing that Rab2 and 14 are required for induction and growth of autophagic vesicles and autolysosomal function and acidification. We also show that Rab5 regulates endocytosis, endomembrane system maturation and the induction and growth of autophagic vesicles. Last, our characterization of Rab6 revealed that it is required for the sorting of lysosomal hydrolases and the insulin receptor upon internalization to avoid its degradation at the lysosome. Both of these functions, provided by Rab6, are required to maintain the delicate balance of autophagic vesicle production and degradation to avoid autophagic stress.

Bibliography

Akbar, M.A., S. Ray, and H. Kramer. 2009. The SM protein Car/Vps33A regulates SNARE-mediated trafficking to lysosomes and lysosome-related organelles. *Molecular biology of the cell*. 20:1705-1714.

Anderson, G.W., H.H. Goebel, and A. Simonati. 2013. Human pathology in NCL. *Biochimica et biophysica acta*. 1832:1807-1826.

Anding, A.L., and E.H. Baehrecke. 2015. Vps15 is required for stress induced and developmentally triggered autophagy and salivary gland protein secretion in Drosophila. *Cell death and differentiation*. 22:457-464.

Ao, X., L. Zou, and Y. Wu. 2014. Regulation of autophagy by the Rab GTPase network. *Cell death and differentiation*. 21:348-358.

Appelqvist, H., P. Waster, K. Kagedal, and K. Ollinger. 2013. The lysosome: from waste bag to potential therapeutic target. *Journal of molecular cell biology*. 5:214-226.

Aspuria, P.J., and F. Tamanoi. 2004. The Rheb family of GTP-binding proteins. *Cellular signalling*. 16:1105-1112.

Axe, E.L., S.A. Walker, M. Manifava, P. Chandra, H.L. Roderick, A. Habermann, G. Griffiths, and N.T. Ktistakis. 2008. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *The Journal of cell biology*. 182:685-701.

Baba, M., M. Osumi, S.V. Scott, D.J. Klionsky, and Y. Ohsumi. 1997. Two distinct pathways for targeting proteins from the cytoplasm to the vacuole/lysosome. *The Journal of cell biology*. 139:1687-1695.

Balderhaar, H.J., and C. Ungermann. 2013. CORVET and HOPS tethering complexes - coordinators of endosome and lysosome fusion. *Journal of cell science*. 126:1307-1316.

Banta, L.M., J.S. Robinson, D.J. Klionsky, and S.D. Emr. 1988. Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. *The Journal of cell biology*. 107:1369-1383.

Beau, I., M. Mehrpour, and P. Codogno. 2011. Autophagosomes and human diseases. *The international journal of biochemistry & cell biology*. 43:460-464.

Benmimoun, B., C. Polesello, L. Waltzer, and M. Haenlin. 2012. Dual role for Insulin/TOR signaling in the control of hematopoietic progenitor maintenance in Drosophila. *Development*. 139:1713-1717.

Bensen, E.S., B.G. Yeung, and G.S. Payne. 2001. Ric1p and the Ypt6p GTPase function in a common pathway required for localization of trans-Golgi network membrane proteins. *Molecular biology of the cell*. 12:13-26.

Betz, C., and M.N. Hall. 2013. Where is mTOR and what is it doing there? *The Journal of cell biology*. 203:563-574.

Bjedov, I., J.M. Toivonen, F. Kerr, C. Slack, J. Jacobson, A. Foley, and L. Partridge. 2010. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. *Cell metabolism*. 11:35-46.

Bonifacino, J.S., and A. Hierro. 2011. Transport according to GARP: receiving retrograde cargo at the trans-Golgi network. *Trends in cell biology*. 21:159-167.

Braulke, T., and J.S. Bonifacino. 2009. Sorting of lysosomal proteins. *Biochimica et biophysica acta*. 1793:605-614.

Brewer, P.D., E.N. Habtemichael, I. Romenskaia, C.C. Mastick, and A.C. Coster. 2014. Insulin-regulated Glut4 translocation: membrane protein trafficking with six distinctive steps. *The Journal of biological chemistry*. 289:17280-17298.

Brown, K.K., and A. Toker. 2015. The phosphoinositide 3-kinase pathway and therapy resistance in cancer. *F1000prime reports*. 7:13.

Carracedo, A., J. Baselga, and P.P. Pandolfi. 2008. Deconstructing feedback-signaling networks to improve anticancer therapy with mTORC1 inhibitors. *Cell cycle*. 7:3805-3809.

Castro, A.F., J.F. Rebhun, G.J. Clark, and L.A. Quilliam. 2003. Rheb binds tuberous sclerosis complex 2 (TSC2) and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner. *The Journal of biological chemistry*. 278:32493-32496.

Chan, S.N., and B.L. Tang. 2013. Location and membrane sources for autophagosome formation - from ER-mitochondria contact sites to Golgi-endosome-derived carriers. *Molecular membrane biology*. 30:394-402.

Chen, Y., and D.J. Klionsky. 2011. The regulation of autophagy - unanswered questions. *Journal of cell science*. 124:161-170.

Chen, Y., and L. Yu. 2013. Autophagic lysosome reformation. *Experimental cell research*. 319:142-146.

Chen, Y., F. Zhou, S. Zou, S. Yu, S. Li, D. Li, J. Song, H. Li, Z. He, B. Hu, L.O. Bjorn, Z. Lipatova, Y. Liang, Z. Xie, and N. Segev. 2014. A Vps21 endocytic module regulates autophagy. *Molecular biology of the cell*. 25:3166-3177.

Cheng, X.W., T. Sasaki, and M. Kuzuya. 2014. The role of cysteinyl cathepsins in venous disorders. *Thrombosis and haemostasis*. 112:216-218.

Chia, P.Z., and P.A. Gleeson. 2011. The regulation of endosome-to-Golgi retrograde transport by tethers and scaffolds. *Traffic*. 12:939-947.

Chia, P.Z., P. Gunn, and P.A. Gleeson. 2013. Cargo trafficking between endosomes and the trans-Golgi network. *Histochemistry and cell biology*. 140:307-315.

Chua, C.E., B.Q. Gan, and B.L. Tang. 2011. Involvement of members of the Rab family and related small GTPases in autophagosome formation and maturation. *Cellular and molecular life sciences : CMLS*. 68:3349-3358.

Conibear, E., and T.H. Stevens. 2000. Vps52p, Vps53p, and Vps54p form a novel multisubunit complex required for protein sorting at the yeast late Golgi. *Molecular biology of the cell*. 11:305-323.

Cornu, M., V. Albert, and M.N. Hall. 2013. mTOR in aging, metabolism, and cancer. *Current opinion in genetics & development*. 23:53-62.

Coutelis, J.B., and A. Ephrussi. 2007. Rab6 mediates membrane organization and determinant localization during Drosophila oogenesis. *Development*. 134:1419-1430.

Coutinho, M.F., L. Matos, and S. Alves. 2015. From bedside to cell biology: a century of history on lysosomal dysfunction. *Gene*. 555:50-58.

Coutinho, M.F., M.J. Prata, and S. Alves. 2012a. Mannose-6-phosphate pathway: a review on its role in lysosomal function and dysfunction. *Molecular genetics and metabolism*. 105:542-550.

Coutinho, M.F., M.J. Prata, and S. Alves. 2012b. A shortcut to the lysosome: the mannose-6-phosphate-independent pathway. *Molecular genetics and metabolism*. 107:257-266.

da Fonseca, T.L., A. Villar-Pique, and T.F. Outeiro. 2015. The Interplay between Alpha-Synuclein Clearance and Spreading. *Biomolecules*. 5:435-471.

Darsow, T., S.E. Rieder, and S.D. Emr. 1997. A multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole. *The Journal of cell biology*. 138:517-529.

Dazert, E., and M.N. Hall. 2011. mTOR signaling in disease. *Current opinion in cell biology*. 23:744-755.

Dennes, A., C. Cromme, K. Suresh, N.S. Kumar, J.A. Eble, A. Hahnenkamp, and R. Pohlmann. 2005. The novel Drosophila lysosomal enzyme receptor protein mediates lysosomal sorting in mammalian cells and binds mammalian and Drosophila GGA adaptors. *The Journal of biological chemistry*. 280:12849-12857.

Deretic, V., and B. Levine. 2009. Autophagy, immunity, and microbial adaptations. *Cell host & microbe*. 5:527-549.

Diao, J., R. Liu, Y. Rong, M. Zhao, J. Zhang, Y. Lai, Q. Zhou, L.M. Wilz, J. Li, S. Vivona, R.A. Pfuetzner, A.T. Brunger, and Q. Zhong. 2015. ATG14 promotes membrane tethering and fusion of autophagosomes to endolysosomes. *Nature*. 520:563-566.

Dibble, C.C., and L.C. Cantley. 2015. Regulation of mTORC1 by PI3K signaling. *Trends in cell biology*.

Dibble, C.C., and B.D. Manning. 2013. Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nature cell biology*. 15:555-564.

Dong, J., and D. Pan. 2004. Tsc2 is not a critical target of Akt during normal Drosophila development. *Genes & development*. 18:2479-2484.

Dou, Z., J.A. Pan, H.A. Dbouk, L.M. Ballou, J.L. DeLeon, Y. Fan, J.S. Chen, Z. Liang, G. Li, J.M. Backer, R.Z. Lin, and W.X. Zong. 2013. Class IA PI3K p110beta subunit promotes autophagy through Rab5 small GTPase in response to growth factor limitation. *Molecular cell*. 50:29-42.

Dragojlovic-Munther, M., and J.A. Martinez-Agosto. 2012. Multifaceted roles of PTEN and TSC orchestrate growth and differentiation of Drosophila blood progenitors. *Development*. 139:3752-3763.

Edwards, S.L., N.K. Charlie, J.E. Richmond, J. Hegermann, S. Eimer, and K.G. Miller. 2009. Impaired dense core vesicle maturation in Caenorhabditis elegans mutants lacking Rab2. *The Journal of cell biology*. 186:881-895.

Efeyan, A., R. Zoncu, S. Chang, I. Gumper, H. Snitkin, R.L. Wolfson, O. Kirak, D.D. Sabatini, and D.M. Sabatini. 2013. Regulation of mTORC1 by the Rag GTPases is necessary for neonatal autophagy and survival. *Nature*. 493:679-683.

Fader, C.M., D. Sanchez, M. Furlan, and M.I. Colombo. 2008. Induction of autophagy promotes fusion of multivesicular bodies with autophagic vacuoles in k562 cells. *Traffic*. 9:230-250.

Fischer von Mollard, G., and T.H. Stevens. 1999. The Saccharomyces cerevisiae v-SNARE Vti1p is required for multiple membrane transport pathways to the vacuole. *Molecular biology of the cell*. 10:1719-1732.

Flinn, R.J., Y. Yan, S. Goswami, P.J. Parker, and J.M. Backer. 2010. The late endosome is essential for mTORC1 signaling. *Molecular biology of the cell*. 21:833-841.

Funderburk, S.F., Q.J. Wang, and Z. Yue. 2010. The Beclin 1-VPS34 complex--at the crossroads of autophagy and beyond. *Trends in cell biology*. 20:355-362.

Galluzzi, L., F. Pietrocola, B. Levine, and G. Kroemer. 2014. Metabolic control of autophagy. *Cell*. 159:1263-1276.

Gao, X., T.P. Neufeld, and D. Pan. 2000. Drosophila PTEN regulates cell growth and proliferation through PI3K-dependent and -independent pathways. *Developmental biology*. 221:404-418.

Garg, A., and L.P. Wu. 2014. Drosophila Rab14 mediates phagocytosis in the immune response to Staphylococcus aureus. *Cellular microbiology*. 16:296-310.

Ge, L., S. Baskaran, R. Schekman, and J.H. Hurley. 2014. The protein-vesicle network of autophagy. *Current opinion in cell biology*. 29:18-24.

Gillingham, A.K., R. Sinka, I.L. Torres, K.S. Lilley, and S. Munro. 2014. Toward a comprehensive map of the effectors of rab GTPases. *Developmental cell*. 31:358-373.

Groenewoud, M.J., and F.J. Zwartkruis. 2013. Rheb and Rags come together at the lysosome to activate mTORC1. *Biochemical Society transactions*. 41:951-955.

Guo, P., T. Hu, J. Zhang, S. Jiang, and X. Wang. 2010. Sequential action of Caenorhabditis elegans Rab GTPases regulates phagolysosome formation during apoptotic cell degradation. *Proceedings of the National Academy of Sciences of the United States of America*. 107:18016-18021.

Guo, Y., D.W. Sirkis, and R. Schekman. 2014. Protein sorting at the trans-Golgi network. *Annual review of cell and developmental biology*. 30:169-206.

Gutierrez, M.G., D.B. Munafo, W. Beron, and M.I. Colombo. 2004. Rab7 is required for the normal progression of the autophagic pathway in mammalian cells. *Journal of cell science*. 117:2687-2697.

Hailey, D.W., A.S. Rambold, P. Satpute-Krishnan, K. Mitra, R. Sougrat, P.K. Kim, and J. Lippincott-Schwartz. 2010. Mitochondria supply membranes for autophagosome biogenesis during starvation. *Cell*. 141:656-667.

Hall, D.J., S.S. Grewal, A.F. de la Cruz, and B.A. Edgar. 2007. Rheb-TOR signaling promotes protein synthesis, but not glucose or amino acid import, in Drosophila. *BMC biology*. 5:10.

Hamasaki, M., N. Furuta, A. Matsuda, A. Nezu, A. Yamamoto, N. Fujita, H. Oomori, T. Noda, T. Haraguchi, Y. Hiraoka, A. Amano, and T. Yoshimori. 2013. Autophagosomes form at ER-mitochondria contact sites. *Nature*. 495:389-393.

Hayashi-Nishino, M., N. Fujita, T. Noda, A. Yamaguchi, T. Yoshimori, and A. Yamamoto. 2009. A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. *Nature cell biology*. 11:1433-1437.

Heard, J.J., V. Fong, S.Z. Bathaie, and F. Tamanoi. 2014. Recent progress in the study of the Rheb family GTPases. *Cellular signalling*. 26:1950-1957.

Hietakangas, V., and S.M. Cohen. 2009. Regulation of tissue growth through nutrient sensing. *Annual review of genetics*. 43:389-410.

Hirota, Y., T. Kuronita, H. Fujita, and Y. Tanaka. 2007. A role for Rab5 activity in the biogenesis of endosomal and lysosomal compartments. *Biochemical and biophysical research communications*. 364:40-47.

Hirota, Y., and Y. Tanaka. 2009. A small GTPase, human Rab32, is required for the formation of autophagic vacuoles under basal conditions. *Cellular and molecular life sciences : CMLS*. 66:2913-2932.

Hong, W., and S. Lev. 2014. Tethering the assembly of SNARE complexes. *Trends in cell biology*. 24:35-43.

Hsu, Y.C., J.J. Chern, Y. Cai, M. Liu, and K.W. Choi. 2007. Drosophila TCTP is essential for growth and proliferation through regulation of dRheb GTPase. *Nature*. 445:785-788.

Huang, S., K. Jia, Y. Wang, Z. Zhou, and B. Levine. 2013. Autophagy genes function in apoptotic cell corpse clearance during C. elegans embryonic development. *Autophagy*. 9:138-149.

Hutagalung, A.H., and P.J. Novick. 2011. Role of Rab GTPases in membrane traffic and cell physiology. *Physiological reviews*. 91:119-149.

Inoki, K., Y. Li, T. Zhu, J. Wu, and K.L. Guan. 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nature cell biology*. 4:648-657.

Itakura, E., C. Kishi-Itakura, and N. Mizushima. 2012. The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes. *Cell*. 151:1256-1269.

Itoh, T., N. Fujita, E. Kanno, A. Yamamoto, T. Yoshimori, and M. Fukuda. 2008. Golgi-resident small GTPase Rab33B interacts with Atg16L and modulates autophagosome formation. *Molecular biology of the cell*. 19:2916-2925.

Jager, S., C. Bucci, I. Tanida, T. Ueno, E. Kominami, P. Saftig, and E.L. Eskelinen. 2004. Role for Rab7 in maturation of late autophagic vacuoles. *Journal of cell science*. 117:4837-4848.

Januschke, J., E. Nicolas, J. Compagnon, E. Formstecher, B. Goud, and A. Guichet. 2007. Rab6 and the secretory pathway affect oocyte polarity in Drosophila. *Development*. 134:3419-3425.

Jewell, J.L., R.C. Russell, and K.L. Guan. 2013. Amino acid signalling upstream of mTOR. *Nature reviews. Molecular cell biology*. 14:133-139.

Jia, G., A.R. Aroor, L.A. Martinez-Lemus, and J.R. Sowers. 2014. Overnutrition, mTOR signaling, and cardiovascular diseases. *American journal of physiology. Regulatory, integrative and comparative physiology*. 307:R1198-1206.

Jiang, P., and N. Mizushima. 2014. Autophagy and human diseases. *Cell research*. 24:69-79.

Jiang, P., T. Nishimura, Y. Sakamaki, E. Itakura, T. Hatta, T. Natsume, and N. Mizushima. 2014a. The HOPS complex mediates autophagosome-lysosome fusion through interaction with syntaxin 17. *Molecular biology of the cell*. 25:1327-1337.

Jiang, T., J.T. Yu, X.C. Zhu, M.S. Tan, H.F. Wang, L. Cao, Q.Q. Zhang, J.Q. Shi, L. Gao, H. Qin, Y.D. Zhang, and L. Tan. 2014b. Temsirolimus promotes autophagic clearance of amyloid-beta and provides protective effects in cellular and animal models of Alzheimer's disease. *Pharmacological research : the official journal of the Italian Pharmacological Society*. 81:54-63.

Jiang, X., M. Overholtzer, and C.B. Thompson. 2015. Autophagy in cellular metabolism and cancer. *The Journal of clinical investigation*. 125:47-54.

Jin, M., and D.J. Klionsky. 2014. Regulation of autophagy: modulation of the size and number of autophagosomes. *FEBS letters*. 588:2457-2463.

Johannes, L., and V. Popoff. 2008. Tracing the retrograde route in protein trafficking. *Cell*. 135:1175-1187.

Juhasz, G., J.H. Hill, Y. Yan, M. Sass, E.H. Baehrecke, J.M. Backer, and T.P. Neufeld. 2008. The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in Drosophila. *The Journal of cell biology*. 181:655-666.

Juhasz, G., and T.P. Neufeld. 2008. Experimental control and characterization of autophagy in Drosophila. *Methods in molecular biology*. 445:125-133.

Kakuta, S., H. Yamamoto, L. Negishi, C. Kondo-Kakuta, N. Hayashi, and Y. Ohsumi. 2012. Atg9 vesicles recruit vesicle-tethering proteins Trs85 and Ypt1 to the autophagosome formation site. *The Journal of biological chemistry*. 287:44261-44269.

Kannan, K., and Y.W. Fridell. 2013. Functional implications of Drosophila insulin-like peptides in metabolism, aging, and dietary restriction. *Frontiers in physiology*. 4:288.

Kim, E., P. Goraksha-Hicks, L. Li, T.P. Neufeld, and K.L. Guan. 2008. Regulation of TORC1 by Rag GTPases in nutrient response. *Nature cell biology*. 10:935-945.

Kim, J., and T.P. Neufeld. 2015. Dietary sugar promotes systemic TOR activation in Drosophila through AKH-dependent selective secretion of Dilp3. *Nature communications*. 6:6846.

Klionsky, D.J., J.M. Cregg, W.A. Dunn, Jr., S.D. Emr, Y. Sakai, I.V. Sandoval, A. Sibirny, S. Subramani, M. Thumm, M. Veenhuis, and Y. Ohsumi. 2003. A unified nomenclature for yeast autophagy-related genes. *Developmental cell*. 5:539-545.

Koike, M., H. Nakanishi, P. Saftig, J. Ezaki, K. Isahara, Y. Ohsawa, W. Schulz-Schaeffer, T. Watanabe, S. Waguri, S. Kametaka, M. Shibata, K. Yamamoto, E. Kominami, C. Peters, K. von Figura, and Y. Uchiyama. 2000. Cathepsin D deficiency induces lysosomal storage with ceroid lipofuscin in mouse CNS neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 20:6898-6906.

Korolchuk, V.I., S. Saiki, M. Lichtenberg, F.H. Siddiqi, E.A. Roberts, S. Imarisio, L. Jahreiss, S. Sarkar, M. Futter, F.M. Menzies, C.J. O'Kane, V. Deretic, and D.C. Rubinsztein. 2011. Lysosomal positioning coordinates cellular nutrient responses. *Nature cell biology*. 13:453-460.

Kovacs, A.L., and H. Zhang. 2010. Role of autophagy in Caenorhabditis elegans. *FEBS letters*. 584:1335-1341.

Kyei, G.B., I. Vergne, J. Chua, E. Roberts, J. Harris, J.R. Junutula, and V. Deretic. 2006. Rab14 is critical for maintenance of Mycobacterium tuberculosis phagosome maturation arrest. *The EMBO journal*. 25:5250-5259.

Lamb, C.A., T. Yoshimori, and S.A. Tooze. 2013. The autophagosome: origins unknown, biogenesis complex. *Nature reviews. Molecular cell biology*. 14:759-774.

Lavery, W., V. Hall, J.C. Yager, A. Rottgers, M.C. Wells, and M. Stern. 2007. Phosphatidylinositol 3-kinase and Akt nonautonomously promote perineurial glial growth in Drosophila peripheral nerves. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 27:279-288.

Li, H., and A.J. Marshall. 2015. Phosphatidylinositol (3,4) bisphosphate-specific phosphatases and effector proteins: A distinct branch of PI3K signaling. *Cellular signalling*.

Li, L., E. Kim, H. Yuan, K. Inoki, P. Goraksha-Hicks, R.L. Schiesher, T.P. Neufeld, and K.L. Guan. 2010. Regulation of mTORC1 by the Rab and Arf GTPases. *The Journal of biological chemistry*. 285:19705-19709.

Liewen, H., I. Meinhold-Heerlein, V. Oliveira, R. Schwarzenbacher, G. Luo, A. Wadle, M. Jung, M. Pfreundschuh, and F. Stenner-Liewen. 2005. Characterization of the human GARP (Golgi associated retrograde protein) complex. *Experimental cell research*. 306:24-34.

Lin, M.K., and M.J. Farrer. 2014. Genetics and genomics of Parkinson's disease. *Genome medicine*. 6:48.

Lindmo, K., A. Simonsen, A. Brech, K. Finley, T.E. Rusten, and H. Stenmark. 2006. A dual function for Deep orange in programmed autophagy in the Drosophila melanogaster fat body. *Experimental cell research*. 312:2018-2027.

Liu, S., and B. Storrie. 2012. Are Rab proteins the link between Golgi organization and membrane trafficking? *Cellular and molecular life sciences : CMLS*. 69:4093-4106.

Long, X., Y. Lin, S. Ortiz-Vega, K. Yonezawa, and J. Avruch. 2005a. Rheb binds and regulates the mTOR kinase. *Current biology : CB*. 15:702-713.

Long, X., S. Ortiz-Vega, Y. Lin, and J. Avruch. 2005b. Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency. *The Journal of biological chemistry*. 280:23433-23436.

Longatti, A., C.A. Lamb, M. Razi, S. Yoshimura, F.A. Barr, and S.A. Tooze. 2012. TBC1D14 regulates autophagosome formation via Rab11- and ULK1-positive recycling endosomes. *The Journal of cell biology*. 197:659-675.

Lorincz, P., Z. Lakatos, T. Maruzs, Z. Szatmari, V. Kis, and M. Sass. 2014. Atg6/UVRAG/Vps34-containing lipid kinase complex is required for receptor downregulation through endolysosomal degradation and epithelial polarity during Drosophila wing development. *BioMed research international*. 2014:851349.

Lu, L., and W. Hong. 2014. From endosomes to the trans-Golgi network. *Seminars in cell & developmental biology*. 31:30-39.

Luo, L., M. Hannemann, S. Koenig, J. Hegermann, M. Ailion, M.K. Cho, N. Sasidharan, M. Zweckstetter, S.A. Rensing, and S. Eimer. 2011. The Caenorhabditis elegans GARP complex contains the conserved Vps51 subunit and is required to maintain lysosomal morphology. *Molecular biology of the cell*. 22:2564-2578.

Luo, Z., and D. Gallwitz. 2003. Biochemical and genetic evidence for the involvement of yeast Ypt6-GTPase in protein retrieval to different Golgi compartments. *The Journal of biological chemistry*. 278:791-799.

Mangahas, P.M., X. Yu, K.G. Miller, and Z. Zhou. 2008. The small GTPase Rab2 functions in the removal of apoptotic cells in Caenorhabditis elegans. *The Journal of cell biology*. 180:357-373.

Manil-Segalen, M., C. Lefebvre, C. Jenzer, M. Trichet, C. Boulogne, B. Satiat-Jeunemaitre, and R. Legouis. 2014. The C. elegans LC3 acts downstream of GABARAP to degrade autophagosomes by interacting with the HOPS subunit VPS39. *Developmental cell*. 28:43-55.

Martinez-Vicente, M. 2015. Autophagy in neurodegenerative diseases: From pathogenic dysfunction to therapeutic modulation. *Seminars in cell & developmental biology*. 40:115-126.

Mauvezin, C., C. Ayala, C.R. Braden, J. Kim, and T.P. Neufeld. 2014. Assays to monitor autophagy in Drosophila. *Methods*. 68:134-139.

Mauvezin, C., P. Nagy, G. Juhasz, and T.P. Neufeld. 2015. Autophagosome-lysosome fusion is independent of V-ATPase-mediated acidification. *Nature communications*. 6:7007.

McEwan, D.G., D. Popovic, A. Gubas, S. Terawaki, H. Suzuki, D. Stadel, F.P. Coxon, D. Miranda de Stegmann, S. Bhogaraju, K. Maddi, A. Kirchof, E. Gatti, M.H. Helfrich, S. Wakatsuki, C. Behrends, P. Pierre, and I. Dikic. 2015. PLEKHM1 regulates autophagosome-lysosome fusion through HOPS complex and LC3/GABARAP proteins. *Molecular cell*. 57:39-54.

Mehrpour, M., A. Esclatine, I. Beau, and P. Codogno. 2010. Overview of macroautophagy regulation in mammalian cells. *Cell research*. 20:748-762.

Menon, S., C.C. Dibble, G. Talbott, G. Hoxhaj, A.J. Valvezan, H. Takahashi, L.C. Cantley, and B.D. Manning. 2014. Spatial control of the TSC complex integrates insulin and nutrient regulation of mTORC1 at the lysosome. *Cell*. 156:771-785.

Miserey-Lenkei, S., F. Waharte, A. Boulet, M.H. Cuif, D. Tenza, A. El Marjou, G. Raposo, J. Salamero, L. Heliot, B. Goud, and S. Monier. 2007. Rab6-interacting protein 1 links Rab6 and Rab11 function. *Traffic*. 8:1385-1403.

Mizuno-Yamasaki, E., F. Rivera-Molina, and P. Novick. 2012. GTPase networks in membrane traffic. *Annual review of biochemistry*. 81:637-659.

Morrison, H.A., H. Dionne, T.E. Rusten, A. Brech, W.W. Fisher, B.D. Pfeiffer, S.E. Celniker, H. Stenmark, and D. Bilder. 2008. Regulation of early endosomal entry by the Drosophila tumor suppressors Rabenosyn and Vps45. *Molecular biology of the cell*. 19:4167-4176.

Moskalev, A.A., and M.V. Shaposhnikov. 2010. Pharmacological inhibition of phosphoinositide 3 and TOR kinases improves survival of Drosophila melanogaster. *Rejuvenation research*. 13:246-247.

Myllykangas, L., J. Tyynela, A. Page-McCaw, G.M. Rubin, M.J. Haltia, and M.B. Feany. 2005. Cathepsin D-deficient Drosophila recapitulate the key features of neuronal ceroid lipofuscinoses. *Neurobiology of disease*. 19:194-199.

Nah, J., J. Yuan, and Y.K. Jung. 2015. Autophagy in Neurodegenerative Diseases: From Mechanism to Therapeutic Approach. *Molecules and cells*. 38:381-389.

Neufeld, T.P. 2008. Genetic manipulation and monitoring of autophagy in Drosophila. *Methods in enzymology*. 451:653-667.

Nordmann, M., M. Cabrera, A. Perz, C. Brocker, C. Ostrowicz, S. Engelbrecht-Vandre, and C. Ungermann. 2010. The Mon1-Ccz1 complex is the GEF of the late endosomal Rab7 homolog Ypt7. *Current biology : CB*. 20:1654-1659.

Ohashi, Y., and S. Munro. 2010. Membrane delivery to the yeast autophagosome from the Golgi-endosomal system. *Molecular biology of the cell*. 21:3998-4008.

Okai, B., N. Lyall, N.A. Gow, J.M. Bain, and L.P. Erwig. 2015. Rab14 regulates maturation of macrophage phagosomes containing the fungal pathogen Candida albicans and outcome of the host-pathogen interaction. *Infection and immunity*. 83:1523-1535.

Pan, P.Y., and Z. Yue. 2014. Genetic causes of Parkinson's disease and their links to autophagy regulation. *Parkinsonism & related disorders*. 20 Suppl 1:S154-157.

Patel, P.H., N. Thapar, L. Guo, M. Martinez, J. Maris, C.L. Gau, J.A. Lengyel, and F. Tamanoi. 2003. Drosophila Rheb GTPase is required for cell cycle progression and cell growth. *Journal of cell science*. 116:3601-3610.

Perez-Victoria, F.J., and J.S. Bonifacino. 2009. Dual roles of the mammalian GARP complex in tethering and SNARE complex assembly at the trans-Golgi network. *Molecular and cellular biology*. 29:5251-5263.

Perez-Victoria, F.J., G.A. Mardones, and J.S. Bonifacino. 2008. Requirement of the human GARP complex for mannose 6-phosphate-receptor-dependent sorting of cathepsin D to lysosomes. *Molecular biology of the cell*. 19:2350-2362.

Perez-Victoria, F.J., C. Schindler, J.G. Magadan, G.A. Mardones, C. Delevoye, M. Romao, G. Raposo, and J.S. Bonifacino. 2010. Ang2/fat-free is a conserved subunit of the Golgi-associated retrograde protein complex. *Molecular biology of the cell*. 21:3386-3395.

Pickrell, A.M., and R.J. Youle. 2015. The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. *Neuron*. 85:257-273.

Pircs, K., P. Nagy, A. Varga, Z. Venkei, B. Erdi, K. Hegedus, and G. Juhasz. 2012. Advantages and limitations of different p62-based assays for estimating autophagic activity in Drosophila. *PloS one*. 7:e44214.

Popovic, D., and I. Dikic. 2014. TBC1D5 and the AP2 complex regulate ATG9 trafficking and initiation of autophagy. *EMBO reports*. 15:392-401.

Potter, C.J., L.G. Pedraza, and T. Xu. 2002. Akt regulates growth by directly phosphorylating Tsc2. *Nature cell biology*. 4:658-665.

Proikas-Cezanne, T., A. Gaugel, T. Frickey, and A. Nordheim. 2006. Rab14 is part of the early endosomal clathrin-coated TGN microdomain. *FEBS letters*. 580:5241-5246.

Pulipparacharuvil, S., M.A. Akbar, S. Ray, E.A. Sevrioukov, A.S. Haberman, J. Rohrer, and H. Kramer. 2005. Drosophila Vps16A is required for trafficking to lysosomes and biogenesis of pigment granules. *Journal of cell science*. 118:3663-3673.

Purcell, K., and S. Artavanis-Tsakonas. 1999. The developmental role of warthog, the notch modifier encoding Drab6. *The Journal of cell biology*. 146:731-740.

Ravikumar, B., S. Imarisio, S. Sarkar, C.J. O'Kane, and D.C. Rubinsztein. 2008. Rab5 modulates aggregation and toxicity of mutant huntingtin through macroautophagy in cell and fly models of Huntington disease. *Journal of cell science*. 121:1649-1660.

Ravikumar, B., C. Vacher, Z. Berger, J.E. Davies, S. Luo, L.G. Oroz, F. Scaravilli, D.F. Easton, R. Duden, C.J. O'Kane, and D.C. Rubinsztein. 2004. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nature genetics*. 36:585-595.

Raymond, C.K., I. Howald-Stevenson, C.A. Vater, and T.H. Stevens. 1992. Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants. *Molecular biology of the cell*. 3:1389-1402.

Reed, S.E., L.R. Hodgson, S. Song, M.T. May, E.E. Kelly, M.W. McCaffrey, C.C. Mastick, P. Verkade, and J.M. Tavare. 2013. A role for Rab14 in the endocytic trafficking of GLUT4 in 3T3-L1 adipocytes. *Journal of cell science*. 126:1931-1941.

Reggiori, F., C.W. Wang, P.E. Stromhaug, T. Shintani, and D.J. Klionsky. 2003. Vps51 is part of the yeast Vps fifty-three tethering complex essential for retrograde traffic from the early endosome and Cvt vesicle completion. *The Journal of biological chemistry*. 278:5009-5020.

Reiser, J., B. Adair, and T. Reinheckel. 2010. Specialized roles for cysteine cathepsins in health and disease. *The Journal of clinical investigation*. 120:3421-3431.

Rintelen, F., H. Stocker, G. Thomas, and E. Hafen. 2001. PDK1 regulates growth through Akt and S6K in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*. 98:15020-15025.

Robinson, J.S., D.J. Klionsky, L.M. Banta, and S.D. Emr. 1988. Protein sorting in Saccharomyces cerevisiae: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Molecular and cellular biology*. 8:4936-4948.

Rong, Y., M. Liu, L. Ma, W. Du, H. Zhang, Y. Tian, Z. Cao, Y. Li, H. Ren, C. Zhang, L. Li, S. Chen, J. Xi, and L. Yu. 2012. Clathrin and phosphatidylinositol-4,5-bisphosphate regulate autophagic lysosome reformation. *Nature cell biology*. 14:924-934.

Rong, Y., C.K. McPhee, S. Deng, L. Huang, L. Chen, M. Liu, K. Tracy, E.H. Baehrecke, L. Yu, and M.J. Lenardo. 2011. Spinster is required for autophagic lysosome reformation and mTOR reactivation following starvation. *Proceedings of the National Academy of Sciences of the United States of America*. 108:7826-7831.

Ruan, Q., A.J. Harrington, K.A. Caldwell, G.A. Caldwell, and D.G. Standaert. 2010. VPS41, a protein involved in lysosomal trafficking, is protective in Caenorhabditis elegans and mammalian cellular models of Parkinson's disease. *Neurobiology of disease*. 37:330-338.

Sancak, Y., L. Bar-Peled, R. Zoncu, A.L. Markhard, S. Nada, and D.M. Sabatini. 2010. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell*. 141:290-303.

Sancak, Y., T.R. Peterson, Y.D. Shaul, R.A. Lindquist, C.C. Thoreen, L. Bar-Peled, and D.M. Sabatini. 2008. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science*. 320:1496-1501.

Sarbassov, D.D., D.A. Guertin, S.M. Ali, and D.M. Sabatini. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 307:1098-1101.

Sato, T.K., T. Darsow, and S.D. Emr. 1998. Vam7p, a SNAP-25-like molecule, and Vam3p, a syntaxin homolog, function together in yeast vacuolar protein trafficking. *Molecular and cellular biology*. 18:5308-5319.

Saucedo, L.J., X. Gao, D.A. Chiarelli, L. Li, D. Pan, and B.A. Edgar. 2003. Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nature cell biology*. 5:566-571.

Scanga, S.E., L. Ruel, R.C. Binari, B. Snow, V. Stambolic, D. Bouchard, M. Peters, B. Calvieri, T.W. Mak, J.R. Woodgett, and A.S. Manoukian. 2000. The conserved PI3'K/PTEN/Akt signaling pathway regulates both cell size and survival in Drosophila. *Oncogene*. 19:3971-3977.

Schleich, S., and A.A. Teleman. 2009. Akt phosphorylates both Tsc1 and Tsc2 in Drosophila, but neither phosphorylation is required for normal animal growth. *PloS one*. 4:e6305.

Schroeder, B., R.J. Schulze, S.G. Weller, A.C. Sletten, C.A. Casey, and M.A. McNiven. 2015. The small GTPase Rab7 as a central regulator of hepatocellular lipophagy. *Hepatology*. 61:1896-1907.

Schulze, R.J., S.G. Weller, B. Schroeder, E.W. Krueger, S. Chi, C.A. Casey, and M.A. McNiven. 2013. Lipid droplet breakdown requires dynamin 2 for vesiculation of autolysosomal tubules in hepatocytes. *The Journal of cell biology*. 203:315-326.

Sevrioukov, E.A., J.P. He, N. Moghrabi, A. Sunio, and H. Kramer. 1999. A role for the deep orange and carnation eye color genes in lysosomal delivery in Drosophila. *Molecular cell*. 4:479-486.

Shacka, J.J., B.J. Klocke, C. Young, M. Shibata, J.W. Olney, Y. Uchiyama, P. Saftig, and K.A. Roth. 2007. Cathepsin D deficiency induces persistent neurodegeneration in the absence of Bax-dependent apoptosis. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 27:2081-2090.

Shacka, J.J., and K.A. Roth. 2007. Cathepsin D deficiency and NCL/Batten disease: there's more to death than apoptosis. *Autophagy*. 3:474-476.

Shen, H.M., and N. Mizushima. 2014. At the end of the autophagic road: an emerging understanding of lysosomal functions in autophagy. *Trends in biochemical sciences*. 39:61-71.

Shetty, K.M., P. Kurada, and J.E. O'Tousa. 1998. Rab6 regulation of rhodopsin transport in Drosophila. *The Journal of biological chemistry*. 273:20425-20430.

Shibutani, S.T., and T. Yoshimori. 2014. A current perspective of autophagosome biogenesis. *Cell research*. 24:58-68.

Shimobayashi, M., and M.N. Hall. 2014. Making new contacts: the mTOR network in metabolism and signalling crosstalk. *Nature reviews. Molecular cell biology*. 15:155-162.

Shirahama-Noda, K., S. Kira, T. Yoshimori, and T. Noda. 2013. TRAPPIII is responsible for vesicular transport from early endosomes to Golgi, facilitating Atg9 cycling in autophagy. *Journal of cell science*. 126:4963-4973.

Shravage, B.V., J.H. Hill, C.M. Powers, L. Wu, and E.H. Baehrecke. 2013. Atg6 is required for multiple vesicle trafficking pathways and hematopoiesis in Drosophila. *Development*. 140:1321-1329.

Siintola, E., S. Partanen, P. Stromme, A. Haapanen, M. Haltia, J. Maehlen, A.E. Lehesjoki, and J. Tyynela. 2006. Cathepsin D deficiency underlies congenital human neuronal ceroid-lipofuscinosis. *Brain : a journal of neurology*. 129:1438-1445.

Singh, R., and A.M. Cuervo. 2011. Autophagy in the cellular energetic balance. *Cell metabolism*. 13:495-504.

Siniossoglou, S., and H.R. Pelham. 2001. An effector of Ypt6p binds the SNARE Tlg1p and mediates selective fusion of vesicles with late Golgi membranes. *The EMBO journal*. 20:5991-5998.

Spilman, P., N. Podlutskaya, M.J. Hart, J. Debnath, O. Gorostiza, D. Bredesen, A. Richardson, R. Strong, and V. Galvan. 2010. Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. *PloS one*. 5:e9979.

Staveley, B.E., L. Ruel, J. Jin, V. Stambolic, F.G. Mastronardi, P. Heitzler, J.R. Woodgett, and A.S. Manoukian. 1998. Genetic analysis of protein kinase B (AKT) in Drosophila. *Current biology : CB*. 8:599-602.

Steele, S., J. Brunton, and T. Kawula. 2015. The role of autophagy in intracellular pathogen nutrient acquisition. *Frontiers in cellular and infection microbiology*. 5:51.

Su, H., B. Liu, O. Frohlich, H. Ma, J.M. Sands, and G. Chen. 2013. Small GTPase Rab14 down-regulates UT-A1 urea transport activity through enhanced clathrin-dependent endocytosis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 27:4100-4107.

Sumakovic, M., J. Hegermann, L. Luo, S.J. Husson, K. Schwarze, C. Olendrowitz, L. Schoofs, J. Richmond, and S. Eimer. 2009. UNC-108/RAB-2 and its effector RIC-19 are involved in dense core vesicle maturation in Caenorhabditis elegans. *The Journal of cell biology*. 186:897-914.

Suzuki, K., and Y. Ohsumi. 2010. Current knowledge of the pre-autophagosomal structure (PAS). *FEBS letters*. 584:1280-1286.

Swetha, M.G., V. Sriram, K.S. Krishnan, V.M. Oorschot, C. ten Brink, J. Klumperman, and S. Mayor. 2011. Lysosomal membrane protein composition, acidic pH and sterol content are regulated via a light-dependent pathway in metazoan cells. *Traffic*. 12:1037-1055.

Tain, L.S., H. Mortiboys, R.N. Tao, E. Ziviani, O. Bandmann, and A.J. Whitworth. 2009. Rapamycin activation of 4E-BP prevents parkinsonian dopaminergic neuron loss. *Nature neuroscience*. 12:1129-1135.

Takahara, T., and T. Maeda. 2013. Evolutionarily conserved regulation of TOR signalling. *Journal of biochemistry*. 154:1-10.

Takats, S., P. Nagy, A. Varga, K. Pircs, M. Karpati, K. Varga, A.L. Kovacs, K. Hegedus, and G. Juhasz. 2013. Autophagosomal Syntaxin17-dependent lysosomal degradation maintains neuronal function in Drosophila. *The Journal of cell biology*. 201:531-539.

Takats, S., K. Pircs, P. Nagy, A. Varga, M. Karpati, K. Hegedus, H. Kramer, A.L. Kovacs, M. Sass, and G. Juhasz. 2014. Interaction of the HOPS complex with Syntaxin 17 mediates autophagosome clearance in Drosophila. *Molecular biology of the cell*. 25:1338-1354.

Takats, S., A. Varga, K. Pircs, and G. Juhasz. 2015. Loss of Drosophila Vps16A enhances autophagosome formation through reduced TOR activity. *Autophagy*:0.

Takeshige, K., M. Baba, S. Tsuboi, T. Noda, and Y. Ohsumi. 1992. Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *The Journal of cell biology*. 119:301-311.

Takino, K., S. Ohsawa, and T. Igaki. 2014. Loss of Rab5 drives non-autonomous cell proliferation through TNF and Ras signaling in Drosophila. *Developmental biology*. 395:19-28.

Tee, A.R., B.D. Manning, P.P. Roux, L.C. Cantley, and J. Blenis. 2003. Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Current biology : CB*. 13:1259-1268.

Thomas, C., and D. Strutt. 2014. Rabaptin-5 and Rabex-5 are neoplastic tumour suppressor genes that interact to modulate Rab5 dynamics in Drosophila melanogaster. *Developmental biology*. 385:107-121.

Thomas, J.D., Y.J. Zhang, Y.H. Wei, J.H. Cho, L.E. Morris, H.Y. Wang, and X.F. Zheng. 2014. Rab1A is an mTORC1 activator and a colorectal oncogene. *Cancer cell*. 26:754-769.

Tisdale, E.J. 1999. A Rab2 mutant with impaired GTPase activity stimulates vesicle formation from pre-Golgi intermediates. *Molecular biology of the cell*. 10:1837-1849.

Tong, C., T. Ohyama, A.C. Tien, A. Rajan, C.M. Haueter, and H.J. Bellen. 2011. Rich regulates target specificity of photoreceptor cells and N-cadherin trafficking in the Drosophila visual system via Rab6. *Neuron*. 71:447-459.

Tsun, Z.Y., L. Bar-Peled, L. Chantranupong, R. Zoncu, T. Wang, C. Kim, E. Spooner, and D.M. Sabatini. 2013. The folliculin tumor suppressor is a GAP for the RagC/D GTPases that signal amino acid levels to mTORC1. *Molecular cell*. 52:495-505.

Vaccari, T., T.E. Rusten, L. Menut, I.P. Nezis, A. Brech, H. Stenmark, and D. Bilder. 2009. Comparative analysis of ESCRT-I, ESCRT-II and ESCRT-III function in Drosophila by efficient isolation of ESCRT mutants. *Journal of cell science*. 122:2413-2423.

Verdu, J., M.A. Buratovich, E.L. Wilder, and M.J. Birnbaum. 1999. Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB. *Nature cell biology*. 1:500-506.

Walkiewicz, M.A., and M. Stern. 2009. Increased insulin/insulin growth factor signaling advances the onset of metamorphosis in Drosophila. *PloS one*. 4:e5072.

Walls, K.C., B.J. Klocke, P. Saftig, M. Shibata, Y. Uchiyama, K.A. Roth, and J.J. Shacka. 2007. Altered regulation of phosphatidylinositol 3-kinase signaling in cathepsin D-deficient brain. *Autophagy*. 3:222-229.

Wang, H., Q. Lu, S. Cheng, X. Wang, and H. Zhang. 2013a. Autophagy activity contributes to programmed cell death in Caenorhabditis elegans. *Autophagy*. 9:1975-1982.

Wang, J., S. Menon, A. Yamasaki, H.T. Chou, T. Walz, Y. Jiang, and S. Ferro-Novick. 2013b. Ypt1 recruits the Atg1 kinase to the preautophagosomal structure. *Proceedings of the National Academy of Sciences of the United States of America*. 110:9800-9805.

Wang, T., Z. Ming, W. Xiaochun, and W. Hong. 2011. Rab7: role of its protein interaction cascades in endo-lysosomal traffic. *Cellular signalling*. 23:516-521.

Warner, T.S., D.A. Sinclair, K.A. Fitzpatrick, M. Singh, R.H. Devlin, and B.M. Honda. 1998. The light gene of Drosophila melanogaster encodes a homologue of VPS41, a yeast gene involved in cellular-protein trafficking. *Genome / National Research Council Canada = Genome / Conseil national de recherches Canada*. 41:236-243.

Wartosch, L., U. Gunesdogan, S.C. Graham, and J.P. Luzio. 2015. Recruitment of VPS33A to HOPS by VPS16 Is Required for Lysosome Fusion with Endosomes and Autophagosomes. *Traffic*. 16:727-742.

Wucherpfennig, T., M. Wilsch-Brauninger, and M. Gonzalez-Gaitan. 2003. Role of Drosophila Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. *The Journal of cell biology*. 161:609-624.

Xie, T., S.J. Li, M.R. Guo, Y. Wu, H.Y. Wang, K. Zhang, X. Zhang, L. Ouyang, and J. Liu. 2015. Untangling knots between autophagic targets and candidate drugs, in cancer therapy. *Cell proliferation*. 48:119-139.

Yang, P., and H. Zhang. 2014. You are what you eat: multifaceted functions of autophagy during C. elegans development. *Cell research*. 24:80-91.

Yang, S., and A.G. Rosenwald. 2014. The roles of monomeric GTP-binding proteins in macroautophagy in Saccharomyces cerevisiae. *International journal of molecular sciences*. 15:18084-18101.

Ye, M., Y. Chen, S. Zou, S. Yu, and Y. Liang. 2014. Ypt1 suppresses defects of vesicle trafficking and autophagy in Ypt6 related mutants. *Cell biology international*. 38:663-674.

Yla-Anttila, P., H. Vihinen, E. Jokitalo, and E.L. Eskelinen. 2009. 3D tomography reveals connections between the phagophore and endoplasmic reticulum. *Autophagy*. 5:1180-1185.

Yousefian, J., T. Troost, F. Grawe, T. Sasamura, M. Fortini, and T. Klein. 2013. Dmon1 controls recruitment of Rab7 to maturing endosomes in Drosophila. *Journal of cell science*. 126:1583-1594.

Yu, L., C.K. McPhee, L. Zheng, G.A. Mardones, Y. Rong, J. Peng, N. Mi, Y. Zhao, Z. Liu, F. Wan, D.W. Hailey, V. Oorschot, J. Klumperman, E.H. Baehrecke, and M.J. Lenardo. 2010. Termination of autophagy and reformation of lysosomes regulated by mTOR. *Nature*. 465:942-946.

Zaidi, N., A. Maurer, S. Nieke, and H. Kalbacher. 2008. Cathepsin D: a cellular roadmap. *Biochemical and biophysical research communications*. 376:5-9.

Zeigerer, A., J. Gilleron, R.L. Bogorad, G. Marsico, H. Nonaka, S. Seifert, H. Epstein-Barash, S. Kuchimanchi, C.G. Peng, V.M. Ruda, P. Del Conte-Zerial, J.G. Hengstler, Y. Kalaidzidis, V. Koteliansky, and M. Zerial. 2012. Rab5 is necessary for the biogenesis of the endolysosomal system in vivo. *Nature*. 485:465-470.

Zhang, J., K.L. Schulze, P.R. Hiesinger, K. Suyama, S. Wang, M. Fish, M. Acar, R.A. Hoskins, H.J. Bellen, and M.P. Scott. 2007. Thirty-one flavors of Drosophila rab proteins. *Genetics*. 176:1307-1322.

Zhang, J., X.H. Yu, Y.G. Yan, C. Wang, and W.J. Wang. 2015. PI3K/Akt signaling in osteosarcoma. *Clinica chimica acta; international journal of clinical chemistry*. 444:182-192.

Zhang, M., L. Chen, S. Wang, and T. Wang. 2009. Rab7: roles in membrane trafficking and disease. *Bioscience reports*. 29:193-209.

Zhang, Y., X. Gao, L.J. Saucedo, B. Ru, B.A. Edgar, and D. Pan. 2003. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nature cell biology*. 5:578-581.

Zoncu, R., L. Bar-Peled, A. Efeyan, S. Wang, Y. Sancak, and D.M. Sabatini. 2011. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. *Science*. 334:678-683.

Zoppino, F.C., R.D. Militello, I. Slavin, C. Alvarez, and M.I. Colombo. 2010. Autophagosome formation depends on the small GTPase Rab1 and functional ER exit sites. *Traffic*. 11:1246-1261.