ABSTRACT

Title of Document: CHARACTERIZATION OF ATG6 FUNCTION IN AUTOPHAGY AND GROWTH CONTROL DURING *DROSOPHILA MELANOGASTER* DEVELOPMENT

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The tumor suppressor Beclin 1 mitigates cell stress by regulating the lysosomal degradation pathway known as autophagy. This process involves formation of intracellular double-membraned vesicles, known as autophagosomes, which engulf proteins and damaged organelles and fuse with lysosomes, where the contents are degraded. It is unclear whether the function of Beclin 1 in autophagy is related to cell transformation in *beclin 1*$^{+/-}$ animals. Using the fruit fly, *Drosophila melanogaster*, I investigated the function of the Beclin 1 ortholog Atg6 in autophagy and growth control. Through transgenic experiments, I found that Atg6, like Beclin 1, induces autophagy by functioning in a complex consisting of the lipid kinase Vps34 and the serine-threonine kinase Vps15. I also generated a strong loss of function mutant, *Atg6*^{1}, and found that Atg6 is required for development. *Atg6* mutant animals contained an excess of blood

cells, which surrounded melanotic tumors prior to death. At the cellular level, Atg6 is required for autophagy and endocytosis, and cells lacking *Atg6* accumulate high levels of the endoplasmic reticulum stress protein Hsc3 and the adaptor protein p62. I also showed that *Atg6* mutant cells displayed mis-regulated nuclear localization of NFκB proteins, transcription factors whose downstream targets include regulators of innate immunity. Significantly, my results suggest that Atg6 may regulate growth independent of its function in autophagy, as mosaic loss of *Atg6* in the eye resulted in over-representation of *Atg6* mutant cells, a phenotype not shared by other autophagy gene mutant mosaics. Finally, through a collaborative effort, our lab identified a novel function for Atg6 in regulation of epithelial cell polarity. This finding is significant, as epithelial tumor cells are known to lose polarity during metastasis. Our studies have provided a significant contribution to the Beclin 1 field, by providing the first characterization of a *Drosophila Atg6* mutant, and demonstrating its function in novel cellular processes.

CHARACTERIZATION OF ATG6 FUNCTION IN AUTOPHAGY AND GROWTH CONTROL DURING DROSOPHILA MELANOGASTER DEVELOPMENT

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Chapter 1

Introduction

I. Genetic Regulation of Growth

The balance between cell growth, cell proliferation, and cell death ensures correct cell, tissue and animal size (Conlon and Raff, 1999). Perturbation of these cell processes can lead to disorders, including uncontrolled growth and cancer (Hanahan and Weinberg, 2000). During animal development, cell proliferation, growth, and death pathways are regulated by both extracellular and intracellular signals. Extracellular signals include hormones, growth factors, and mitogens, which regulate growth and proliferation by binding to membrane receptors, initiating downstream intracellular signaling cascades (van der Geer et al., 1994). In addition to growth factors, nutrients also regulate cell growth (Shamji et al., 2003). Cells sense nutrient availability and increase in mass by synthesizing proteins. In the absence of nutrients, anabolism ceases and catabolic pathways are induced to provide energy. The pathways that coordinate these diverse signals are highly conserved among diverse organisms.

A. Cell Proliferation

Growth factors regulate metazoan cell proliferation and growth via receptor tyrosine kinases (RTK). Binding of a growth factor ligand to the extracellular side of its RTK induces oligomerization of the intracellular domains of the receptor. Upon dimerization, the activated RTK is bound by the adaptor protein Grb2 through its Src homology 2 (SH2) domain, which recruits a guanine nucleotide exchange factor (GEF),

Son of sevenless (Sos), to the membrane (Bonfini et al., 1992; Chardin et al., 1993). Sos activates the small GTPase Ras by stimulating exchange of GDP for GTP (Chardin et al., 1993). GTP-bound activated Ras plays a central role in cell proliferation, transmitting the signal downstream to the mitogen activated protein (MAP) kinase cascade, starting with direct activation of the MAP kinase kinase kinase Raf (Hattori et al., 1992; Moodie et al., 1993; Pomerance et al., 1992). The signal is transduced via a series of phosphorylations through the MAP kinase cascade to the nucleus, where proliferation genes, including the G_1 phase cyclins, are turned on (Lavoie et al., 1996). Alternatively, MAP kinase signaling can be initiated by G-protein coupled receptors, which can activate Ras family GTPases (Luttrell, 2002).

Ras has also been shown to bind directly to the p110 catalytic subunit of the class I phosphatidylinositol 3-kinase (PI3K) (Rodriguez-Viciana et al., 1994). PI3K is a central regulator of cell growth, which also regulates proliferation via activation of the serine/threonine kinase Akt. Following Ras binding and activation of PI3K, Akt is recruited to the plasma membrane by the phospholipid product of PI3K, phosphatidylinositol (3,4,5)-triphosphate (PIP_3), to which Akt is bound via its plekstrin homology (PH) domain (Franke et al., 1997). There are numerous downstream targets of Akt phophorylation, including regulators of protein synthesis, metabolism, growth, and proliferation (Manning and Cantley, 2007). Akt regulates cell proliferation by phosphorylating cell cycle regulators. Cell cycle targets of Akt include glycogen synthase kinase 3-β (GSK3β), which phosphorylates cyclin D1, targeting it for proteasomal degradation, and FOXO, a transcription factor that regulates expression of the cyclin dependent kinase inhibitor p21 (Brunet et al., 1999; Cross et al., 1995; Diehl et al., 1998).

B. Cell Growth

The class I PI3K pathway is also a central regulator of cell growth. Upon growth factor binding to transmembrane RTKs, PI3K subunits become localized near the membrane and are activated by phosphorylation (Carpenter et al., 1990; Kaplan et al., 1987; Whitman et al., 1987). Next, they form heterodimers and these active PI3K complexes phosphorylate phospholipid substrates, which include phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI-4-P), and phosphatidylinositol 4,5 bisphosphate (PIP_2) (Whitman et al., 1988; Carpenter et al., 1990). These membrane-bound phospholipids recruit PH domain-containing proteins to the plasma membrane to propagate signaling through phosphorylation of downstream effectors (Franke et al., 1997).

In mammals, there are two subfamilies of class I PI3K proteins. Class IA PI3Ks consist of a p110 catalytic subunit, of which there are three isoforms, and a p85 regulatory subunit, of which there are five isoforms (Engelman et al., 2006). Class IB PI3Ks are composed of either a p101 or a p84 regulatory subunit, of which there are 3 isoforms, and a single p110 catalytic subunit. The two subfamilies of Class I PI3Ks differ based on the receptors that activate them. Class IA PI3Ks are activated by RTKs, including insulin and insulin like growth factor (IGF) receptors, epidermal growth factor receptor (EGFR), and platelet derived growth factor (PDGF) receptor, while class IB PI3Ks are activated by G-protein coupled receptors (GPCR).

Binding of either insulin or IGF1 to cell surface receptors induces autophosphorylation of the dimerized subunits (Kasuga et al., 1982). This leads to recruitment of a class IA PI3K p85/p110 complex to the plasma membrane (Gillham et

al., 1999). Binding of PI3K by the RTK is facilitated by insulin receptor substrate (IRS) proteins, which act as adaptors by binding to both in proximity to the membrane (White, 1998). The signal is transduced from the RTK through the adaptor protein by phosphorylation. The phospholipid substrate of PI3K, PIP_2, is converted to PIP_3, a second messenger that recruits Akt to the membrane, where it is phosphorylated by the serine/threonine kinases PDK1 and PDK2 (Vivanco and Sawyers, 2002). Downstream of Akt, the kinase target of rapamycin (TOR) regulates growth by stimulating protein synthesis in the presence of nutrients, and inhibiting autophagy, a cell autonomous catabolic pathway (Fig. 1-1) (Kim et al., 2002; Noda and Ohsumi, 1998; Oldham et al., 2000). Activated Akt phosphorylates the GTPase activating protein (GAP) tuberous sclerosis complex protein 2 (TSC2), leading to its inhibition and subsequent accumulation of the active GTP-bound form of the GTPase Rheb (Zhang et al., 2003). Upon activation of TOR by Rheb, TOR phosphorylates p70 S6 kinase, inhibiting the translational repressor 4E-BP1(Burnett et al., 1998). In a parallel, Rheb-independent pathway, Akt can also activate TOR via repression of the proline-rich Akt substrate of 40kDa (PRAS40) (Sancak et al., 2007). PRAS40 binds to TOR, and mutation of the Akt phosphorylation site on PRAS40 leads to TOR activation (Sancak et al., 2007; Vander Haar et al., 2007).

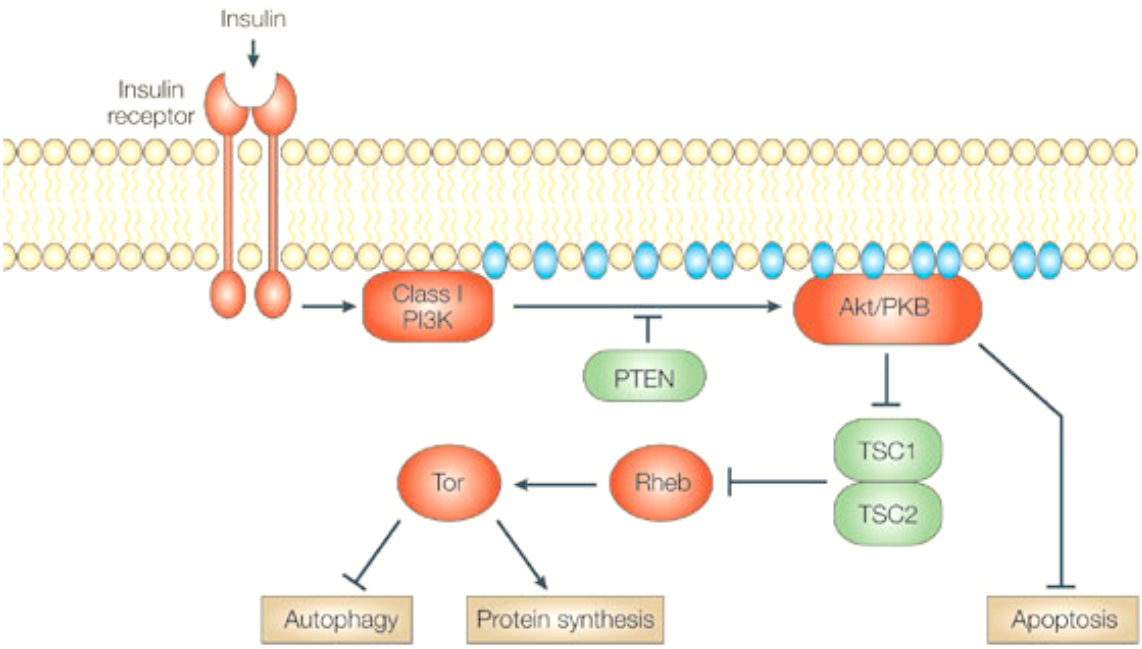

Figure 1-1. The Class I PI3K signaling pathway regulates growth by inhibiting autophagy and apoptosis. Class I PI3K, Akt, Rheb, and TOR are all negative regulators of autophagy (red). PTEN positively regulates autophagy by inhibiting activation of Akt by membrane lipids. TSC1 and TSC2 are also positive regulators of autophagy, via repression of Rheb (Baehrecke, 2005).

There are multiple regulatory nodes in the PI3K pathway to prevent constitutive activation and uncontrolled growth. The phosphatase and tensin homolog (PTEN) terminates pathway signaling by converting PIP_3 to PIP_2, blocking recruitment and activation of Akt (Maehama and Dixon, 1998). This central role in growth regulation makes PTEN a potent tumor suppressor, and it is mutated in many cancers (Keniry and Parsons, 2008). Downstream of Akt, the TSC1-TSC2 complex also serves an inhibitor of cell growth (Tee et al., 2002). The TSC1-TSC2 complex activates Rheb GTPase activity, leading to GTP-hydrolysis, and conversion of Rheb to its inactive, GDP-bound form (Inoki et al., 2003). As negative regulators of TOR signaling, both PTEN and the TSC1-TSC2 complex positively regulate autophagy, a catabolic process essential to maintaining homeostasis. Without negative regulation of TOR, cell growth could proceed uncontrolled, a phenomenon which could contribute to cancer.

II. Growth signaling gone awry

A. Oncogenes

PI3K pathway activation

The PI3K pathway is essential to maintaining tissue homeostasis within an organism. However, if left unchecked, ectopic activation of PI3K signaling can lead to oncogenesis. Activating mutations in PI3K have been identified in both the p110 catalytic subunit and the p85 regulatory subunit. The p110α gene contains two mutational hotspots, one in the kinase domain and another in the helical domain, which are frequently mutated in a variety of cancers (Zhao and Vogt, 2008a). Many point mutations in the kinase domain lead to elevated lipid kinase activity and activation of downstream

proliferation and survival pathways (Zhao and Vogt, 2008b). The p110α gene is also frequently amplified in cancer. Specifically, amplification of p110α leads to increased gene expression and kinase activity in ovarian cancer (Shayesteh et al., 1999). Often, the signal is transduced via Akt. However, some cancers display activation of PI3K without Akt phosphorylation and activation (Stemke-Hale et al., 2008). Some p85 mutations are also associated with cell transformation. Mutations in the SH2 domain of p85α have been hypothesized to relieve inhibition of p110α by p85 by weakening the interaction between the two subunits (Jaiswal et al., 2009).

Ras

Mutations in the small GTPase Ras are the most common in human cancers, with approximately 30% of cancers containing activating mutations in one of three Ras genes (Downward, 2003). Point mutations have been identified in each of the three Ras genes, H-ras, K-ras, and N-ras, and the incidence of mutation in each gene varies among tumor types. K-ras mutations are the most prevalent, and are often associated with pancreatic, lung, and colorectal cancers, while N-ras mutations are found in myeloid leukemias and in approximately 20% of melanomas (Bos, 1989). Due to the pleiotropic nature of Ras GTPase activity, activating mutations in Ras can exert oncogenic effects through a number of downstream effectors, including Raf and PI3K. Raf, a serine/threonine kinase transduces the signal from Ras to the MAPK cascade via the kinase Erk, whose targets include regulators of growth and cell cycle entry (Howe et al., 1992).

B-cell leukemia/ lymphoma-2 (BCL-2) family proteins

Genetically regulated programmed cell death is essential for both physiological maintenance of cell homeostasis and also in response to stress or damage. Bcl-2 was identified at a B-cell lymphoma-associated chromosomal breakpoint, where a gene rearrangement placed it under control of the immunoglobulin heavy chain promoter and resulted in overexpression of Bcl-2 (Tsujimoto and Croce, 1986). Bcl-2 was later characterized as a proto-oncogene, as Bcl-2 expression conferred resistance to cell death following growth factor withdrawal and induced tumor formation in mice (Reed et al., 1988; Reed et al., 1990; Vaux et al., 1988). Further, knock down of Bcl-2 in leukemia cells resulted in apoptosis (Reed et al., 1990). Anti-apoptotic function was attributed to Bcl-2 in 1990, when Hockenbery et al. found that mouse pro-B lymphocyte cells transfected with human Bcl-2, inducible by interleukin-3 (IL-3), did not display the membrane blebbing, nuclear condensation, and DNA fragmentation associated with apoptosis in control cells following IL-3 withdrawal (Hockenbery et al., 1990). Furthermore, Bcl-2 was found to localize to the inner mitochondrial membrane, where it was later shown to block apoptosis by suppressing release of cytochrome *c* and subsequent caspase activation (Hockenbery et al., 1990; Yang et al., 1997).

In the years following the discovery of the pro-survival Bcl-2 protein, several other proteins sharing similar domain structure were identified and grouped into the Bcl-2 family. The Bcl-2 family of proteins is made up of anti- and pro-apoptotic proteins, all of which contain at least one Bcl-2 homology (BH) domain (Adams and Cory, 1998). In addition to Bcl-2, the anti-apoptotic family members include Bcl-X_L, Bcl-w, and myeloid cell leukemia sequence 1 (Mcl-1). All of the anti-apoptotic Bcl-2 family members contain

three to four BH domains, which are required for suppression of apoptosis and are essential for interactions with other Bcl-2 family proteins. The pro-apoptotic Bcl-2 family members are sub-divided into multi-domain and single domain groups. Bax, Bcl-2 antagonist killer 1 (Bak), and Bcl-2 related ovarian killer (Bok) each contain three BH domains. Bax and Bak reside in proteolipid pores at the outer mitochondria membrane where they function to induce mitochondrial outer membrane permeabilization (MOMP), triggering the release of cytochrome *c* from the mitochondria (Liu et al., 1996). The largest class of Bcl-2 family proteins is composed of several members, all of which contain a single BH3 domain. BH3-only proteins can induce apoptosis directly, by activating Bax and Bak, or indirectly, by binding to anti-apoptotic Bcl-2 proteins, relieving repression on pro-apoptotic Bcl-2 proteins (Chipuk and Green, 2008).

B. Tumor suppressors

Rb and p53

Cells require a diverse network of tumor suppressor proteins in order to counteract the potent growth, proliferation, and survival signals propagated by onco-proteins. Tumor suppressors are induced in response to DNA damage, serve as checkpoints in the cell cycle, and maintain cell-to-cell contacts to prevent metastasis.

The concept of tumor suppression by genetic mechanisms was first presented in 1969, when Harris and colleagues reported that a factor from noncancerous cells could prevent tumor formation when fused to cancerous cells and reintroduced into animals (Harris et al., 1969). The first tumor suppressor identified was the gene responsible for the rare ocular cancer retinoblastoma. In 1971, Knudson hypothesized that tumor

formation required recessive mutation in the causative gene, and characterization of the retinoblastoma mutation supported this "two hit" model of tumor suppression (Knudson, 1971). The retinoblastoma gene, RB1, was later mapped and its product, pRb, was determined to function as a transcriptional repressor of cell cycle regulators (Hiebert et al., 1992). More recent studies revealed a more complex role for pRb, as it has since been shown to function as a regulator of apoptosis and differentiation, and has also been found to be mutated in cases of bladder cancer and osteogenic sarcoma (Chen et al., 2009).

The most commonly mutated tumor suppressor gene, tumor protein p53 (TP53), was discovered independently by two groups, both of whom found that it immunoprecipitated with the oncogenic Simian virus 40 (SV40) large T-cell antigen in transformed mouse cells (Lane and Crawford, 1979; Linzer and Levine, 1979). Like RB1, p53 functions in diverse cellular processes through transcriptional regulation of genes required for apoptosis, cell cycle arrest, differentiation, DNA repair, senescence, and metabolism (Riley et al., 2008). Disruption of any of these pathways can have oncogenic implications, underscoring the critical tumor suppressive function of p53. Activation of p53 can occur in response to DNA damage, cell stress, and hyperproliferation, and the cellular response to these injuries is mediated by at least 100 downstream target genes. Depending on the genes induced at a given time by p53, cells coordinate multiple signals on the way to cell cycle arrest or apoptosis.

Cell polarity regulators

Epithelial cell cancers, known as carcinomas, are the most common type of cancer. Normal epithelial cells are polarized, that is they display asymmetric distribution

of proteins at their apical, basal, and lateral surfaces (St Johnston and Ahringer, 2010). Mis-localization of cell polarity proteins has been associated with over-growth (Huang and Muthuswamy, 2010). Among those proteins regulating epithelial cell polarity in mammals are Scribble (Scrib), lethal giant larvae (Lgl), and discs large (Dlg), which reside at the basolateral membrane. The apical membrane is home to two polarity complexes. Crumbs (Crb), protein associated with Lin7 1 (Pals1), and Pals associated tight junction protein (Patj), comprise the Crumbs polarity complex, and partitioning defective 3 (Par3), Par6, and atypical protein kinase C (aPKC), comprise the Par complex (Fig. 1-2). Additionally, structural proteins such as integrins and cadherins are essential to maintaining contact with neighboring cells.

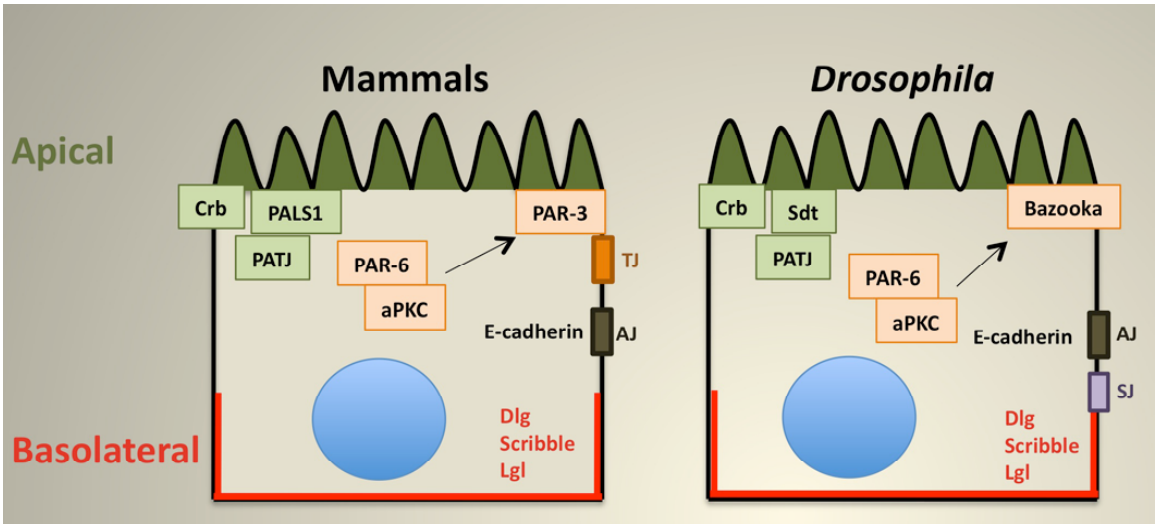

Figure 1-2. Epithelial cell polarity complexes. In both mammalian and *Drosophila* epithelial cells, the Par and Crumbs polarity complexes are localized to the apical membrane, while the Scribble complex localizes to the basolateral membrane. In mammals, tight junctions (TJ) form a permeability barrier between neighboring cells. In both organisms, E-cadherin localizes to lateral points of cell-to-cell contact, known as adherens junctions (AJ). In *Drosophila*, Dlg is required for septate junction (SJ) structure. Septate junctions, like tight junctions, are important for prevention of diffusion of molecules between neighboring cells (modified from St Johnston and Ahringer, 2010).

Lgl, Dlg, and Scrib were initially discovered in *Drosophila melanogaster*, where they were found to regulate cell proliferation, in addition to polarity. *Lgl* was identified as the first *Drosophila* tumor suppressor gene in 1967 (reviewed in Bilder, 2004). Five years later, *dlg* was discovered in a screen for mutations that affected morphology of the larval imaginal discs, which are precursors to adult epithelial tissues (Stewart et al., 1972). Scribble was implicated as a tumor suppressor following experiments in genetically mosaic follicular epithelium of *Drosophila* ovaries (Bilder et al., 2000). Clonal mutations in *lgl*, *dlg*, and *scrib* all produce similar phenotypes in epithelial cells. Larvae mutant for any one of these genes contained layered, disorganized epithelial cells, displayed loss of apico-basal cell polarity, and showed overgrowth of imaginal discs and brain (Bilder et al., 2000). These phenotypes led to the characterization of *lgl*, *dlg*, and *scrib* as neoplastic, rather than hyperplastic, tumor suppressors because mutants in these genes exhibit epithelial disorganization in addition to overproliferation (Bilder, 2004). Genetic epistasis analysis indicated that *lgl*, *dlg*, and *scrib* function in the same pathway, as each was required for proper localization of the other proteins. Despite these elegant studies of *Drosophila* cell polarity markers and their role in neoplastic tumor formation, few human cancers have been directly linked to misregulation of cell polarity proteins.

It has long been observed that cell polarity is lost in the process of transformation though the molecular mechanisms behind cell polarity disruption during tumorigenesis have been unclear (Lee and Vasioukhin, 2008). One of the hallmarks of advanced tumorigenesis is an epithelial to mesenchymal transition (EMT), which occurs when epithelial cell polarity and cell adhesion are lost, contributing to metastasis (Thiery, 2002). Recent experiments have shown that loss of cell polarity early can contribute to

transformation. Analysis of breast tumors revealed reduced expression of *scrib* RNA and mislocalization of Scribble from cell-cell junctions to the cytoplasm (Zhan et al., 2008; Zhang et al., 2008). Furthermore, experiments in 3D breast epithelial acini, used to model *in vivo* breast acini, showed that Scribble is required for oncogene induced apoptosis, regulating activation of the GTPase Rac via the GEF β-PIX at adherens junctions (Zhan et al., 2008). These data suggest that loss of Scribble can directly contribute to, and is not simply a consequence of, transformation. In addition to breast cancer, some cervical and colon cancers also display mislocalization and/or downregulation of Scribble (Huang and Muthuswamy, 2010). The cervical cancer-causing human papillomavirus (HPV) targets Scribble and Dlg1 for degradation, mediated by the ubiquitin ligase E6 viral oncoproteins, which interact with Scribble and Dlg1, as well as the well characterized tumor suppressors p53 and Rb (Nakagawa and Huibregtse, 2000).

Microarray and proteomic analyses of tumors have shown variable changes to cell polarity regulators, depending on the stage of cancer and affected tissue (Huang and Muthuswamy, 2010). While Scribble, Lgl, Crumbs, and Par3 are often down-regulated in tumor derived cell lines and cancerous tissues, some cancers exhibit increased levels of polarity proteins. For example, Par6 overexpression is associated with estrogen receptor (ER) positive breast cancers, and Par6 contribution to transformation is likely due to its function as a MAP kinase activator via interaction with aPKC and the GTPase cell division cycle 42 (Cdc42) (Nolan et al., 2008). In addition to changes in expression levels, subcellular localization of polarity proteins is also altered in cancer. Lgl1 becomes localized to the cytoplasm during ovarian tumorigenesis, and Dlg1 is mislocalized to the cytoplasm in colon and cervical carcinomas, resulting in reduced Dlg1 at cell-cell

junctions (Humbert et al., 2008). As the epithelial polarity modules regulate multiple downstream signaling pathways, among them cell proliferation and death, their proper localization is critical to maintain control of these pathways and keep oncogenic signaling in check.

Beclin 1, a haploinsufficient tumor suppressor

The Beclin 1 tumor suppressor was identified as a Bcl-2 interacting protein (Liang et al., 1998). Aita et al. then mapped the *beclin 1* gene to a breast and ovarian cancer susceptibility locus on chromosome 17, and found that 41% of breast carcinoma cell lines screened contained a deletion of *beclin 1* (Aita et al., 1999). Subsequent studies revealed that transfected Beclin 1 slowed proliferation of MCF7 human breast carcinoma cells, which display loss of heterozygosity at the 17q21 locus, and contain no endogenous Beclin 1 (Liang et al., 1999). Further, mice injected with MCF7 cells expressing Beclin 1 displayed significantly lower incidence of tumorigenesis compared to mice injected with MCF7 cells that did not express Beclin 1 (Liang et al., 1999).

Functional characterization of Beclin 1 uncovered a role in the lysosomal delivery process known as autophagy. Human Beclin 1 shares approximately 30% amino acid sequence identity with the yeast *Saccharomyces cerevisiae* protein Vps30/Atg6 (herein referred to as Atg6), and Beclin 1 expression in Atg6 deficient yeast or MCF7 cells rescued a defect in starvation-induced autophagy in both cell types (Liang et al., 1999). Restoration of autophagy in breast carcinoma cells, coupled with the reduction in tumorigenicity, suggested that the potential tumor suppressive role of Beclin 1 might be attributed to its function in autophagy.

Though early studies with Beclin 1 suggested that it might function in tumor suppression, it did not qualify as a tumor suppressor by Knudson's two-hit model. Fifty percent of breast, 75% of ovarian, and 40% of prostate cancers display monoallelic deletion of *beclin 1*, but sequence analysis of breast carcinoma cells did not identify any "second hit" point mutations in *beclin 1* (Aita et al., 1999). Knockout mouse studies revealed that monallelic loss of *beclin 1* resulted in increased spontaneous tumorigenesis compared to animals with two functional copies of *beclin 1* (Qu et al., 2003; Yue et al., 2003). *beclin 1*$^{+/-}$ mice were affected by lymphomas, lung carcinomas, and liver carcinomas. Molecular and biochemical analyses revealed that all tumors in *beclin 1*$^{+/-}$ mice contained one wild type *beclin 1* allele and wild type Beclin 1 protein, ruling out spontaneous loss of heterozygosity and demonstrating that *beclin 1* is a haploinsufficient tumor suppressor gene (Qu et al., 2003, Yue et al., 2003).

III. Autophagy regulation of cell and tissue homeostasis

Macroautophagy (autophagy) delivers cytoplasmic components, such as long-lived proteins and damaged organelles, to the lysosome for degradation (Deter and De Duve, 1967; (Takeshige et al., 1992). In the absence of nutrients, growth arrests and cells adapt to the absence of either growth factors or amino acids by inducing autophagy. Protein turnover by the lysosome enables recycling of amino acids to be utilized for protein synthesis, while breakdown of damaged organelles prevents accumulation of toxic reactive oxygen species in the cell (Yang et al., 2006; Zhang et al., 2007). This highly conserved process occurs in organisms as diverse as yeast, fruit flies, and mammals in response to nutrient starvation and cell stress (Meléndez and Neufeld, 2008).

A. Discovery of autophagy genes

Cell morphological changes associated with autophagy were first observed in the liver (Deter and De Duve, 1967), but it wasn't until a series of experiments in the 1990s in the budding yeast *Saccharomyces cerevisiae* that the mechanisms of autophagy were uncovered. The first study showed that defects in vacuolar proteolysis led to accumulation of autophagic bodies under conditions of nutrient restriction (Takeshige et al., 1992). These autophagic bodies, now known as autophagosomes, are composed of a double membrane that surrounds cytosolic components targeted for degradation in the vacuole, the yeast equivalent of the lysosome. This study was followed by 2 independent genetic screens in yeast; one for mutants defective in degradation of autophagic bodies (Tsukada and Ohsumi, 1993), and the other for mutants that accumulate the cytosolic protein fatty acid synthase (Thumm et al., 1994). These concurrent studies led to the discovery of the 14 autophagy (*apg)* genes and the 3 autophagocytosis (*AUT*) complementation groups, which are required for autophagy. Additionally, a screen for mutants in the cytoplasm to vacuole targeting pathway (CVT) uncovered genetic overlap between this pathway and autophagy (Harding et al., 1995). The CVT pathway functions in transport of the vacuolar protein aminopeptidase I (API). Thirty-one autophagy-related genes (now called *Atg* genes) have been discovered and most are conserved in animals (Klionsky et al., 2003).

B. Regulation of autophagy

Two lipid kinase signaling pathways converge to regulate autophagy. The class I PI3K/insulin signaling pathway negatively regulates autophagy induction via TOR

regulation of Atg13 and the kinase Atg1. The class III PI3K/vacuolar protein sorting 34 (Vps34) complex that includes p150/Vps15 and Beclin 1/Atg6 positively regulates autophagosome formation, and the Atg conjugation pathways regulate autophagosome maturation and fusion with the lysosome (Fig. 1-3).

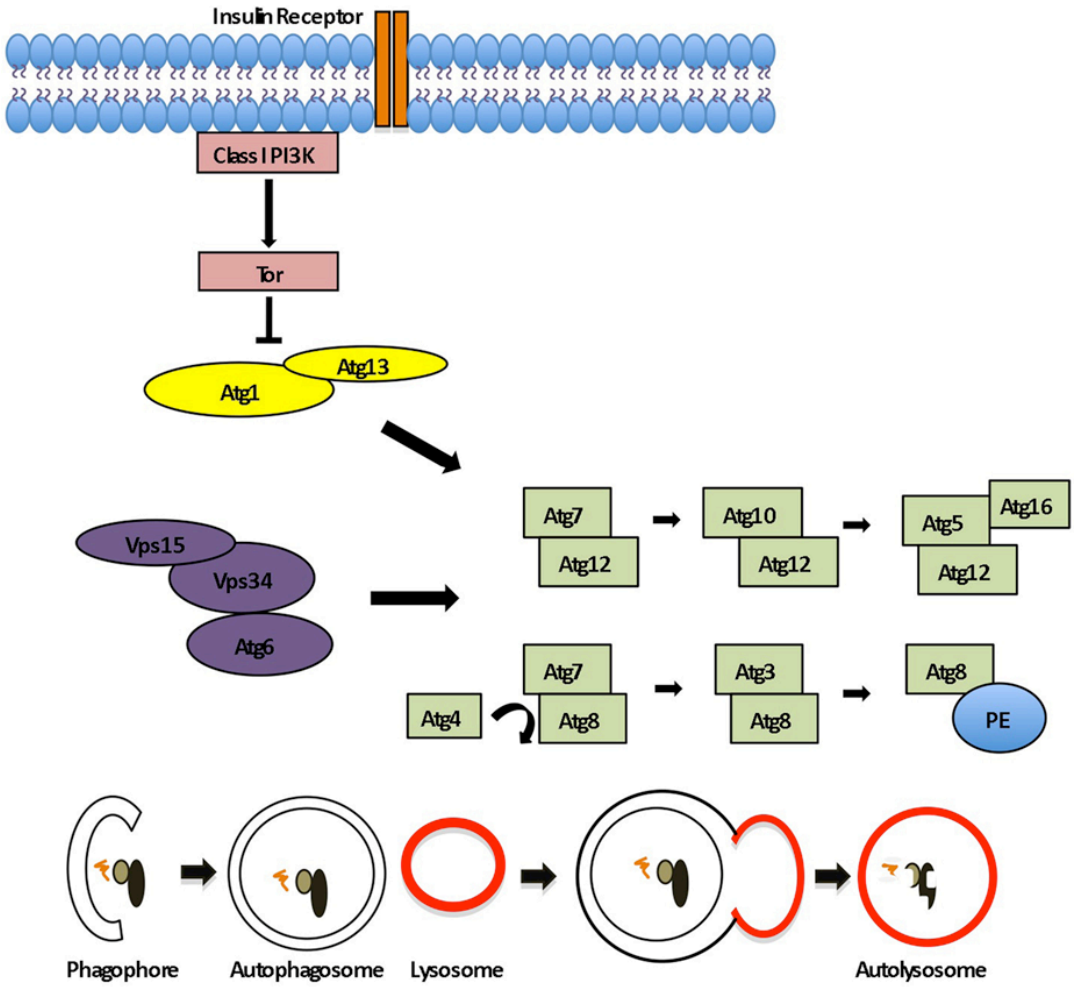

Figure 1-3. Regulation of autophagy. Class I PI3K negatively regulates autophagy via TOR kinase. Downstream of Atg1-Atg13, a complex containing the Class III PI3K Vps34, the serine-threonine kinase Vps15, and Atg6 positively regulates autophagosome formation. A double membrane forms around proteins and organelles to be degraded. Two ubiquitin-like conjugation pathways regulate elongation of the autophagosome membrane. Atg7 activates Atg12 and Atg8 in an ATP-dependent manner. This is followed by covalent conjugation of Atg12 to Atg5 and Atg8 to the lipid phosphatidylethanolamine (PE). Atg5 binds noncovalently to Atg16 and Atg8-PE becomes anchored in the autophagosome membrane. The outer membrane of the autophagosome fuses with the lysosome, releasing the inner membrane and sequestered contents into the lysosome for degradation (From Hill and Baehrecke, 2009).

Atg1, the first *Atg* gene characterized in yeast, encodes a protein kinase. Mutant analysis showed that Atg1 kinase activity is required for autophagosome formation and viability during starvation (Matsuura et al., 1997). Atg1 binds to Atg13 and this complex functions early in autophagosome formation. Regulation of the Atg1-Atg13 complex is under control of TOR. Under nutrient rich conditions, TOR hyperphosphorylates Atg13, reducing its binding affinity for Atg1. In response to nutrient starvation, TOR becomes inactivated, alleviating repression of Atg13 (Kamada et al., 2000).

Regulation of autophagy by TOR is more complex in multicellular eukaryotes, where the class I PI3K pathway signals upstream of TOR to induce cell growth and inhibit autophagy. The mechanisms of PI3K/TOR regulation of autophagy have been largely elucidated from genetic studies in the fruit fly, *Drosophila melanogaster*. Mutations in either *tor* or *dp110*, the catalytic subunit of PI3K, lead to reduced cell growth and induction of autophagy in well fed *Drosophila* larvae, while ectopic expression of either class I PI3K or TOR in *Drosophila* larvae inhibits starvation-induced autophagy (Scott et al., 2004). In addition to its requirement for starvation-induced autophagy, Atg1 activity is also vital in *Drosophila* development, as animals lacking *Atg1* function die before completing development (Scott et al., 2004).

Studies in yeast identified 2 ubiquitin-like conjugation pathways that function downstream of Atg1 activity. These conjugation pathways are composed of several Atg proteins, all of which are required for autophagosome formation. Atg7 was identified as an E1-like enzyme that activates Atg12 and Atg8 in 2 distinct conjugation pathways (Ichimura et al., 2000; Mizushima et al., 1998; Tanida et al., 1999). Atg12 and Atg8 are then covalently conjugated to the E2-like proteins Atg10 and Atg3 (Ichimura et al., 2000;

Shintani et al., 1999). This is followed by conjugation of Atg12 to Atg5 and lipidation of Atg8 with phosphatidylethanolamine (PE) (Ichimura et al., 2000; Mizushima et al., 1998). The Atg5-Atg12 complex interacts with multimerized Atg16 (Mizushima et al., 1999). These protein conjugation reactions occur in the membrane of the forming autophagosome, mediating membrane expansion around cytosolic components destined for degradation. Both conjugation pathways are conserved in higher eukaryotes and components of these pathways are required for autophagy in *Drosophila*, *C. elegans*, and mammals (Meléndez and Neufeld, 2008).

A third complex of autophagy related genes recovered from yeast screens were first identified in a screen for mutants defective in vacuolar protein sorting (Bankaitis et al., 1986). First identified as Vps30, Atg6 was found to interact in 2 distinct complexes, both of which include the class III PI3K, Vps34, and the protein kinase Vps15 (Kihara et al., 2001b). Vps15 activity is required for Vps34 function. The single known target of Vps34 is the lipid phosphatidylinositol (PI), which is converted to PI(3)P upon phosphorylation by Vps34. PI(3)P functions in recruitment of proteins to membranes, supporting a general role for Vps34 in intracellular membrane trafficking. A complex including Atg6, Vps34, Vps15, and Vps38 is required for vacuolar protein sorting, while a second complex containing Atg6, Vps34, Vps15, and Atg14 localizes to the preautophagosomal structure (PAS) and is required for autophagosome formation (Kihara et al., 2001b).

Similar Vps34 complexes have been described in mammalian cells, where several additional proteins have been shown to interact with Vps34 and Atg6 (Beclin 1 in mammals). In addition to interacting with the anti-apoptotic protein Bcl-2, biochemical

experiments confirmed that Beclin 1 also interacts with Vps34 and p150, the mammalian homolog of Vps15, and that this complex is required for starvation-induced autophagy (Kihara et al., 2001a; Liang et al., 1999; Liang et al., 1998; Petiot et al., 2000). Additionally, the tumor suppressors UVRAG and Bif-1, and the previously uncharacterized Ambra1, have been shown to interact physically with Beclin 1 (Fimia et al., 2007; Liang et al., 2006; Takahashi et al., 2007). An Atg14 homolog was recently discovered and UVRAG was determined to be the homolog of Vps38 (Itakura et al., 2008). Both interact with Beclin 1, but never in the same complex. These results suggest a mechanism similar to yeast where 2 distinct complexes function in autophagy and endocytosis.

C. Physiological Functions of Autophagy

Cell stress-induced autophagy

The best characterized function of autophagy is recycling of macromolecules under conditions of nutrient restriction. Experiments in model organisms have provided valuable information about the physiological implications of autophagy impairment during starvation. If restricted from feeding during development, the nematode *Caenorhabditis elegans* enters a period of quiescence known as the dauer stage, and it remains there until environmental conditions improve (Klass and Hirsh, 1976). Dauer entry can also be induced by inhibition of the insulin signaling pathway (Kimura et al., 1997). Utilizing temperature sensitive alleles of the *C. elegans* insulin-like receptor gene *daf-2* and RNAi against *bec-1*, the homolog of *beclin 1,* Mélendez et al. showed that autophagy is required for dauer formation, as animals with reduced *bec-1* expression

suffered abnormal dauer formation and eventual death (Meléndez et al., 2003). In *Drosophila*, autophagy is induced in the larval fat body, analogous to the liver, in response to starvation (Scott et al., 2004). This organ is essential for nutrient storage, and induction of autophagy in response to starvation may promote survival by providing essential macromolecules to maintain vital cellular processes.

This pro-survival function is conserved in mammals, where cell culture studies have shown that autophagy is induced following growth factor withdrawal, prolonging survival of cells for up to 20 weeks (Lum et al., 2005). Furthermore, autophagy is required for these cells to survive, as RNAi-mediated knockdown of autophagy genes resulted in cell death within four days following growth factor withdrawal. Autophagy is also essential for survival of neonatal mice, which endure a short period of starvation following birth before they begin suckling from their mothers (Kuma et al., 2004).

In addition to recycling macromolecules to enable a cell to survive starvation, autophagy also comes to the rescue in the face of other cell stresses, such as hypoxia. Reactive oxygen species (ROS) are generated by mitochondria in response to low levels of oxygen (hypoxia). Zhang et al. showed that mitochondrial autophagy is induced in embryonic fibroblasts (MEFs) grown under hypoxic conditions. This mitochondrial turnover blocked ROS accumulation and prevented cell death (Zhang et al., 2008).

Developmental autophagy

Studies in *Drosophila* revealed a developmental function for autophagy. Several tissues in the developing fruit fly, including the larval fat body, midgut, and salivary glands, utilize autophagy. This programmed autophagy differs from starvation-induced

autophagy in that it is regulated by steroid hormone signaling. Wandering third instar larvae undergo programmed autophagy in the fat body before metamorphosis to the adult. The steroid hormone 20-hydroxyecdysone (ecdysone) has been shown to induce autophagy in this tissue, and expression of a dominant negative ecdysone receptor in the fat body strongly inhibits autophagy (Rusten et al., 2004). Like starvation-induced autophagy, developmental autophagy can be blocked by class I PI3K activation, and gain of function studies showed that ecdysone signaling induced autophagy by down-regulating PI3K activity (Rusten et al., 2004).

Similarly, an ecdysone pulse at the end of the third instar triggers autophagy and eventual elimination of the larval midgut. Destruction of this tissue occurs as the adult midgut forms around the larval structure. The ecdysone responsive transcription factor, E93, is required for proper formation of autophagosomes, and E93 mutants show evidence of incomplete midgut degradation (Lee et al., 2002). As the larval midgut is inaccessible to phagocytes once the adult midgut is formed, autophagy is induced, in the presence of active caspases, to degrade the tissue (Denton et al., 2009). RNAi-mediated knockdown of *Atg1*, but not RNAi targeting the effector caspase *decay*, led to inhibition of midgut degradation (Denton et al., 2009).

Cell Death

The larval salivary glands also undergo programmed autophagy before degradation and subsequent formation of adult salivary glands. Histological analysis has shown that autophagic vacuoles begin to form within the salivary glands a few hours before they are completely degraded (Lee and Baehrecke, 2001). Though dying salivary

gland cells exhibit DNA fragmentation and caspase activity, two hallmarks of apoptosis, they are eliminated in the absence of phagocytic cells. This led to the classification of salivary gland cell death as autophagic cell death.

Like midgut cell death, salivary gland cell death requires expression of ecdysone responsive genes. A developmentally regulated pulse of ecdysone induces expression of the transcription factor E93. While direct targets of E93 have yet to be identified, microarray analysis of dying salivary glands showed that several *Atg* genes and caspases were induced immediately prior to cell death with autophagy, and that the response of several of these genes was attenuated in salivary glands of *E93* mutants (Lee et al., 2003). Genetic experiments showed that caspases and autophagy are required for salivary gland cell death, and that ectopic expression of Atg1 is able to induce early gland degradation independent of caspase activation (Berry and Baehrecke, 2007; Martin and Baehrecke, 2004). In the latter study, autophagy induction was linked to growth arrest through down-regulation of class I PI3K signaling, and ectopic PI3K induction was sufficient to prevent normal gland degradation.

Caspases have also been shown to regulate autophagy and cell death in *Drosophila* ovaries, where starvation can trigger death of egg chambers prior to their normal developmental cell death during late stage oogenesis. An RNAi based assay was used to identify known cell death genes that regulate starvation-induced autophagy in a larval blood cell line and the function of these genes in autophagy was tested *in vivo* (Hou et al., 2008). Death caspase-1 (Dcp-1) and Bruce, an inhibitor of apoptosis (IAP), were among the known cell death regulators found to function in autophagy in ovaries. Dcp-1 is required for induction of autophagy in degenerating egg chambers, while Bruce plays

an inhibitory role under nutrient rich conditions. Consistent with caspase activity, degenerating egg chambers also show evidence of DNA fragmentation, and this requires autophagy, as knock down of *Atg1* or *Atg7* leads to reduced DNA fragmentation (Hou et al., 2008). Autophagy was also recently implicated in developmental programmed cell death in the egg chamber during mid-oogenesis and in nurse cell death during late oogenesis (Nezis et al., 2009; Nezis et al., 2010). This cell death occurred in the presence of caspases and was characterized by DNA fragmentation. Interestingly, Bruce was degraded by autophagy in the nurse cells, and this process required functional Vps34 and Atg1 (Nezis et al., 2010). This result was the first example of cell death regulation by autophagic degradation of an apoptosis regulator.

A recent model of neuronal cell death in *C. elegans* has linked autophagy to necrosis. Gain of function alleles of *mec-4(d)*, which encodes an ion channel subunit, are neurotoxic in early larval stages and this cell death is necrotic (Hall et al., 1997). Neuronal cell death in *mec-4(d)* requires expression of the Atg1 ortholog UNC-51, and a time course revealed that autophagy is highly induced in neurons during the early stages of necrosis, but is down-regulated in later stages (Samara et al., 2008). The authors hypothesize that high levels of autophagy trigger necrotic cell death and propose that inhibition of autophagy might protect neurons from cell death after ischemic stroke. Autophagy genes were first shown to regulate cell death in mammals in 2004. Treatment of fibroblast-derived L929 cells with the caspase-8 inhibitor zVAD induced autophagic cell death within 2 days (Yu et al., 2004). Regulation of this autophagic cell death was under control of the death domain containing receptor interacting serine-threonine kinase (RIP), and this cell death required Atg7 and Beclin 1 expression (Yu et al., 2004). The

Bcl-2 protein family, known for its role in regulation of apoptosis, also functions to regulate autophagy. Cells double mutant for the pro-apoptotic Bcl-2 family members Bax and Bak died by autophagic cell death when treated with the apoptosis inducing drug etoposide (Shimizu et al., 2004). This cell death required Beclin 1 expression, and was further enhanced by overexpression of anti-apoptotic Bcl-2 or Bcl-X_L after etoposide treatment of Bax/Bak double mutant cells. These studies suggest that autophagy can compensate for a lack of apoptosis to induce cell death when survival is no longer an option.

V. The Beclin 1 Complex

Since the implication of Beclin 1 in tumor suppression, multiple studies have sought to identify other proteins with which it might interact in order to ascertain the mechanism of Beclin 1 function in the cell. The Beclin 1/Vps34/Vps15 complex is evolutionarily conserved from yeast to mammals. Despite their seemingly stable interaction with Beclin 1, Vps34 and Vps15/p150, have so far not been ascribed any tumor suppressor function. In yeast, the core complex functions in autophagy and vacuolar protein sorting, and the proteins Atg14 and Vps38 confer specificity of the complex to one process or the other. Mammalian homologs of Atg14 and Vps38 have recently been identified, and their functions suggest an increasingly complex role for Beclin 1 in the cell. Details gleaned from studying the specificity of Beclin 1 signaling in different tissues under variable conditions are likely to lead to more clues about the tumor suppressor function of Beclin 1.

A. UVRAG

The mammalian Vps38 homolog, also known as ultraviolet radiation resistance-associated gene (UVRAG), was not recognized as such when it was first discovered. UVRAG was identified as a member of the Beclin 1 complex in mammalian 293T cells expressing glutathione S-transferase (GST)-tagged viral Bcl-2, where it was purified along with Beclin 1 and Vps34 (Liang et al., 2006). UVRAG was previously identified in xeroderma pigmentosum cells that exhibited partial UV-resistance, and it was mapped to a region of human chromosome 11 that is mutated in breast and colon cancers (Perelman et al., 1997). Like Beclin 1, UVRAG is monoallelically mutated in colon cancer cells (Ionov et al., 2004). Expression of UVRAG in HCT116 colon carcinoma cells resulted in suppression of tumorigenicity when the cells were injected into nude mice (Liang et al., 2006). Furthermore, overexpressed UVRAG induced autophagy in mouse fibroblasts, and this activity required interaction with Beclin 1 via coiled-coil domains within both proteins (Liang et al., 2006). Liang et al. also observed reduced autophagosome formation in MCF7 cells expressing wild type Beclin 1 and UVRAG lacking the coiled coil Beclin 1 binding domain, indicating that interaction between these proteins is critical for autophagy induction (Liang et al., 2006). PSI-BLAST sequence analysis later identified UVRAG as a homolog of yeast Vps38 (Itakura et al., 2008). The function of UVRAG in autophagy was thus surprising, as yeast Vps38 is not required for autophagy, but associates with the Beclin 1 complex exclusively to regulate vacuolar protein sorting (Kihara et al., 2001b).

Further characterization of UVRAG revealed a role in endocytic trafficking. Affinity purification of GST-tagged C-terminus of UVRAG from 293T cells and mass

spectrometry analysis identified two class C complex Vps proteins, Vps16 and Vps11, as UVRAG-interacting proteins (Liang et al., 2008). Originally identified in yeast, the class C Vps proteins regulate homotypic vacuole formation, protein sorting, and tethering of endosomes to the vacuole/lysosome (Nickerson et al., 2009). UVRAG colocalizes with C-Vps proteins at early endosomes and facilitates trafficking of endocytic cargo to lysosomes for degradation (Liang et al., 2008). Binding to C-Vps proteins is critical for this function, as cells expressing a UVRAG protein lacking the Vps16 binding site had displayed a decreased rate of endocytosis compared to cells expressing wild type UVRAG, measured by degradation of fluorescent EGF (Liang et al., 2008).

C-Vps proteins and UVRAG also regulate autophagic intracellular traffic. UVRAG facilitates recruitment of Vps16 to autophagosomes and promotes maturation to the late endosome/lysosome compartment. RNAi-mediated knockdown of UVRAG resulted in reduced colocalization of Vps16 with the autophagy marker LC3 in HeLa cells, while overexpression of UVRAG in HCT116 cells doubled the rate of autophagosome to lysosome fusion (Liang et al., 2008). While interaction with Beclin 1 is required for autophagosome formation, it is not required for UVRAG-induced maturation. Conversely, interaction between C-Vps proteins and UVRAG are required for autophagosome maturation, but not UVRAG-induced autophagosome formation. These results indicate that UVRAG regulates autophagy at two independent steps, and its function in the cell depends on its binding partners.

B. Atg14

Simultaneous publications reported the identification of the mammalian ortholog of Atg14 (Matsunaga et al., 2009; Sun et al., 2008; Zhong et al., 2009) Atg14 like protein (Atg14L), shares only 15% sequence identity with yeast Atg14. Taking an affinty purification approach, Zhong et al. found that Atg14L interacted with the Beclin 1/Vps34 complex *in vivo*. A complex containing Beclin 1, Vps34, Vps15, and an Atg14L was purified from mouse liver, brain, and thymus expressing green fluorescent protein (GFP)-tagged Beclin 1 in place of endogenous Beclin 1 (Zhong et al., 2009). Similar affinity purification and co-IP experiments were carried out in human cells lines and yielded similar results (Itakura et al., 2008; Matsunaga et al., 2009). Like yeast Atg14, Atg14L is required for autophagosome formation and it localizes to early autophagosomal precursors known as isolation membranes (Itakura et al., 2008).

C. Rubicon

In addition to identification of Atg14L, the experiments described above also identified a novel Beclin 1 interacting protein. Rubicon was identified by both Zhong et al. and Matsunaga et al. in biochemical pull down experiments. Both groups observed Rubicon interaction with Beclin 1, Vps34, Vps15, and UVRAG, but not with Atg14L, suggesting the presence of at least two distinct Beclin 1 complexes in mammals (Zhong et al., 2009; Matsunaga et al., 2009). Rubicon is a negative regulator of autophagy, as RNAi-mediated knockdown of Rubicon led to increased turnover of LC3 and a higher rate of autophagosome maturation, measured by quantification of lysosomal associated membrane protein (LAMP)-1-positive, GFP-LC3 positive puncta following lysosomal

inhibitor treatment (Matsunaga et al., 2009). Like UVRAG, Rubicon also regulates endosome maturation, albeit negatively. Overexpression of Rubicon prevents fusion of the endocytic machinery with the lysosome, resulting in enlarged endosomal structures with accumulated internalized EGF receptors (Matsunaga et al., 2009).

D. Proteins that transiently interact with Beclin 1

A third tumor suppressor, Bax-interacting factor 1 (Bif-1) was found to interact with the Beclin 1 complex in a study investigating the function of this Endophilin B family protein in intracellular membrane formation (Takahashi et al., 2007). Bif-1 was originally identified as a regulator of the pro-apoptotic Bcl-2 family member Bax (Cuddeback et al., 2001; Takahashi et al., 2005). Bif-1 interacts with the Beclin 1 complex via binding to UVRAG, and it localizes to autophagosomes in simian derived COS7 cells during nutrient withdrawal (Takahashi et al., 2007). Regulation of autophagy by Bif-1 is at the step of autophagosome formation, and specifically PI(3)P formation, as starvation-induced Vps34 lipid kinase activity was reduced in *Bif-1*$^{-/-}$ MEFs compared to wild type cells. Generation of knock out mice revealed a tumor suppressive function for Bif-1, as *Bif-1*$^{-/-}$ mice had an 89.7% incidence of tumor formation by 1 year, compared to 14.3% incidence for wild type mice (Takahashi et al., 2007). Despite the proposed tumor suppressive function of Bif-1 via regulation of autophagy, subsequent Beclin 1 affinity purification experiments in mouse tissues failed to identify Bif-1 as a member of the Beclin 1 complex (Zhong et al., 2009). This suggested that the interaction between Bif-1, UVRAG, and Beclin 1 described by Takahashi et al. was transient in nature and cell type/condition dependent.

A similar phenomenon was observed for the autophagy/Beclin 1 regulator 1 (Ambra 1) protein. Ambra 1 was identified in a mouse screen for regulators of nervous system development, and a yeast two-hybrid screen isolated Beclin 1 as a binding partner of Ambra 1 (Fimia et al., 2007). Co-immunoprecipitation experiments showed that Ambra 1 interacts with both Beclin 1 and Vps34 in 2FTGH cells, derived from human fibroblasts, and in developing mouse brain. RNAi-mediated knockdown of Ambra 1 expression in 2FTGH cells resulted in reduced autophagosome formation following rapamycin treatment or nutrient withdrawal, and overexpression of Ambra 1 led to an increase in basal and rapamycin-induced autophagy, in a Beclin 1 dependent manner (Fimia et al., 2007). Homozygous disruption of *Ambra 1* via gene trap led to embryonic lethality, preceded by overproliferation of cells in the neuroepithelium. Ambra 1 overexpression rescued this proliferative phenotype in a Beclin 1 dependent manner, and Ambra 1 was proposed to regulate neural tube development via autophagic control of cell proliferation. However, like Bif-1, Ambra 1 was not identified in *in vivo* affinity purification experiments (Zhong et al., 2009). This could suggest a neuronal tissue specific function for Beclin 1/Ambra 1 complexes in regulating cell proliferation during development, and does not account for the widely observed tumor suppressive function of Beclin1 in other tissues.

E. Regulation of intracellular trafficking by specific Beclin 1 complexes

Like yeast, mammals contain multiple Beclin 1 complexes, which regulate different intracellular trafficking pathways based on which proteins interact with the core complex members, Beclin 1, Vps34, and Vps15. The Beclin 1/Atg14L complex function

in autophagosome formation is conserved from yeast to mammals. A second Beclin 1 complex, containing the Vps38 homolog UVRAG but not Atg14, regulates endocytic trafficking at the stage of vesicle fusion (Liang et al., 2008). While initial experiments with UVRAG suggested that it is required for autophagosome formation, others have provided evidence that it functions exclusively in endocytosis and does not regulate autophagy (Itakura et al., 2008). Rubicon can also interact with a Beclin 1, Vps34, Vps15, and UVRAG to form a third complex. When present, Rubicon inhibits maturation of autophagosomes and endosomes. As UVRAG, like Beclin 1, is a tumor suppressor, its function in endocytosis suggests that the tumor suppressive function of this complex may not solely be attributed to autophagy.

VI. Drosophila models of overgrowth

Several *Drosophila* tumor suppressor genes have been identified (Hariharan and Bilder, 2006). Most of these genes were identified in genetic screens, and characterization of these genes has revealed diverse functions such as regulation of cell polarity and endocytosis. *Drosophila* tumor suppressors are divided into two types. Hyperplastic tumor suppressors cause over-proliferation of epithelial cells when inactivated, but these cells maintain normal shape, polarity, and the ability to differentiate. Mutations in neoplastic tumor suppressors cause loss of epithelial organization, in addition to cell over-proliferation and invasion of neighboring cells. Additionally, cells with mutations in neoplastic tumor suppressors are unable to terminally differentiate (Hariharan and Bilder, 2006).

A. Hyperplastic tumor suppressor genes

Among the hyperplastic tumor suppressor genes are the class I PI3K pathway regulators, *Tsc1*, *Tsc2*, and *pten*, all of which are also human tumor suppressor genes. As these genes negatively regulate cell growth via inhibition of PI3K pathway signaling, inactivating mutations result in increased cell growth, increased rate of cell proliferation, and increased tissue growth (Gao et al., 2000; Gao and Pan, 2001; Ito and Rubin, 1999). A second group of hyperplastic tumor suppressors function in the Hippo (Hpo) signaling pathway, which controls organ size. Hpo is a kinase, which regulates apoptosis and cell proliferation via negative regulation of the transcription factor Yorkie (Yki) (Harvey et al., 2003; Huang et al., 2005; Wu et al., 2003). When bound to its adaptor protein Salvador (Sav), Hpo phosphorylates the kinase Warts (Wts), which in turn phosphorylates Yki, preventing its binding to target genes in the nucleus (Wu et al., 2003; Dong et al., 2007). Downstream target genes of Yki include *Drosophila inhibitor of apoptosis I* (*diap1*), *cyclin E* (*cycE*), and the microRNA *bantam* (Huang et al., 2005; Nolo et al., 2006; Thompson and Cohen, 2006). Flies mosaic for mutations in *hpo*, *sav*, or *wts* display large epithelial overgrowths, which are the result of apoptosis resistance, an increased growth rate, and a corresponding increase in proliferative rate of mutant cells over wild type cells (Tapon et al., 2002; Wu et al., 2003).

B. Neoplastic tumor suppressor genes

Neoplastic tumor suppressors are subdivided into two types: junctional scaffold regulators and endocytic regulators (Hariharan and Bilder, 2006). The junctional scaffold neoplastic tumor suppressors, Lgl, Dlg, and Scrib, regulate epithelial cell polarity. Larvae

mutant for any one of these genes contain layered, disorganized epithelial cells, exhibit loss of apico-basal cell polarity, and display massive overgrowth of imaginal discs and brain (Bilder et al., 2000). Genetic epistasis analysis indicated that *lgl*, *dlg*, and *scrib* function in the same pathway, as each is required for proper localization of the other proteins (Bilder et al., 2000). Scrib and Dlg co-localize at septate junctions, which are the basal points of lateral cell-to-cell contact (Bilder and Perrimon, 2000); Bilder et al., 2000). Lgl localization is more diffuse. While some Lgl co-localizes at septate junctions with Scrib and Dlg, its overall distribution spans the entire lateral plasma membrane surface (Bilder et al., 2000). Loss of function mutations in *scrib* result in mis-localization of adherens junctions proteins to the basolateral membrane (Bilder and Perrimon, 2000). Thus, the Scrib polarity module maintains cell polarity by regulating formation and dictating localization of proteins at cell-to-cell junctions. There is also evidence that these cell polarity regulators function as negative regulators of cell proliferation. Clonal loss of *scrib* or *lgl* in the developing eye induces expression of Cyclin E and ectopic cell proliferation in larvae (Brumby and Richardson, 2003; Grzeschik et al., 2007).

The endocytic neoplastic tumor suppressors *avalanche* (*avl*), *Rab5*, *erupted* (*ept*), and *vps25* regulate different steps of endocytosis, but loss of function mutations in these genes results in accumulation of undegraded signaling molecules that regulate cell proliferation pathways. An *avl* mutant was isolated in a mosaic screen for genes that regulate epithelial morphogenesis in ovarian follicle cells (Lu and Bilder, 2005). Within the mosaic follicular epithelium, *avl* mutant follicle cells are multilayered and display altered apico-basal polarity. A similar phenotype was also observed in the eye imaginal disc epithelium, where *avl* cells also displayed mislocalization of adherens junctions and

formed tumorous overgrowths (Lu and Bilder, 2005). These phenotypes were attributed to a defect in endocytic turnover of the apical determinant Crumbs and the Notch receptor, which accumulated at the surface of *avl* mutant cells (Lu and Bilder, 2005). Mosaic loss of Rab5, which regulates formation of early endosomes, resulted in a similar phenotype in eye discs and follicle cells, and accumulation of Notch and Crumbs (Lu and Bilder, 2005).

Ept and Vps25 function at later steps in the endolysosomal degradation pathway. Ept is a subunit of ESCRT I, and Vps25 is a subunit of ESCRT II, both of which function in fusion of multivesicular bodies with lysosomes. Clonal loss of function mutations in *ept* or *vps25* result in non-autonomous cell proliferation in the eye (Moberg et al., 2005; Vaccari and Bilder, 2005). These phenotypes are associated with cell autonomous disruption of apico-basal polarity and Notch accumulation in endosomal compartments (Moberg et al., 2005; Vaccari and Bilder, 2005). In the case of *ept*, Notch accumulation in endosomes resulted in increased expression of the Janus kinase (JAK)- Signal transducer and activator of transcription (STAT) pathway ligand Unpaired (Upd), a cytokine which can induce non-autonomous proliferation of neighboring cells (Moberg et al., 2005). Furthermore, *ept* mutant cells, in the background of apoptosis inhibition, exhibit activation of the transcription factor STAT92E, which induces cell autonomous proliferation and tissue overgrowth (Gilbert et al., 2009). In *vps25* mosaic eye discs, accumulation of Notch in mutant cells led to increased Upd expression. In apoptosis competent tissue, secreted Upd signals neighboring cells to proliferate, while cell autonomous signals induce death of mutant cells (Herz et al., 2006; Thompson et al.,

2005). Like *ept* mutant cells, *vps25* mutant cells can overgrow and form epithelial tumors when apoptosis is inhibited in neighboring cells (Thompson et al., 2005).

Though *Drosophila* tumorigenesis can be induced by a single gene mutation, a phenomenon not observed in human cancers, the fly represents a good model for studying tumor suppressors nonetheless. Genetic mutagenesis screens have been essential to identifying a number of growth and proliferation regulators with functional homologs in mammals. The ability to study the cell biological effects of mutations in multiple genes in mosaic tissues provides an advantage over a knockout mouse approach.

VI. *Drosophila* autophagy genes

A. Regulation of autophagy induction by Atg1 and Atg13

Following the discovery of the *Atg* genes in yeast, several homologs were identified in other organisms, including *Drosophila*. Seventeen autophagy gene homologs have been identified in *Drosophila*, and most have been characterized as autophagy regulators in the fly (Chang and Neufeld, 2009, 2010). At the step of autophagy induction, homologs of the kinases Atg1 and Atg13 have been identified. Both Atg1 and Atg13 are required for starvation induced autophagy and development, as *Atg1* and *Atg13* mutants die prior to metamorphosis (Scott et al., 2004; Chang and Neufeld, 2009). Their function as activators of autophagy has been conserved as Atg1 overexpression is sufficient to induce autophagy in the larval fat body (Scott et al., 2007). The fact that *Drosophila* Atg1 and Atg13 constitutively interact suggests a different regulatory mechanism than in yeast, where the two proteins only interact under conditions of nutrient deprivation (Kamada et al., 2000). In yeast, phosphorylation of Atg13 by TOR

prevents interaction with Atg1 under nutrient rich conditions. TOR also negatively regulates autophagy induction in *Drosophila* via phosphorylation of Atg1 and Atg13, but Atg1 overexpression is sufficient to relieve this repression and induce autophagy under nutrient rich conditions (Chang and Neufeld, 2009; Scott et al., 2007). Furthermore, Atg1 kinase activity is required for autophagy induction in *Drosophila*, though the mechanism of induction is unknown (Chang and Neufeld, 2009).

B. Autophagosome nucleation by Atg6, Vps34, and *Vps15*

Early autophagosome formation/ vesicle nucleation is regulated by the Atg6 complex in yeast. The core members of this complex, Atg6, Vps34, and Vps15, are conserved in *Drosophila*. *Drosophila* Atg6 shares 20% amino acid identity with yeast Atg6 and 49% overall identity, including 71% identity within the evolutionarily conserved domain, with human Beclin 1. Vps34 shares 30% amino acid identity with yeast Vps34 and 53% identity with human Vps34. Finally, *Drosophila* Vps15, also known as immune response deficient 1 (Ird1), shares 22% identity with yeast Vps15 and 40% identity with human Vps15. Sequence analysis has also identified putative homologs of UVRAG, Rubicon, and Atg14.

C. Atg conjugation pathways

The Atg conjugation pathway regulators, which function in autophagosome membrane expansion, are the most well characterized *Drosophila* autophagy genes. RNAi-mediated knockdown of *Atg5*, *Atg7*, and *Atg12* in the larval fat body revealed a defect in starvation-induced autophagy, indicating functional conservation of these genes

in *Drosophila* (Scott et al., 2004). Moreover, sub-cellular localization experiments with epitope tagged proteins revealed co-localization of Atg5 with human LC3 and *Drosophila* Atg8, and of LC3/Atg8 with lysosomes, further indicating conservation of cellular function (Rusten et al., 2004; Scott et al., 2004). Loss of function mutants of *Atg7*, the E1-like protein that activates Atg12 for conjugation to Atg5 and Atg8 for conjugation to PE, have a defect in starvation-induced autophagy in the fat body and developmental autophagy in the midgut (Juhász et al., 2007). However, unlike *Atg1* mutants, *Atg7* mutants survive to adulthood. These animals are short lived and sensitive to oxidative stress (Juhász et al., 2007). These results suggest that autophagy is not absolutely necessary during development and other developmentally lethal *Atg* mutations may affect other processes in the organism.

The Atg8 conjugation pathway is also conserved in *Drosophila*. Two *Atg8* homologs, *Atg8a* and *Atg8b*, exist in *Drosophila*, and both localize to autophagosomes (Scott et al., 2007). Because of the availability of mutants, most autophagy studies have focused on *Atg8a.* Like *Atg7* mutants, *Atg8a* mutants have autophagy defects during starvation and development, but survive to adulthood (Berry and Baehrecke, 2007; Scott et al., 2007). Tissue specific RNAi experiments and mutant analysis showed that Atg3, whose yeast homolog is an E2 like protein that participates in conjugation of Atg8 to PE, is required for autophagy in *Drosophila*, as knockdown of Atg3 resulted in impairment of starvation-induced autophagy (Juhász et al., 2003; Scott et al., 2007).

When this study was initiated, few *Drosophila* autophagy genes had been functionally characterized, due to the lack of mutant alleles for many of the genes. There were no existing mutants for any of the Atg6 complex components. Because of the

function of mammalian Beclin 1 in tumor suppression, functional characterization of this pathway in the context of a whole organism is essential to understanding the complex genetic interactions with other pathways in the cell. As a genetically tractable organism that provides the ability to observe mutant cells next to wild type cells in the same tissue, *Drosophila melanogaster* is an excellent system for investigating the complex Beclin 1 functions in a physiological context.

Chapter 2

Induction of autophagy by the Vps34 complex in *Drosophila*

Abstract

The phosphatidylinositol (3)-kinase Vps34 has been implicated in vacuolar protein sorting and autophagy in the budding yeast *Saccharomyces cerevisiae*. Vps34 functions in these processes by generating PI(3)P, a phospholipid that recruits proteins to assemble at nascent intracellular membrane structures. Vps34 is present in at least 2 distinct complexes in yeast, where different binding partners dictate its function in autophagy or vacuolar protein sorting. In addition to Vps34, all known Vps34 core complexes are composed of the serine-threonine kinase Vps15 and autophagy protein 6 (Atg6). The binding of either Vps38 or Atg16 to this core complex specifies function in either vacuolar protein sorting or autophagy. The *Drosophila melanogaster* genome encodes orthologs of Vps34, Vps15 and Atg6, but their functions in intracellular signaling pathways were previously uncharacterized. Here we describe functional characterization of the Vps34 complex in *Drosophila*, where biochemical and cellular analyses show that these proteins form a complex *in vivo* and regulate autophagosome formation.

Introduction

The process of autophagy is initiated by formation of an isolation membrane, or phagophore, the source of which is unclear (Kirisako et al., 1999). Some studies have

suggested that this structure forms de novo in the cytoplasm, while others have concluded that nucleation occurs from membranes of existing organelles, such as the endoplasmic reticulum, Golgi, or mitochondria (Axe et al., 2008; Hailey et al., 2010; Hayashi-Nishino et al., 2009; van der Vaart et al., 2010). Recent data even suggest that the plasma membrane is involved in autophagosome biogenesis (Ravikumar et al., 2010). Phagophore formation is followed by double membrane expansion and sequestration of cytoplasmic contents to be degraded (Hamasaki and Yoshimori, 2010). Upon completion of vesicle formation, the outer membrane of the autophagosome fuses with the lysosomal membrane, depositing the inner autophagosomal membrane and engulfed contents into the lysosome for degradation.

The regulators of each step of autophagy were initially identified in genetic screens in yeast (Klionsky et al., 2003). Upstream of autophagosome formation, a complex consisting of Atg1 and Atg13 is required for autophagy induction (Kamada et al., 2000). This complex is negatively regulated by TOR phosphorylation of Atg13 in the presence of nutrients (Kamada et al., 2010). In yeast, Atg1 and Atg13 interact only under starvation conditions, but this interaction is constitutive in *Drosophila* (Chang and Neufeld, 2009). In response to starvation, TOR is inactivated, leading to activation of Atg1, which phosphorylates Atg13. Atg1 kinase activity is required for autophagy induction in yeast and flies (Matsuura et al., 1997). However, the biochemical mechanism of Atg1-Atg13 complex activity on autophagosome formation is unknown.

A second complex, containing Vps34, Vps15, and Atg6, is required for autophagosome formation. This complex was initially identified in a yeast screen for mutants in vacuolar protein sorting and was later isolated in another screen for mutants

defective in autophagosome formation (Bankaitis et al., 1986; Tsukada and Ohsumi, 1993). Through phosphorylation of phosphatidylinositol (PI) to generate PI(3)P, Vps34 activity regulates intracellular membrane formation, and this function is dependent on its serine-threonine kinase cofactor, Vps15 (Stack et al., 1993). PI(3)P is critical to vesicular trafficking, as it recruits proteins containing FYVE (conserved in Fab1, YOTB, Vac1, and EEA1) and Phox homology (PX) domains to nascent membranes (Ellson et al., 2002; Stenmark et al., 2002). These membranes include autophagosome isolation membranes, which serve as precursors to double membrane autophagosomes prior to membrane expansion (Kirisako et al., 1999; (Simonsen et al., 2004). In yeast, Vps34 and Vps15 form a stable complex with Atg6. This complex is required for autophagy and trafficking of hydrolases to the vacuole, the functional equivalent of the lysosome in animal cells. Specificity of function is conferred by alternative binding partners. When bound to Atg14, the Vps34 complex regulates starvation-induced autophagy, while binding to Vps38 dictates function in vacuolar protein sorting (Kihara et al., 2001a). While studies in yeast have been critical to elucidating the details of genetic regulation of autophagy, a genetic and biochemical approach in a multicellular organism is essential to understanding autophagy regulation in a physiological context, as this will enhance our understanding of the role of this complex in diseases.

Drosophila melanogaster is an excellent system to carry out genetic experiments investigating the function of the Vps34 complex in a developing animal model. Autophagy is induced in the larval fat body in response to starvation, and also contributes to histolysis of the larval fat body, midgut, and salivary glands during metamorphosis (Scott et al., 2004; Rusten et al., 2004; Lee and Baehrecke, 2001; Lee et al., 2002). To

determine the function of the Vps34 complex *in vivo*, we generated transgenic flies that ectopically express Vps34, Vps15, and Atg6. Utilizing both genetic and biochemical approaches, we found that these proteins physically interact, and the function of this complex in autophagy is conserved in *Drosophila*. Furthermore, in addition to regulating autophagosome formation, Vps34 was also found to regulate endocytosis.

Results

Co-expression of Vps34 complex components induces autophagy in the larval fat body

The fat body is the *Drosophila* equivalent of the mammalian liver, which functions in metabolism and nutrient storage (Canavoso et al., 2001). Prior to metamorphosis, *Drosophila* develop through three larval instars. The first, second, and early third instars are characterized by periods of feeding, which provide the developing animal with energy to support growth. Further, nutrient stores are built during the larval instars, and this helps to sustain the animal through the non-feeding stages of metamorphosis. If a larva is deprived of nutrients, the cells of the fat body undergo autophagy, promoting survival until restoration of nutrients (Scott et al., 2004). To test whether the Vps34 complex was sufficient to induce autophagy independent of nutrient availability, Vps34, Vps15 and Atg6 were ectopically expressed in the fat body using the Gal4 upstream activating sequence (UAS) expression system (Brand and Perrimon, 1993).

Several transgenic lines were generated to enable ectopic expression of wild type and epitope tagged Atg6 and Vps34 in a tissue specific manner. Among the constructed

lines are 19 different UAS-Vps34 lines and 54 UAS-Atg6 lines. Included in those lines are 9 N-terminal and 10 C-terminal TAP-tagged UAS-Vps34 lines, 9 N-terminal and 10 C-terminal GFP-tagged UAS-Atg6 lines, and 9 N-terminal and 10 C-terminal Myc-tagged UAS-Atg6 lines (Fig. 2-1). I have also obtained untagged and FLAG-tagged UAS-Vps15 transgenic flies (provided by L.Wu).

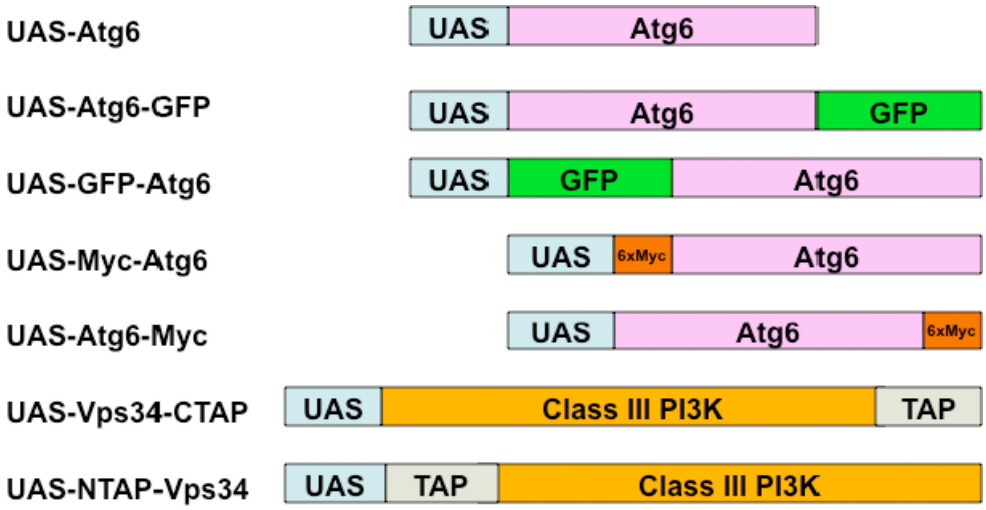

Figure 2-1: Transgenic constructs for cellular and biochemical characterization of the Vps34 complex. *Atg6* cDNA was cloned into the vector pUAST for ectopic expression of the wild type proteins. *Atg6* cDNA was cloned into the Gateway vectors pTGW (N-term GFP), pTWG (C-term GFP), pTMW (N-term 6xMyc), and pTWM (C-term 6xMyc), which allowed Gal4-driven tissue specific expression of epitope tagged Atg6. *Vps34* cDNA was cloned into pUAST-NTAP and pUAST-CTAP, which provide a tandem affinity purifcation tag. All plasmid constructs were utilized in P-element mediated transformation, to generate transgenic flies.

Autophagosome formation was monitored by GFP-Atg8a localization. Atg8a is conjugated to phosphatidylethanolamine (PE) and localizes to the autophagosome membrane through fusion with the lysosome (Ichimura et al., 2000). Under nutrient rich conditions, Atg8a localization is diffuse and cytoplasmic. Upon autophagy induction, Atg8a localizes in puncta, which mark autophagosomes (Kirisako et al., 1999). Fat body autophagosome number increased in control larvae, which expressed heat shock inducible GFP-Atg8, from an average of 13 GFP-positive puncta per field under nutrient rich conditions to an average of 58 puncta per field following 4 hours of starvation (Fig. 2-2A, E, M). When either Atg6, Vps34, or Vps15 was individually over-expressed in the fat body of feeding larvae, via the larval serum protein 2 (*Lsp2*)-Gal4 driver, single transgene expression was not sufficient for significant induction of autophagy (Fig. 2-2A-H, M). However, co-expression of either Atg6 and Vps34 or Vps15 and Vps34 resulted in a significant increase in GFP-Atg8a puncta formation in feeding larvae (Fig. 2-2I, J, M).

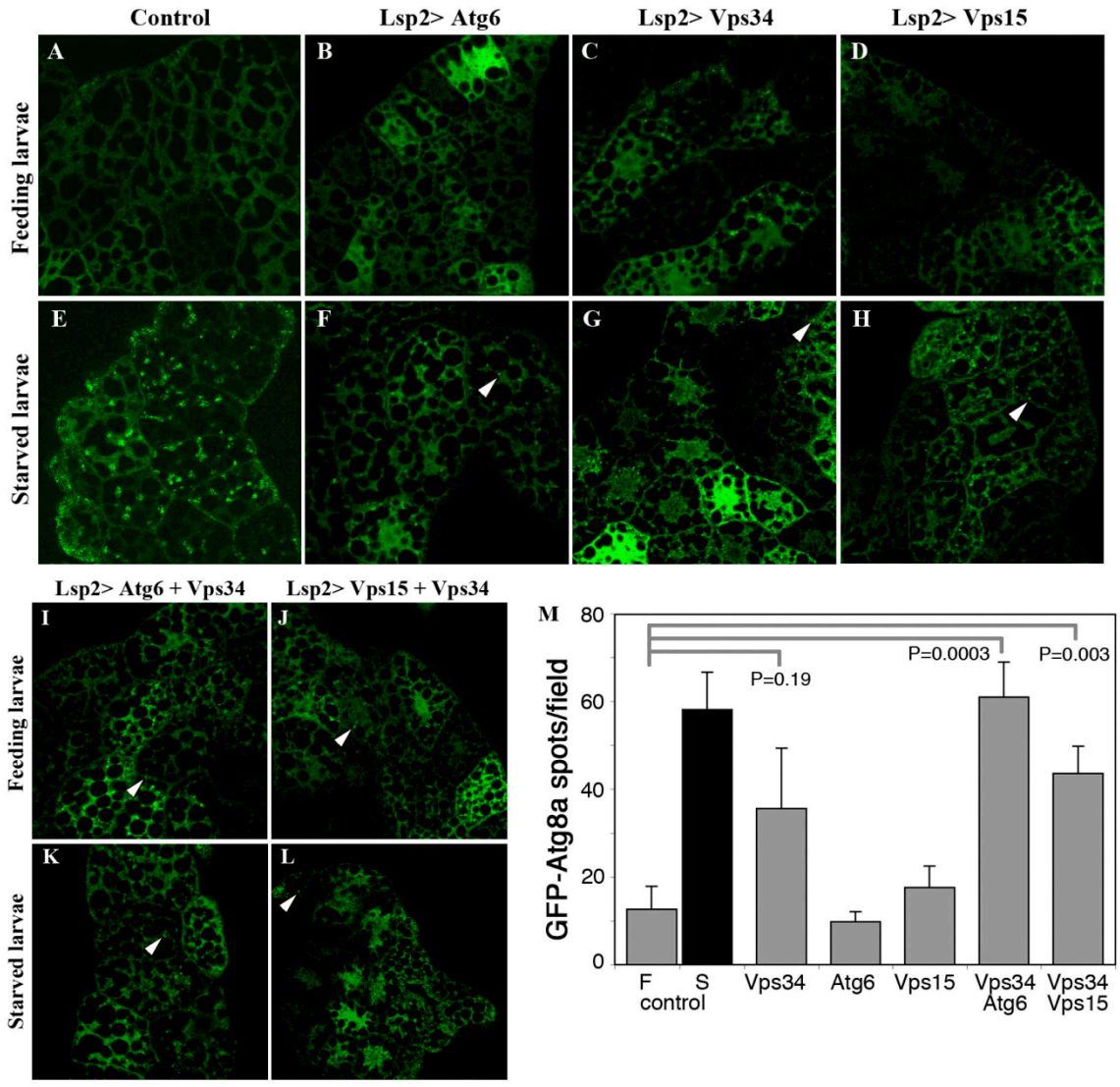

Figure 2-2. Ectopic co-expression of Vps34 complex members induces autophagosome formation in the larval fat body. Animals were raised at 25°C, then third instar larvae were shifted to 37°C for 1 hour, followed by a 3-hour recovery incubation at 25°C. Larvae were subjected to 4 hours of starvation, including the 1 hour heat shock period. Following incubation, the fat body was dissected and mounted in Slowfade Gold reagent (Invitrogen) for imaging with a Zeiss LSM confocal microscope. (**A-L)** Third instar larval fat body expressing heat shock driven GFP-Atg8a. (**M)** Quantification of GFP-Atg8 puncta in feeding larvae compared to control feeding and starved larvae. GFP-positive puncta were counted in 3 fields of view per fat body from 10 animals and the average number of spots per field per animal was calculated. Statistical significance was determined by a paired t-test, and bars represent standard error. For control samples, "F" denotes feeding larvae and "S" denotes starved larvae. **Genotypes:** (A, E) *Hs-GFP-*

Atg8a/+; Lsp2-Gal4/+ (B, F) *w; UAS-Atg6/Hs-GFP-Atg8a; Lsp2-Gal4/+*, (C, G) *w; Hs-GFP-Atg8a/+; Lsp2-Gal4/UAS-Vps34-TAP*, (D, H) *w; Hs-GFP-Atg8a/UAS-Vps15; Lsp2-Gal4/+*, (I, K) *w; UAS-Atg6/Hs-GFP-Atg8a; UAS-Vps34-TAP/Lsp2-Gal4*, (J, L) *w; UAS-Vps15/Hs-GFP-Atg8a; UAS-Vps34/Lsp2-Gal4*.

Next, we utilized the acidomotropic dye, Lysotracker Red, to monitor formation of acidic structures in the fat body, including multivesicular bodies and lysosomes. Consistent with GFP-Atg8a experiments, Lysotracker positive puncta appeared throughout the fat body of both feeding and starved larvae co-expressing either Vps34 and Atg6 or Vps34 and Vps15, but not in animals expressing either Vps34, Atg6, or Vps15 individually (Fig. 2-3). Taken together, the GFP-Atg8a and Lysotracker results indicate that Vps34 complex function in autophagy is conserved in *Drosophila*, and these proteins are capable of inducing autophagy in non-starved conditions when co-expressed together.

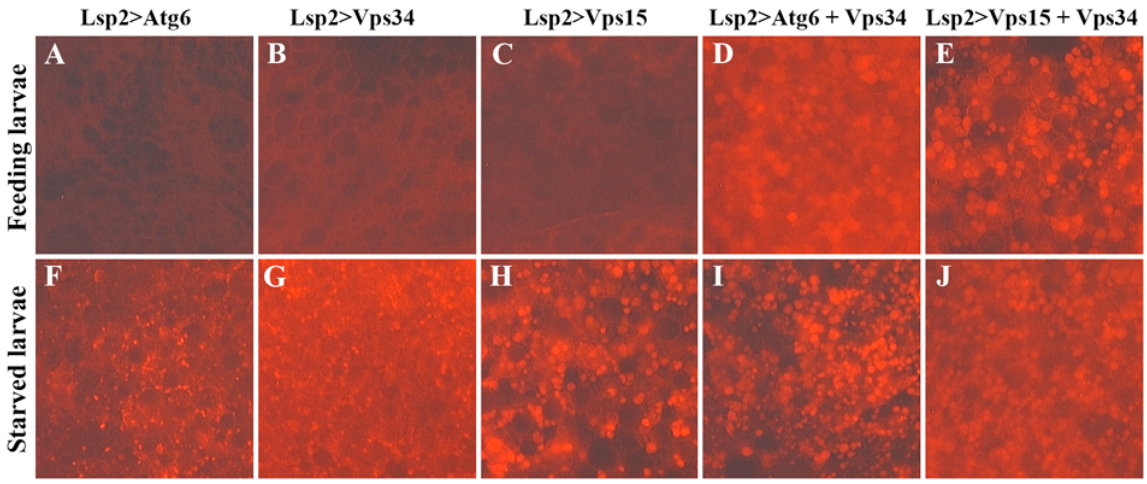

Figure 2-3. Co-expression of Vps34 complex members induces autophagy in the larval fat body. Lysotracker Red staining, which labels acidic structures including autolysosomes, of fat body from third instar larvae. Fat body was dissected from feeding or starved larvae, expressing Vps34 complex transgenes driven by Lsp2-Gal4, and stained with Lysotracker Red. Tissue was mounted in phosphate buffered saline and imaged with the 40X objective of a Zeiss Axiophot. **Genotypes:** (A, F) *w; UAS-Atg6; Lsp2-Gal4*, (B, G) *w; UAS-Vps34-TAP/+; Lsp2-Gal4/+*, (C, H) *w; UAS-Vps15; Lsp2-Gal4*, (D, I) *w; UAS-Atg6/UAS-Vps34-TAP; Lsp2-Gal4/+*, (E, J) *w; UAS-Vps15/UAS-Vps34-TAP; Lsp2-Gal4/+*.

Atg6 redistributes to punctate structures in the fat body following starvation

Studies in mammalian cells have shown that Beclin1/Atg6 localizes to the endoplasmic reticulum and *trans*-Golgi network (TGN) (Kihara et al., 2001a). While Vps34 is co-localized with Atg6 in the ER and TGN, it also localizes to late endosomes independently of Atg6 (Kihara et al., 2001a). Transgenic UAS-Atg6-GFP-flies were generated for protein localization experiments (Fig. 2-1). It is important to note that the UAS-Atg6-GFP transgene rescues *Atg6* null mutants (Chapter 3), indicating that this protein functions *in vivo*. Atg6-GFP localization was diffuse in the fat body of feeding larvae, consistent with low basal autophagy levels in fed animals (Fig 2-4A). Following starvation, Atg6-GFP localized in puncta of variable sizes in the fat body, and most of these puncta were exclusive of the acidic Lysotracker-stained structures whose formation was induced by starvation (Fig. 2-4F). The redistribution of Atg6-GFP into punctate, non-lysosomal structures following starvation suggests that it localizes to autophagosomes prior to fusion with the lysosome.

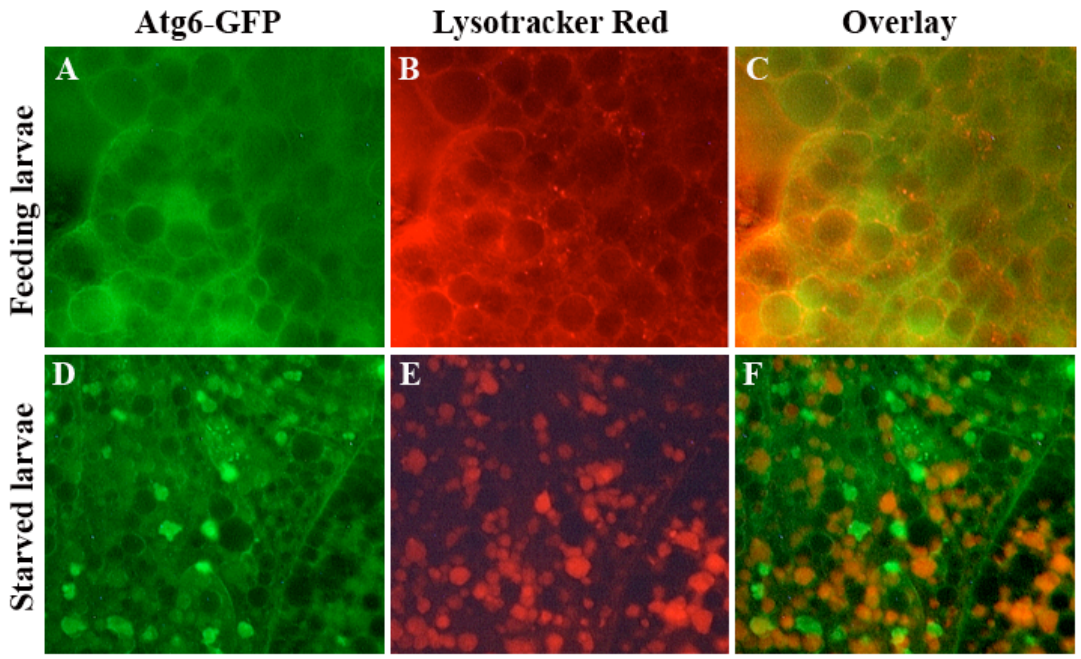

Figure 2-4. Atg6 redistributes to punctate structures following starvation. Lysotracker Red staining of fat body from feeding or starved (4h) larvae expressing Atg6-GFP. Following starvation, fat body was dissected, mounted in phosphate buffered saline, and imaged with the 40X objective of a Zeiss Axiophot. **Genotype:** *w; UAS-Atg6-GFP/+; Lsp2-Gal4/+*.

Vps34 binds to Atg6 and Vps15 *in vivo*

A biochemical approach was taken to determine whether Vps34, Atg6, and Vps15 physically interact in a complex in *Drosophila*. Due to the lack of antibodies that recognize these proteins in *Drosophila*, epitope tags were utilized to isolate these proteins from cell lysates (Fig. 2-1). Tandem affinity purification (TAP)-tagged Vps34 was co-expressed with FLAG-tagged Vps15 and Myc-tagged Atg6 in larvae. Transgenes were driven via the UAS/Gal4 system, using heat shock (hs)-Gal4 to drive ubiquitous expression. Utilizing an antibody against human Vps34, which recognizes the *Drosophila* protein, Vps34 complexes were immunoprecipitated from whole animal protein extracts, collected from feeding and starved larvae. Myc-Atg6 co-immunoprecipitated with both endogenous Vps34 and Vps34-TAP, and this interaction was independent of nutrient availability (Fig. 2-5A). Furthermore, Vps15-FLAG interacted with Vps34-TAP under starved conditions, but not in well-fed animals (Fig. 2-5B). By contrast, Vps15-FLAG did not co-immunoprecipitate with Myc-Atg6 in either condition (Fig. 2-5B). Vps15-FLAG levels appeared very low and could only be detected when immunoprecipitated with Vps34 under starvation conditions (Fig 2-5B). These results suggest that Vps34 and Atg6 constitutively interact, while Vps15 interacts with this complex via Vps34 in a nutrient dependent manner.

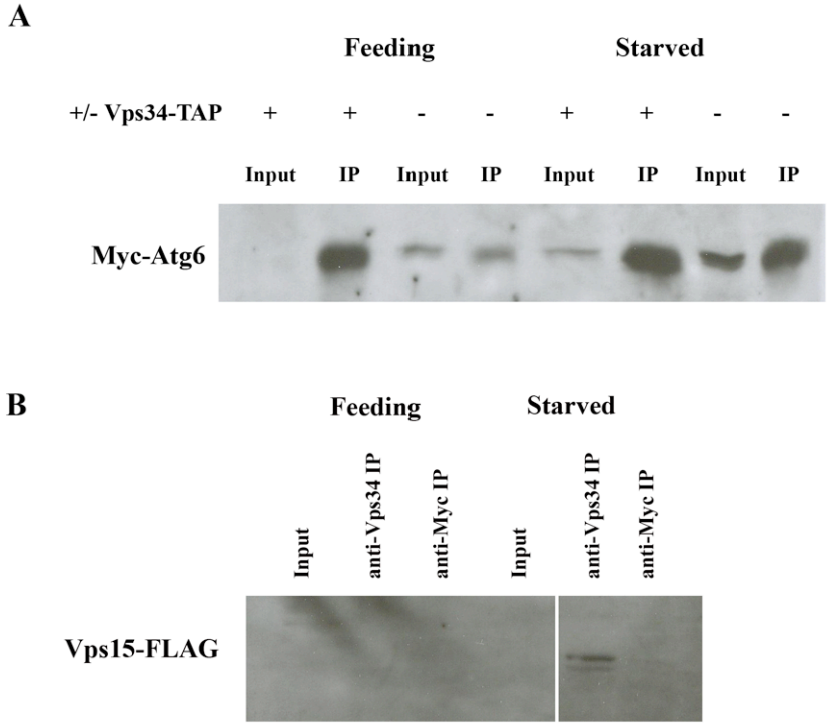

Figure 2-5. Vps34 interacts biochemically with Atg6 and Vps15 *in vivo*. Vps34, Atg6, and Vps15 were epitope tagged and ectopically expressed via the UAS/GAL4 system. UAS-transgenes were induced ubiquitously by heat shock (hs)-Gal4. Larvae were heat shocked for one hour at 37°C, followed by a 3 hour incubation at 25°C. During this 4-hour time course, larvae were raised in the presence or absence of food. **(A)** Co-immunoprecipitation of Myc-Atg6 with Vps34. Vps34 was immunoprecipitated with an antibody raised against human Vps34, in feeding or starved third instar larvae co-expressing Myc-Atg6 and Vps15-FLAG in the presence or absence of Vps34-TAP. Atg6 was detected via Western blot with an α-Myc antibody. **(B)** CoIP of Vps15 with Vps34-TAP or Myc-Atg6. Vps34 was immunoprecipitated with an antibody raised against human Vps34, and Myc-Atg6 was immunoprecipitated with an α-c-Myc antibody, in feeding or starved third instar larvae co-expressing Vps34-TAP, Myc-Atg6, and Vps15-FLAG. Vps15-FLAG was detected with an α-FLAG antibody. **Genotypes:** (A) "+Vps34-TAP" *w; UAS-Myc-Atg6/UAS-Vps15-FLAG; UAS-Vps34-TAP/Hs-Gal4*, "-Vps34-TAP" *w; UAS-Myc-Atg6/UAS-Vps15-FLAG; Hs-Gal4/+*, (B) *w; UAS-Myc-Atg6/UAS-Vps15-FLAG; UAS-Vps34-TAP/Hs-Gal4.*

Discussion

While the genes regulating autophagy have been known for more than a decade, the functions of these genes in other pathways are yet to be characterized in multicellular eukaryotes. In particular, the Vps34 complex functions in the CVT pathway, vacuolar protein sorting, and autophagy in yeast, and its specificity of function depends on which proteins are bound to the core components. The function of the *Drosophila* Vps34 complex was previously uncharacterized. Although *Vps15* was identified in a screen for regulators of NFκB signaling in response to bacterial infection, its function in the Vps34 complex was not studied (Wu et al., 2007).

We investigated the functions of Vps34, Atg6, and Vps15 in autophagy using a genetic approach. Over-expression of either Vps34, Atg6, or Vps15 alone was not sufficient to induce autophagy in the fat body of fed third instar larvae. This suggests that a stoichiometric ratio of these proteins is required for autophagosome formation. It is possible that over-expressed Vps34 is unable to generate additional PI(3)P in the absence of ectopic Vps15 activity. Similarly, PI(3)P may be limiting when Atg6 or Vps15 are individually over-expressed in the fat body of fed larvae containing endogenous levels of Vps34. Co-expression of either Vps34 with Atg6 or Vps34 with Vps15 induced high levels of autophagy in feeding larvae, as visualized by GFP-Atg8a localization and lysotracker staining (Fig. 2-2 and 2-3). Due to Vps34 over-expression, one would expect higher levels of PI(3)P, and the presence of over-expressed Atg6 or Vps15, in addition to Vps34, may be sufficient for recruitment of other proteins to the nascent autophagosome. I did not test whether co-expression of Vps15 and Atg6 was sufficient for autophagy induction in fed larvae. However, this experiment, in addition to mutant analysis, may

allow us to determine whether PI(3)P is limiting for ectopic autophagy induction. Furthermore, we confirmed that Vps34 and Atg6 constitutively interact *in vivo*, while Vps15 binds only to Vps34 under starvation conditions (Fig. 2-5). Constitutive interaction of Vps34 with Atg6 suggests a possible role for these proteins in cellular processes other than autophagy.

In support of a non-autophagic function for the Vps34 complex, our collaborator found that Vps34 is required for both autophagy and endocytosis in *Drosophila* (Juhász et al., 2008). Functional Vps34 generates PI(3)P, which recruits FYVE-domain containing proteins to intracellular membranes. Clonal loss of *Vps34* blocked GFP-FYVE perinuclear puncta formation in the larval fat body. These puncta were identified as early endosomes due to their co-localization with Rab5. Starvation of larvae resulted in redistribution of FYVE to cytoplasmic puncta that co-localized with GFP-Atg8a, indicating that PI(3)P is involved in formation of both autophagosomes and endosomes (Juhász et al., 2008). Further, the function of Vps34 in endocytosis was confirmed by observing endocytic uptake of a Texas Red (TR)-avidin conjugate by Garland cells, which are highly endocytic nephrocytic cells. *Vps34* mutant cells failed to endocytose the TR-avidin tracer (Juhász et al., 2008).

Endocytosis regulators have previously been shown to function in autophagy. Specifically, subunits of the endosomal sorting complex required for transport (ESCRT) pathway are required for autophagy in *Drosophila* (Rusten et al., 2007). The ESCRTs regulate formation of multivesicular bodies and sorting of endocytosed proteins to the lysosome for degradation. There are three ESCRT complexes, and all are required for autophagy, as cells mutant for *vps22* (ESCRT I), *vps25* (ESCRT II), or *vps32* (ESCRT

III) accumulate un-degraded GFP-Atg8a puncta in the fat body (Rusten et al., 2007). This phenomenon occurs in both starved and fed larvae, and is due to a defect in fusion of autophagosomes with the endolysosomal machinery (Rusten et al., 2007). To test whether *Vps34* function in autophagy was related to its function in endocytosis, our collaborators generated flies with mutations in both *Vps34* and the ESCRT II subunit *vps28*. While GFP-Atg8a puncta accumulated in *Vps28* mutant larval fat body cells, they never formed in *Vps34* mutant cells (Juhász et al., 2008). Like *vps28* mutant cells, *vps28, Vps34* double mutant cells accumulated autophagosomes in fed and starved larvae, albeit at much lower levels than *vps28* single mutant cells (Juhász et al., 2008). These results indicated that the function of Vps34 in autophagy, at the step of autophagosome formation, was distinct from its role in endocytic trafficking. Further, though autophagosomes fuse with the endocytic machinery, Vps34 does not appear to be a rate-limiting factor in this event.

Significant questions remain about differential regulation of intracellular trafficking pathways by the Vps34 complex. While Vps15 and Atg6 are required for autophagy and vacuolar protein sorting in yeast, early studies of the Vps34 complex in mammalian cells suggested that Beclin 1/Atg6 did not participate in endocytic trafficking (Furuya et al., 2005; Zeng et al., 2006). This was supported by cellular localization experiments, which indicated that only 50% of intracellular Vps34 interacted with Atg6 at the trans-Golgi network, while the other 50% was localized to late endosomes (Kihara et al., 2001a). Despite this early model for Beclin 1-independent regulation of endocytosis by Vps34, the identification of a Vps38 homolog, UVRAG, as a Beclin 1-interacting protein in mammals, suggested that Beclin 1 functions in endocytosis after all (Liang et al., 2008). Thoresen et al. recently provided additional data in support of Beclin

1 function in endocytic trafficking (Thoresen et al., 2010). Using an RNAi-mediated knockdown approach in mammalian cells, the authors found that the core Vps34 complex, composed of Vps34, Vps15, and Beclin 1, was required for endocytosis of ligand-bound EGFR. The Beclin 1-interacting proteins UVRAG and Bif-1 were also required for EGFR endocytosis and degradation, but the autophagy specific protein Atg14L was not (Thoresen et al., 2010).

Experiments in mammalian cell lines have so far provided contradictory models of Vps34 complex regulation of endocytic trafficking. In order to advance our understanding of complex function *in vivo*, these proteins must be investigated in whole animal models. Research on the Beclin 1-interacting tumor suppressor protein UVRAG indicated that the function of this complex in endocytosis might contribute to tumor suppression (Liang et al., 2008). *Drosophila melanogaster* is an ideal animal model for investigating the cellular functions of the Vps34 complex. While the aforementioned experiments indicated functional conservation of Vps34 in *Drosophila*, full functional charcterization of its binding partner, Atg6, is necessary to fully understand now autophagy and endocytosis function together in the cell. By generating a mutant in the Beclin 1 ortholog Atg6, we can utilize elegant genetic tools, such as the ability to make tissue mosaics, to study both autophagy and endocytosis in the context of a developing animal.

Acknowledgements

We thank L.P. Wu, T.P. Neufeld and the Bloomington Stock Center for transgenic *Drosophila* lines, Alexey Veraksa for the UAS-CTAP and UAS-NTAP plasmids, the

Drosophila Genomics Resource Center (DGRC) for the pTMW, pTWM, pTGW, and pTWG Gateway expression vectors, and J.M. Backer for the Vps34 antibody.

Materials and Methods

Generation of Transgenic Flies

UAS-Atg6: Atg6 cDNA (CG5429) was cloned from the pOT2 vector (LD35669) into pUAST, using XhoI and EcoRI restriction sites. The resultant construct, pUAST-Atg6 was sent to Duke University Model Systems Genomics for P-element transformation into *w1118*.

Myc-Atg6: Atg6 cDNA was cloned into pENTR/D-TOPO (Invitrogen) using the primers 5'CACCATGAGTGAGGCGGAA 3' and 5' TCACGGTGACACAAACTGTG 3'. Atg6 cDNA was then recombined into the *Drosophila* Gateway vector pTMW using LR clonase (Invitrogen). The resultant construct, pTMW-Atg6 was sent to Best Gene, Inc. for P-element transformation into *w1118*.

Atg6-GFP: Atg6 cDNA was cloned into pENTR/D-TOPO (Invitrogen) using the primers 5'CACCATGAGTGAGGCGGAA 3' and 5'CGGTGACACAAACTGTGAAG 3'. Atg6 cDNA was then recombined into the *Drosophila* Gateway vector pTWG using LR clonase (Invitrogen). The resultant construct, pTWG-Atg6 was sent to Best Gene, Inc. for P-element transformation into *w1118*.

PI3K-TAP: TOPO-TA cloning was used to clone the PI3K59F cDNA (CG5373), using the primers 5' GGGGTACCCCAAAAATGGACC 3' (introduce 5' KpnI site) and 5'CTAGGATTACTCGCTCCTCC 3' for N-terminal fusion and 5'GCAGATCTGATATCATCGCC 3' and 5'GGGTACCCTTCCGCCAGTAT

(introduce 3' KpnI site) for C-terminal fusion. To generate N-terminal UAS-TAP-PI3K, PI3K59F was cloned from TOPO-PI3K(N) into pUAST-NTAP (from A.Veraksa) using the KpnI and XbaI restriction sites. C-terminal UAS-PI3K-TAP was generated by cloning PI3K59F from TOPO-PI3K(C) using the EcoRI and KpnI restriction sites. pUAST-NTAP-PI3K and pUAST-PI3K-CTAP were sent to Best Gene, Inc. for P-element transformation into *w1118*.

GFPAtg8 Quantification

Crosses were carried out at 25°C and adults were removed from vials 24h after cross was set up. Larvae were heat shocked for 1h at 37°C, then incubated at 25°C for 3 hours under feeding or starvation conditions. For starvation experiments, incubations were done in a dish with wet tissue paper. For feeding experiments, feeding larvae were scooped, along with food, into a dish for incubations. Larval fat body was dissected in 1X phosphate buffered saline, 3 hours after completion of heat shock, and mounted in *SlowFade Gold* antifade reagent (Invitrogen). Fat body samples were imaged with a Zeiss Confocal Laser Scanning Microscope using a 40X objective. For each animal, 3 fields per fat body were imaged and punctate GFP spots were counted. Quantification represents average number of punctate GFP positive spots per field per animal (n=10).

Lysotracker staining

Fat body was dissected from larvae in 1X PBS, and incubated with Lysotracker Red (Invitrogen), diluted 1:10,000 in 1X PBS, for 2 minutes at room temperature. Tissue was rinsed with 1X PBS, then mounted for immediate imaging with a Zeiss Axiophot.

Immunoprecipitation and Western Blot

(Heat shock/starvation conditions same as for GFPAtg8 analysis) Larvae were homogenized in lysis buffer [50mM HEPES, pH 7.4, 150mM KCl, 6.5% glycerol, 0.5mM DTT, 0.1% Triton X-100, complete protease inhibitor tablet (Roche)] and incubated on ice for 15 minutes. Samples were spun at 10,000 x g for 10 minutes at 4°C to collect protein, which was stored at -80°C. For each IP, 300µg of protein was precleared with 50uL of Protein A sepharose 4B 50% slurry (Zymed) on ice for 1h. Precleared lysate was incubated with 2µg of antibody at 4°C for 1h [mouse α-c-Myc (9e10): sc-40 Santa Cruz Biotechnology, mouse α-FLAG M2 (Sigma), rabbit α-hVps34 (Jonathan Backer)]. 50ul of Protein A sepharose was added, followed by an additional 1h incubation at 4°C. Beads were collected by centrifugation (10,000 x g) for 30 seconds, washed with lysis buffer, and resuspended in 25ul of Laemmli buffer. Antigen-antibody complexes were released from beads with a 10 minute incubation at 95°C, followed by centrifugation at 10,000 x g for 5 minutes at 4°C. Protein was separated by SDS-PAGE on a 7.5% acrylamide gel and transferred to an Immobilon-P PVDF membrane (Millipore). Membranes were blocked in 3% milk/Tris-buffered saline + 0.1% Triton-X 100 (TBST) for 1h at room temperature and washed 3 x 10 minutes in TBST. Blots were incubated with primary antibody for 1h at room temperature, followed by 3 10-minute washes in TBST [α-c-Myc 1:200 in 2% BSA/TBST, α-FLAG M2 1:2000 in 3% milk/TBST, α-hVps34 1:1000 in 3% milk/TBST]. Blots were incubated in goat α-mouse-HRP or goat α-rabbit-HRP secondary antibody, diluted 1:2000 in 3% milk/TBST, for 1h at room temperature. Blots were washed in TBST, then incubated with ECL reagent (Amersham) and exposed with Hyperfilm ECL (Amersham).

Chapter 3

Loss of Atg6 causes defects in endocytosis and autophagy, and leads to cell overgrowth

Abstract

Beclin 1, the mammalian homolog of the autophagy related protein 6 (Atg6), functions in autophagy and endosomal trafficking. Mutations in *beclin 1* are associated with sporadic breast, ovarian, and prostate cancers. These pathologies have been attributed to a defect in autophagy that leads to protein stress, p62 accumulation, activation of NFκB, accumulation of reactive oxygen species, and genome instability. In order to study the physiological defects and cellular phenotypes associated with *beclin 1* loss, we generated an *Atg6* null mutation in *Drosophila*. This model system allowed us to characterize the function of *Atg6* in the context of a developing animal, and use genetic approaches to identify interactions with other signaling pathways. We found that *Atg6* is required during development, as mutant larvae died at the end of larval development with excess blood cells. Furthermore, mosaic analyses in epithelial tissues showed that *Atg6* mutant cells displayed a loss of cell polarity and invaded neighboring wild type cells. These defects appear to be independent of autophagy and the lipid kinase Vps34, as cells mutant for autophagy gene *Atg1* or *Vps34* did not display similar phenotypes.

Introduction

Autophagy is a catabolic process that sequesters proteins and organelles into double-membraned autophagosomes for delivery to the lysosome, and has been linked to

cancer (Kisen et al., 1993). In particular, the mammalian tumor suppressor Beclin 1 functions in autophagy (Liang et al., 1999). Monallelic loss of *beclin 1* in mice resulted in increased spontaneous tumorigenesis compared to animals with two functional copies of *beclin 1* (Qu et al., 2003; Yue et al., 2003). *beclin 1*$^{+/-}$ mice were affected by lymphomas, lung carcinomas, and liver carcinomas. Initial studies suggested that the autophagy-promoting function of Beclin 1 might be tumor suppressive, as Beclin 1 expression in MCF7 breast carcinoma cells rescued an autophagy defect and reduced tumorigenicity (Liang et al., 1999).

Beclin 1 functions in a complex with the class III PI3-kinase Vps34 and its cofactor p150/Vps15 to regulate autophagosome formation (Kihara et al., 2001b; (Sun et al., 2008). In addition to the core members of this complex, several other proteins interact with Beclin 1 to regulate later steps in autophagy and endocytosis. Among these proteins are the anti-apoptotic Bcl-2 and Bcl-XL, the autophagy protein Atg14L and autophagy inhibitor Rubicon, tumor suppressors UVRAG and Ambra-1, and the endocytosis related protein Bif-1 (Liang et al., 1998; Itakura et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009; Liang et al., 2006; Fimia et al., 2007; Takahashi et al., 2007).

Investigation of the tumor suppressive function of autophagy has shown that autophagy is induced in response to metabolic stress in tumor cells overexpressing Bcl-2 or deficient in Bax and Bak (Degenhardt et al., 2006). Furthermore, autophagy impairment in *beclin 1*$^{+/-}$ immortalized baby mouse kidney (iBMK) epithelial cells overexpressing Bcl-2 resulted in sensitivity to ischemic metabolic stress and increased tumorigenesis (Degenhardt et al., 2006). These cells possess genome instability, including elevated levels of DNA double-stranded breaks, abnormal centrosomes, and

aneuploidy (Mathew et al., 2007). In the absence of Bcl-2 expression, however, apoptosis was functional and *beclin 1*$^{+/-}$ cells did not accumulate these genome irregularities (Mathew et al., 2007). Similarly, *Atg5* deficient iBMK cells expressing Bcl-2 were also more sensitive to metabolic stress and exhibited increased genome damage compared to *Atg5*$^{+/+}$ cells, suggesting that the process of autophagy, and not simply Beclin 1 function in an alternative pathway, suppresses tumorigenesis by protecting cells from genome damage induced by metabolic stress (Mathew et al., 2007).

Experiments in mouse models of mammary epithelial tumorigenesis also support a role for autophagy in tumor suppression via protection from metabolic stress-induced genome instability (Karantza-Wadsworth et al., 2007). *beclin*$^{+/-}$, Bcl-2 expressing immortalized mouse mammary epithelial cells (iMMECs) formed mammary tumors when injected into immune-compromised nude mice at a faster rate than *beclin 1*$^{+/+}$ iMMECs. Tumors that formed from *beclin 1*$^{+/-}$ cells displayed reduced autophagy levels, DNA damage, and gene amplification, suggesting that metabolic stress leads to genome damage and tumor progression in the absence of autophagy and apoptosis (Karantza-Wadsworth et al., 2007).

Mathew et al. (2009) presented a possible biochemical mechanism for transformation of autophagy defective cells, which involved accumulation of and signaling by the cell stress protein p62 (Mathew et al., 2009). p62 is an adaptor protein, which binds to atypical protein kinase C (aPKC) and modulates binding to other proteins that control diverse signaling pathways including tumor necrosis factor alpha (TNF-α) receptor interacting protein (RIP), TNF receptor-associated factor 6 (TRAF6), LC3, and ubiquitin (Moscat et al., 2006). In autophagy, p62 functions as an adaptor, which binds to

the autophagosome-associated protein LC3/Atg8 and brings ubiquitinated proteins to the autophagosome for degradation by the lysosome (Komatsu et al., 2007). However, in autophagy defective cells, p62 accumulates and appears to contribute to either transformation or death (Komatsu et al., 2007; Mathew et al., 2009).

p62 was first linked to tumorigenesis in studies focusing on its regulation of the nuclear factor kappa-B (NFκB) pro-survival pathway, which is activated by oncogenic Ras during cell transformation (Duran et al., 2008; Mayo et al., 1997). p62 is required for Ras-induced lung cancer, and it promotes tumor formation in response to Ras signaling through activation of IκB kinase (IKK) (Duran et al., 2008). Specifically, p62 expression is induced by oncogenic Ras via the PI3K pathway, and it mediates activation and self-ubiquitination of the E3 ligase TRAF6. This leads to activation of IKK by an unknown mechanism, followed by degradation of the NFκB inhibitor IκB. The NFκB transcription factor family, which includes five proteins in mammals, promotes cell survival and proliferation through a myriad of downstream targets (Pahl, 1999). Murine gene targeting studies revealed hematopoietic hyperplasia as a result of loss of function mutations of the NFκB gene RelB or deletion of the C-terminal transcriptional transactivation domain of the c-Rel protein (Carrasco et al., 1998; Weih et al., 1995)

Consistent with early p62 studies, Mathew et al. (2009) observed elevated p62 levels in autophagy deficient tumor cells (Mathew et al., 2009). Under metabolic stress conditions, autophagy was required for turnover of p62. Heterozygous disruption of *beclin 1* or deletion of *Atg5* in iBMK cells overexpressing Bcl-2 resulted in elevated p62 levels, ROS accumulation, and genomic instability (Mathew et al., 2009). *In vivo*, the lungs, heart, and liver of *beclin* $1^{+/-}$ mice, as well as spontaneous tumors from these

animals, displayed p62 accumulation. Overexpression of p62 in *Atg5*$^{-/-}$ iBMK cells led to increased tumor growth in nude mice and DNA damage pathway induction compared to *Atg5*$^{-/-}$ cells overexpressing GFP. Gene expression analysis of *Atg5*$^{-/-}$ tumors overexpressing p62 showed down-regulation of immunity pathways, including Toll-like receptor signaling, compared to *Atg5*$^{-/-}$ tumors expressing GFP. Surprisingly, the NFκB pathway was suppressed by p62 expression in *Atg5*$^{-/-}$ tumors, and this was confirmed in both *beclin 1*$^{+/-}$ and *Atg5*$^{-/-}$ cells with an IL-6-luciferase reporter (Mathew et al., 2009). This suggested an alternate model for suppression of tumorigenesis by autophagy, whereby autophagy is required for turnover of p62 under conditions of metabolic stress, and failure to do so leads to p62 accumulation and inhibition of NFκB signaling downstream by an unknown mechanism. Inhibition of canonical NFκB activation by this mechanism may then lead to tumorigenesis because of inflammation associated with accumulating cell stress and tissue damage.

Drosophila has a single Beclin 1 ortholog, Atg6, which shares 71% amino acid identity with the evolutionarily conserved domain of mammalian Beclin 1, and 50% overall identity. Atg6 interacts with the Class III PI3K Vps34 *in vivo*, and co-expression of Atg6 with Vps34 or Atg6 with the Vps34-interacting serine-threonine kinase Vps15 is sufficient to induce autophagy in the developing larva (Juhász et al., 2008; Chapter 2). Autophagy is induced in the larval fat body, a nutrient storage organ, in response to starvation (Scott et al., 2004). Histolysis of the larval fat body and salivary glands involves steroid regulated autophagy, which clears these tissues before adult structures are formed during metamorphosis (Rusten et al., 2004; Lee and Baehrecke, 2001). Both Vps34 and Vps15 are required for autophagy and adult viability in *Drosophila* (Juhász et

al., 2008; Lindmo et al., 2008; Wu et al., 2007). However, the lack of a null *Atg6* mutant has precluded full functional analysis of the Vps34 complex in the context of a developing animal.

Here we characterize a *Drosophila Atg6* null mutant. We used ends-out gene targeting to knock out the open reading frame of *Atg6* (Rong and Golic, 2000). Animals lacking *Atg6* die prior to metamorphosis and contain melanotic blood cell tumors. Consistent with the role of Beclin 1 as a tumor suppressor, loss of *Atg6* causes over-production of blood cells (hemocytes). Furthermore, clonal loss of *Atg6* in the follicular epithelium leads to altered cell polarity and invasion of neighboring cells. These phenotypes are not present in *Vps34* or *Atg1* mutant cells, suggesting a novel, autophagy independent function for *Atg6* in maintenance of cell architecture.

Results

Generation of *Atg6*^{1} mutant animals

Flybase reports a P-element allele of *Atg6*, P{PZ}Atg6^{00096}(Fig. 3-1A), which is located in the 5' untranslated region (UTR), in a region that overlaps with another gene on the opposite strand. Preliminary experiments showed that autophagy function is maintained in these animals (data not shown). Based on this result, and the fact that the transposable element does not disrupt the open reading frame, it was necessary to generate a true null mutant for *Atg6* for further functional characterization. To generate strong loss of function mutations in *Atg6*, we utilized the ends-out gene targeting approach (Rong and Golic, 2000). With this method, homologous recombination was used to target the genomic locus and remove the sequence of interest, replacing it with a

w+ minigene eye-color marker carried on a donor construct that also includes 3.5 kb of homologous sequence on each side of *Atg6* (Fig. 3-1A). Flip (FLP) recombinase and I-Sce I endonuclease were used to induce DNA double stranded break and homologous recombination at the *Atg6* locus. The resultant line, $Atg6^{1}$, has a complete deletion of the *Atg6* open reading frame (ORF), replaced with *w+* (Fig. 3-1A and B). Reverse transcription (RT)-PCR confirmed the absence of *Atg6* expression in homozygous third instar larvae (Fig. 3-1C). Flanking gene expression was unperturbed in $Atg6^{1}$ homozygous larvae (Fig. 3-1C). Animals homozygous for $Atg6^{1}$ die during the late third instar/early pupal stage of development (Fig. 3-1D). $Atg6^{1}$ is also associated with late larval lethality when crossed in trans with the *Df(3R) Exel 6197* deficiency for this region. The majority (85%) of animals lacking *Atg6* die during the third larval instar, while 15% pupariate, but die during prior to eclosion (Fig. 3-1D). Expression of a GFP-Atg6 transgene, via ubiquitously expressed *actin*-Gal4 rescued lethality of $Atg6^{1}$.

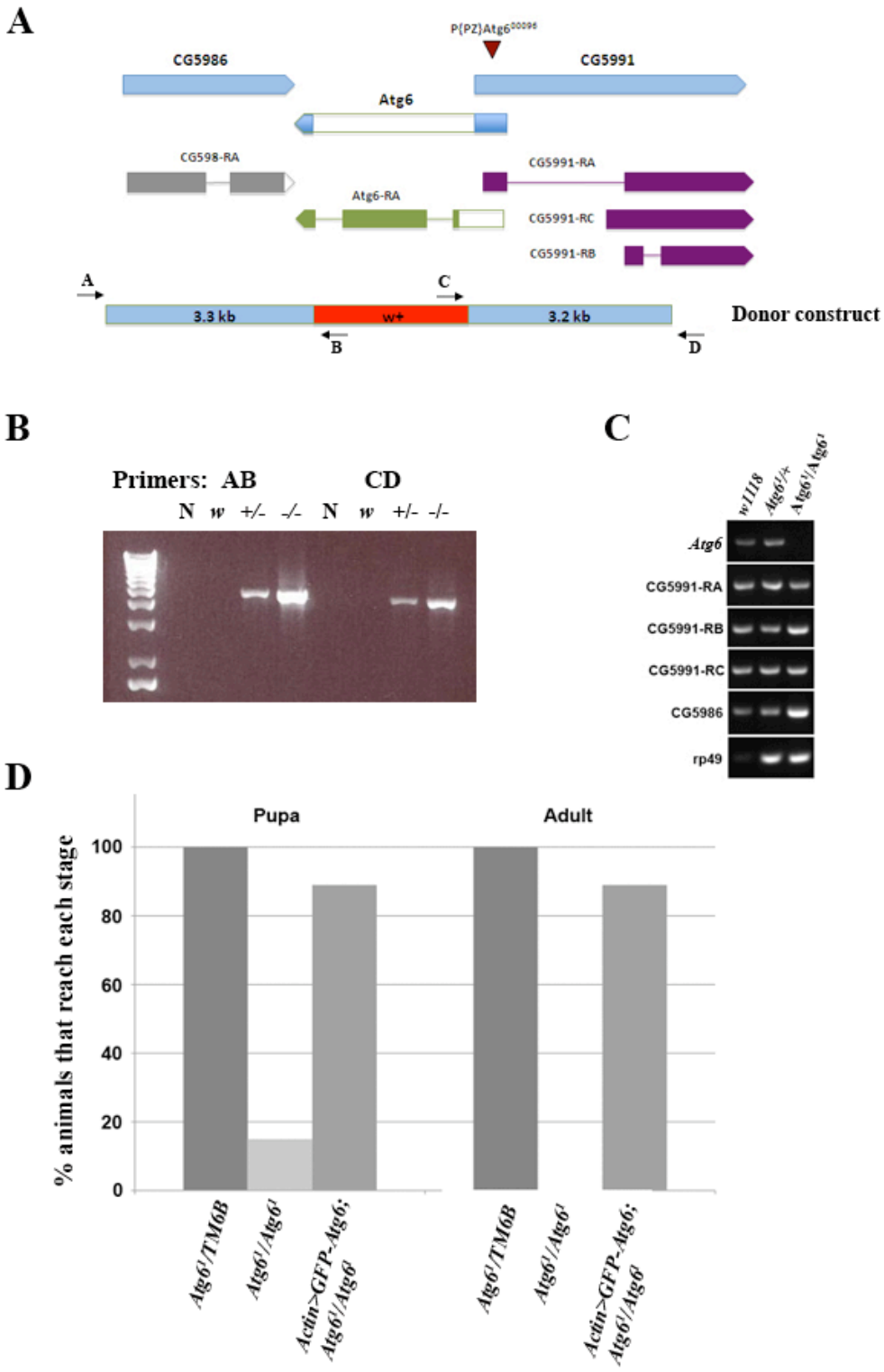

Figure 3-1. Ends out targeting of *Atg6*. (A) *Atg6* genomic locus and donor targeting construct. The donor construct consisted of a *w+* minigene flanked by 3.5 kb of genomic sequence (blue) from each side of *Atg6*.

Flip recombination target (FRT) sites and I-Sce I endonuclease recognition sites were present on each side of flanking sequence, to facilitate double stranded break and homologous recombination at the target site. The resultant flies contained the *w+* minigene (red) in place of the *Atg6* ORF (white). **(B)** PCR to confirm insertion of *w+* at the *Atg6* genomic locus. N= no template control, *w= w1118*, +/- = $Atg6^{1}$/+, -/- = $Atg6^{1}/Atg6^{1}$. Genomic DNA was collected from third instar larvae. Primers A-D, pictured in (A), were used in PCR. **(C)** RT-PCR of *Atg6* and flanking gene transcripts and ribosomal protein 49 (rp49), which served as a positive control for loading, and absence of rp49 in *w1118* sample likely due to human error. RNA was collected from third instar larvae. **(D)** Lethal phase analysis of *Atg6* mutant animals. **Genotypes:** *w; Sp/CyO;* $Atg6^{1}$*/TM6B* (n=337), *w; Sp/CyO;* $Atg6^{1}/Atg6^{1}$ (n=145)*, w; Actin-Gal4{25FO1}/UAS-GFP-Atg6;* $Atg6^{1}/Atg6^{1}$ (n=119).

***Atg6* is required for autophagy and endocytosis**

Atg6, when co-expressed with either Vps34 or Vps15, is sufficient to induce autophagy in the third instar larval fat body (Juhász et al. 2008). To determine whether *Atg6* is required for starvation-induced autophagy, localization of the autophagosome marker GFP-Atg8 was monitored. Atg8a, the *Drosophila* homolog of mammalian LC3, displays diffuse cytoplasmic localization in feeding larvae, but becomes incorporated into autophagosome membranes during starvation and is visualized as intracellular puncta (Scott et al., 2004). Quantification of GFP-Atg8a puncta in the fat body of starved third instar larvae showed that control animals, expressing GFP-Atg8a in the fat body, contained an average of 86 puncta per field per animal (Fig. 3-2A and C), while $Atg6^{1}$ larval fat body contained an average of 22 puncta per field per animal (Fig. 3-2B and C).

In addition to its function in autophagy, Vps34 also has a well-characterized function in endocytosis. Vps34 phosphorylates the lipid phosphatidylinositol (PI) to generate PI(3)P. PI(3)P then recruits FYVE domain containing proteins to form intracellular membranes. Vps34 activity can be monitored using a transgenically expressed protein consisting of GFP fused to the FYVE domain of hepatocyte growth factor regulated tyrosine kinase substrate (Hrs), an early endosome specific protein (Gillooly et al., 2000). We utilized FLP recombinase-mediated recombination at FLP recombination target (FRT) sites to generate $Atg6^{1}$ mitotic clones in the fat body, resulting in tissue composed of phenotypically wild type (control) and *Atg6* mutant cells. When expressed in the larval fat body of control cells (marked by red), GFP-FYVE localized to perinuclear puncta that are presumed to be endosomes (Fig. 3-2 D-F). By contrast, homozygous *Atg6* mutant cells (marked by the absence of red) did not have

GFP-FYVE puncta (Fig. 3-2 D-F). To test whether *Atg6* functions in endocytosis, uptake of a Texas Red (TR)-avidin endocytic tracer was monitored in larval fat body. Following a 20-minute pulse with TR-avidin and 10-minute chase, control cells (lacking GFP) contained TR-avidin throughout, while homozygous *Atg6* mutant cells (GFP positive), did not incorporate the tracer (Fig. 3-2I). These results indicate that *Atg6* is required for both autophagy and endocytosis *in vivo*.

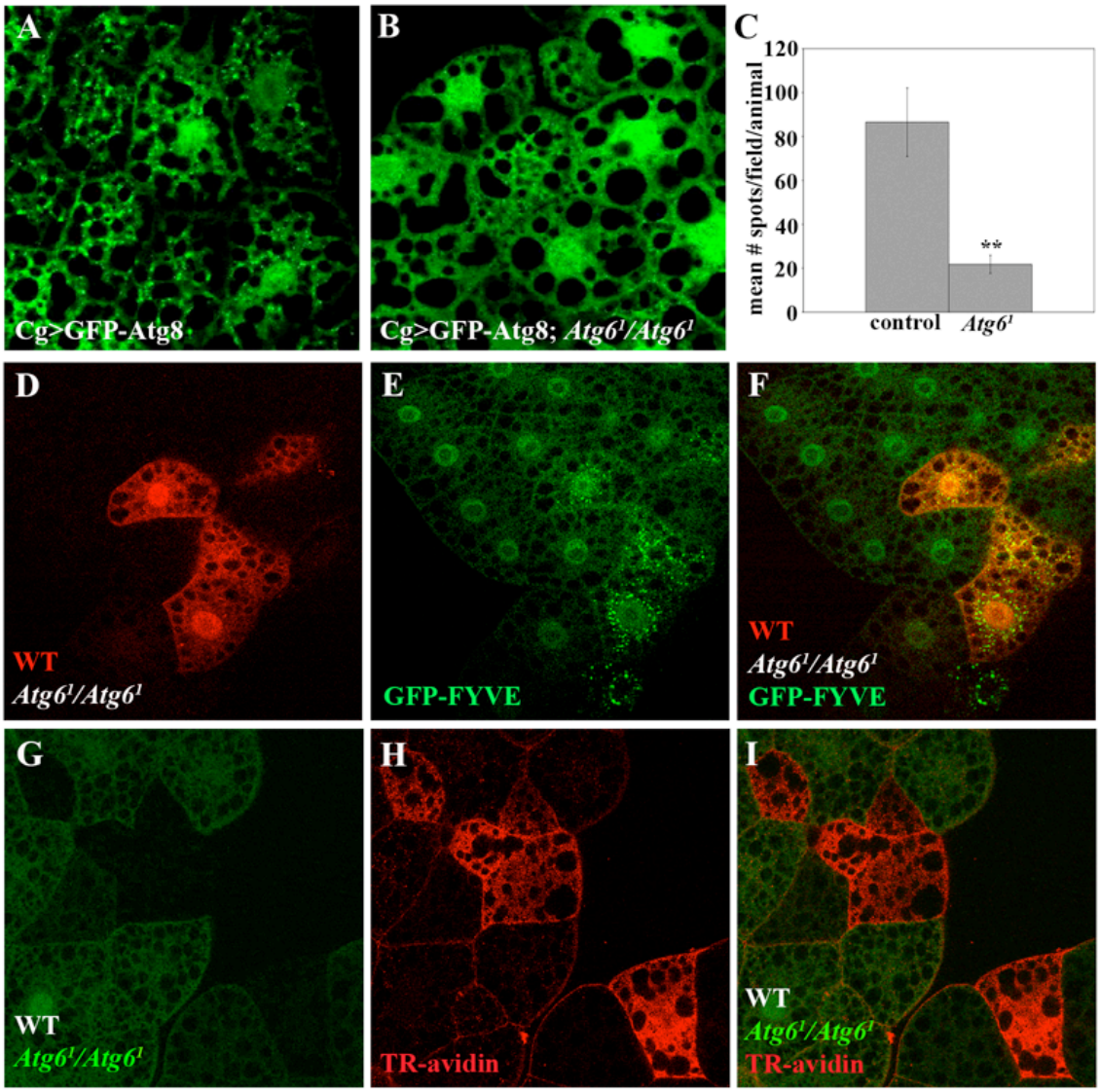

Figure 3-2. *Atg6* is required for starvation-induced autophagy and endocytosis in the larval fat body. **(A-B)** GFP-Atg8 localization in the fat body of control (A) and *Atg6* mutant (B) larvae subjected to starvation for 4 hours at 25°C. GFP-Atg8 expression was driven by the fat body driver *Collagen* (Cg)-Gal4. **(C)** Quantification of GFP-Atg8 puncta in control and *Atg6* mutant fat body. Control= Cg>GFP-Atg8, $Atg6^{1}$ = $Atg6^{1}/Atg6^{1}$ Fat body was dissected from starved larvae, fixed in 4% formaldehyde, and processed for imaging with a Zeiss LSM 510 confocal microscope. GFP-positive puncta were counted in 3 fields per animal and averaged. For both genotypes, n=9, bars represent standard error, and P= 0.0015. A two tailed student's t-test was used to calculate statistical significance. **(D-F)** GFP-FYVE localization in *Atg6* fat body clones. **(D)** Mitotic clones were induced in the larval fat body by heat shock (hs)-FLP expression. Wild

type cells are marked by the presence of mCherry, while homozygous *Atg6* mutant cells are marked by the absence of mCherry. **(E)** GFP-2xFYVE is a reporter of Vps34 activity and marks PI(3)P structures. **(F)** Overlay of D and E, showing GFP-FYVE puncta at a perinuclear compartment in wild type cells. **(G-I)** Texas Red-avidin uptake in *Atg6* mosaic fat body. **(G)** $Atg6^{1}$ clones were induced using the mosaic analysis with a repressible cell marker (MARCM) approach ((Lee and Luo, 1999). *Atg6* mutant cells are marked by the presence of GFP, while WT cells are marked by the absence of GFP. **(H)** Fat body was dissected from third instar larvae and incubated *ex vivo* with TR-avidin (80μg/mL) for 20 minutes, then chased with phosphate buffered saline (PBS) + 0.5% bovine serum albumin (BSA) for 10 minutes prior to fixation in 4% formaldehyde. **(I)** Overlay of G and H, showing TR-avidin uptake in wild type, but not *Atg6* mutant cells. **Genotypes:** (A) *w; UAS-GFP-Atg8a/Cg-Gal4;+*, (B) *w;UAS-GFP-Atg8a/Cg-Gal4;* $Atg6^{1}/Atg6^{1}$. (D-F) *y,w,hs-FLP; Cg-Gal4 UAS-GFP-2XFYVE/+; FRT82B UAS-mCherry/FRT82B* $Atg6^{1}$, (G-I) *y,w,hs-FLP; Actin-Gal4{25F01}/UAS-GFP; FRT82B Tubulin-Gal80/FRT82B* $Atg6^{1}$.

Loss of *Atg6* leads to blood cell over-production and melanotic mass formation

We analyzed *Atg6* mutants for defects. While the parental w^{1118} and heterozygous $Atg6^{1}$ animals exhibited no obvious phenotypes (Fig. 3-3 A,B), homozygous $Atg6^{1}$ mutant larvae displayed striking melanotic mass blood cell tumors before death (Fig. 3-3C). Significantly, this blood cell tumor phenotype was rescued by ubiquitous expression of Atg6 (Fig. 3-3D).

Melanotic mass formation has been associated with mutations in pathways that regulate hematopoeisis, as over-production of hemocytes, the *Drosophila* equivalent of macrophages, can lead to melanotic mass formation (Minakhina and Steward, 2006). To investigate whether hemocytes are the source of melanotic masses in $Atg6^{1}$ larvae, the hemocyte and lymph gland specific *hemolectin* (*hml*)*Δ*-Gal4 driver was used to drive GFP expression in control and $Atg6^{1}$ mutant larvae. Immunohistochemical analyses of paraffin sections with a GFP antibody revealed that these masses were indeed composed of hemocytes (Fig. 3-3G), and suggested that homozygous $Atg6^{1}$ mutant larvae contained more blood cells than either parental w^{1118}or heterozygous $Atg6^{1}$ larvae (Fig. 3-3 E,F). Quantification of larval hemocytes revealed that homozygous $Atg6^{1}$ mutant larvae contained nearly ten times as many hemocytes as parental w^{1118} larvae, while heterozygous $Atg6^{1}$ larvae contained about twice as many hemocytes as control w^{1118} larvae (3-3D). These data indicate that Atg6 functions to control hemocyte numbers in the developing animal.

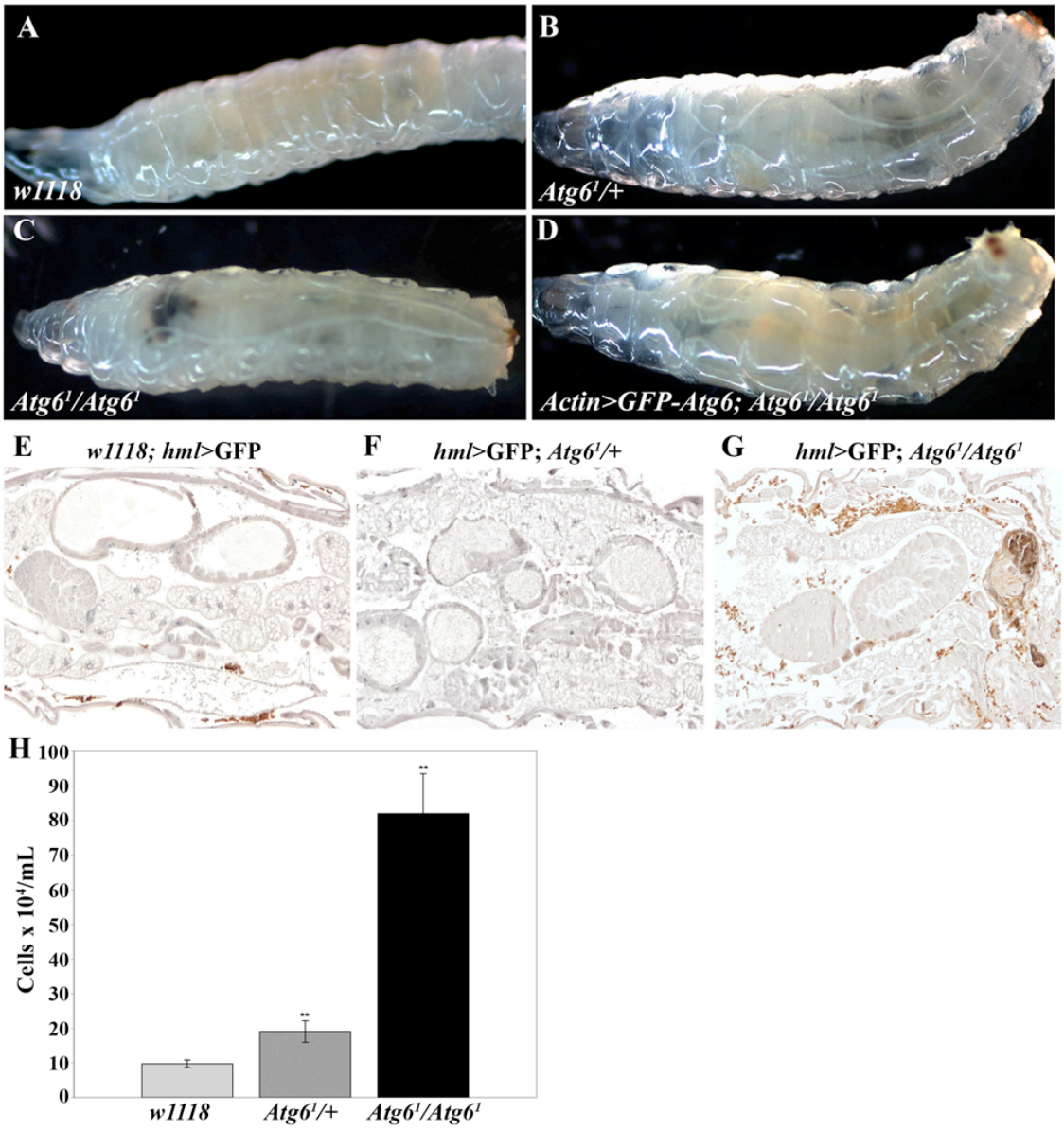

Figure 3-3. *Atg6* mutant larvae contain melanotic masses and an excess of hemocytes. (A-D) White light images of wandering third instar larvae. **(E-G)** Hemocytes were visualized by immunohistochemistry in third instar larvae expressing GFP, driven by *hml*Δ-Gal4. Wandering third instar larvae of similar age (+/- 3 hours) were fixed and embedded in paraffin wax for histological analysis. Sections were stained with a rabbit α-GFP antibody (Novus) and the signal (brown) was detected colorimetrically. Counterstaining with Weigert's hematoxylin allowed visualization of other tissues. **(H)** Quantification of hemocytes from wandering third instar larvae of similar age (+/- 3 hours). Individual larvae were bled into 20μL of 1X PBS, and 10μL was loaded into a hemacytometer. For each genotype, n= 20 animals and bars represent standard

error. A one tailed t-test was used for statistical analysis and the P-values relative to *w1118* are: $Atg6^{1}$/+, P= 0.004; $Atg6^{1}/Atg6^{1}$, P= 2.6 x 10^{-6}. **Genotypes:** (A) *w1118*, (B) *w; +;* $Atg6^{1}$/+, (C) *w; Sp/Cyo;* $Atg6^{1}/Atg6^{1}$, (D) *w; UAS-GFP-Atg6/Actin-Gal4{25F01};* $Atg6^{1}/Atg6^{1}$, (E) *w; hmlΔ-Gal4, UAS-GFP/+*, (F) *w; hmlΔ-Gal4, UAS-GFP/+;* $Atg6^{1}$/+, (G) *w; hmlΔ-Gal4, UAS-GFP;* $Atg6^{1}/Atg6^{1}$.

***Atg6* mutant cells accumulate cell stress markers and display activation of the NFκB proteins Relish and Dif**

Autophagy deficient cells and tumors have previously been shown to accumulate endoplasmic reticulum (ER) stress protein markers, and it has been hypothesized that cell stress in autophagy deficient cells can contribute to transformation (Mathew et al., 2009). To test whether *Atg6* mutant cells exhibited signs of ER stress, we generated mitotic mutant clones of cells in the larval fat body. We stained this tissue with an antibody against the ER chaperone heat shock cognate 3 (Hsc3), the *Drosophila* homolog of GRP78/BiP that is up-regulated in response to ER stress during the unfolded protein response (Ryoo et al., 2007). Homozygous $Atg6^{1}$ mutant fat body cells (lacking GFP) accumulated high levels of Hsc3, while neighboring control cells (GFP positive) did not accumulate Hsc3 (Fig. 3-4A). To investigate whether the accumulation of Hsc3 was due to a defect in autophagy, we generated clones of homozygous mutant *vps15* and *Atg1* cells. Vps15 functions in autophagosome formation by interaction with Vps34, while Atg1 regulates induction of autophagy upstream of the Vp34 complex. Though Hsc3 levels were slightly higher in homozygous *vps15* mutant cells than in neighboring control cells, there did not appear to be a significant induction of ER stress (Fig. 3-4B). Similarly, homozygous *Atg1* mutant cells did not display elevated levels of Hsc3 (Fig. 3-4C). These results suggest that ER stress induction in cells lacking *Atg6* is independent of autophagy impairment.

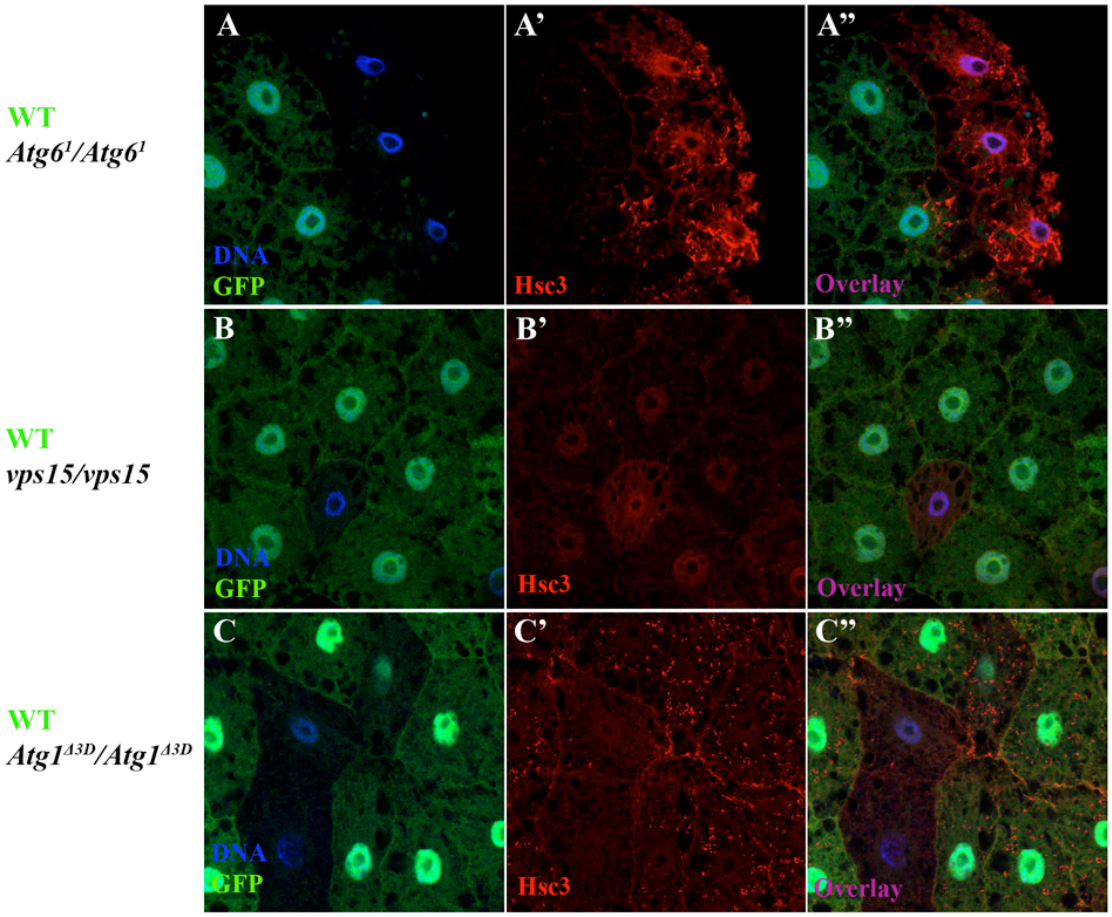

Figure 3-4. *Atg6* is required to mitigate ER stress, independent of its function in autophagy. Induction of the ER chaperone Hsc3 was monitored in mitotic clones, induced by hs-FLP expression, in the larval fat body. Clones were induced by 1hr heat shock of embryos at 37°C. Fat body was dissected from 3rd instar larvae and fixed for staining with guinea pig α-Hsc3 antibody. **Genotypes:** (A) *y,w, hs-FLP;+; FRT82B Atg6¹/FRT82B Ubiquitin-GFP*, (B) *y,w hs-FLP;+; FRT82B vps15/FRT82B Ubiquitin-GFP*, (C) *y,w hs-FLP; +; Atg1Δ3D FRT80B/Ubiquitin-GFP FRT80B*.

The adaptor protein p62 has been implicated as the culprit in tumorigenesis caused by mono-allelic loss of *beclin 1* (Mathew et al., 2009). Like its mammalian ortholog p62, *Drosophila* Ref(2)P is an autophagy substrate that accumulates in cells when autophagy is inhibited (Nezis et al., 2008). Homozygous $Atg6^{1}$ mutant fat body cells (lacking GFP) accumulated large Ref(2)P puncta in the cytoplasm, while neighboring control GFP-positive cells failed to express Ref(2)P (Fig. 3-5A). Similarly, cells with reduced function in either *vps15* (Fig. 3-6B), *Atg1* (Fig. 3-5C), or *vps34* (Fig. 3-5D) accumulated Ref(2)P, while neighboring control cells did not contain any Ref(2)P puncta.

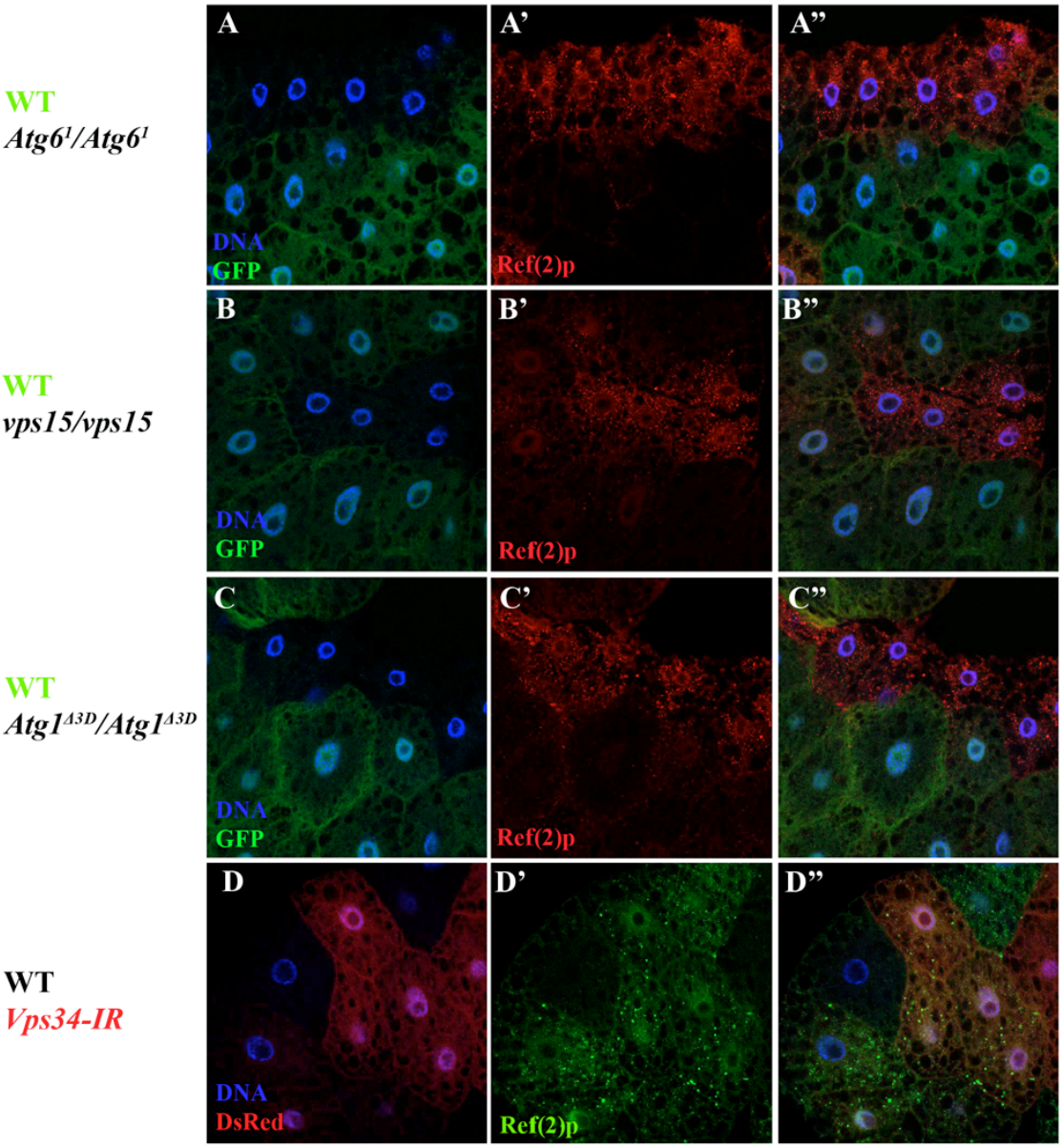

Figure 3-5. Disruption of autophagy in cells lacking Atg6, Vps15, Atg1, or Vps34 leads to accumulation of Ref(2)P. Fat body clones were generated in third instar larvae, and tissues were stained with rabbit α-Ref(2)P antibody. Images were collected on a Zeiss LSM 710 confocal microscope. **(A-C)** *Atg6*^{1}, *vps15*, and *Atg1*$^{\Delta 3D}$ clones were induced in the third instar larval fat body with hs-FLP. Clones were marked by the absence of GFP. **(D)** *vps34* expression was knocked down clonally via RNAi. *Vps34-IR* "flip out" clones were generated by FLP-mediated removal of the CD2 cassette, resulting in *Vps34-IR* clones expressing DsRed. **Genotypes:** (A) *y,w, hs-FLP;+; FRT82B Atg6*^{1}*/FRT82B Ubiquitin-GFP*, (B) *y,w hs-*

FLP;+; FRT82B vps15/FRT82B Ubiquitin-GFP, (C) *y,w hs-FLP; +; Atg1$^{\Delta 3D}$ FRT80B/Ubiquitin-GFP FRT80B*. (D) *y,w hs-FLP; UAS-Vps34-IR/Actin>CD2>GAL4 UAS-DsRed; +*.

p62 regulates NFκB signaling, and whether this regulation is positive or inhibitory depends on the tissue (Duran et al. 2008, Mathew et al., 2009). *Drosophila* has 3 NFκB proteins, Relish, Dif, and Dorsal, which regulate innate immune defense against pathogen infection. In response to infection, NFκB proteins translocate to the nucleus of fat body cells to induce transcription of downstream target genes (Ip et al., 1993; Lemaitre et al., 1995; Stöven et al., 2000). Control larval fat body cells (GFP positive) expressed Relish and Dif exclusively in the cytoplasm, while homozygous $Atg6^{1}$ mutant cells (lacking GFP) displayed nuclear localization of both Relish and Dif (Fig. 3-6A, 3-7A). Dorsal was localized to the nucleus in both control and *Atg6* mutant cells (data not shown). To determine whether the nuclear localization of Relish and Dif was related to the disruption of autophagy in *Atg6* mutant cells, we generated either *vps15*, or *Atg1* mutant fat body cells. Relish and Dif were localized in the nucleus of homozygous *vps15* mutant fat body cells (Fig. 3-6B, 3-7B), but were localized in the cytoplasm of *Atg1* mutant cells (Fig. 3-6C, 3-7C). These data suggest that the Vps34 complex, made up of Vps34, Atg6, and Vps15, functions in a non-autophagic process to control the activation of NFκB signaling.

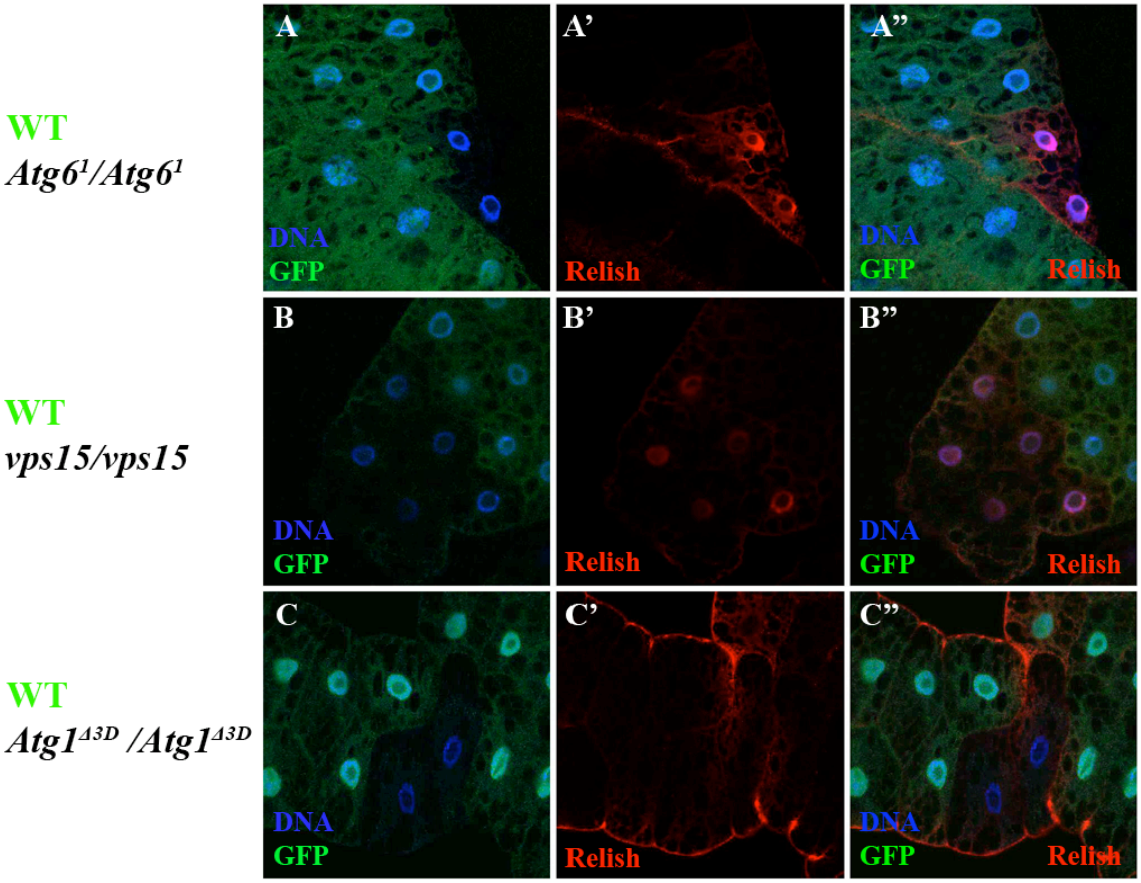

Figure 3-6. The NFκB protein Relish localizes to the nucleus of *Atg6* mutant fat body clones. Staining of mosaic third instar larval fat body with rabbit α-Relish antibody. Mitotic clones were induced with hs-FLP. Mutant cells were marked by the absence of GFP. DAPI labels nuclei. **Genotypes:** (A) *y,w, hs-FLP;+; FRT82B Atg6^{1}/FRT82B Ubiquitin-GFP*, (B) *y,w hs-FLP;+; FRT82B vps15/FRT82B Ubiquitin-GFP*, (C) *y,w hs-FLP; +; Atg1$^{\Delta 3D}$ FRT80B/Ubiquitin-GFP FRT80B*.

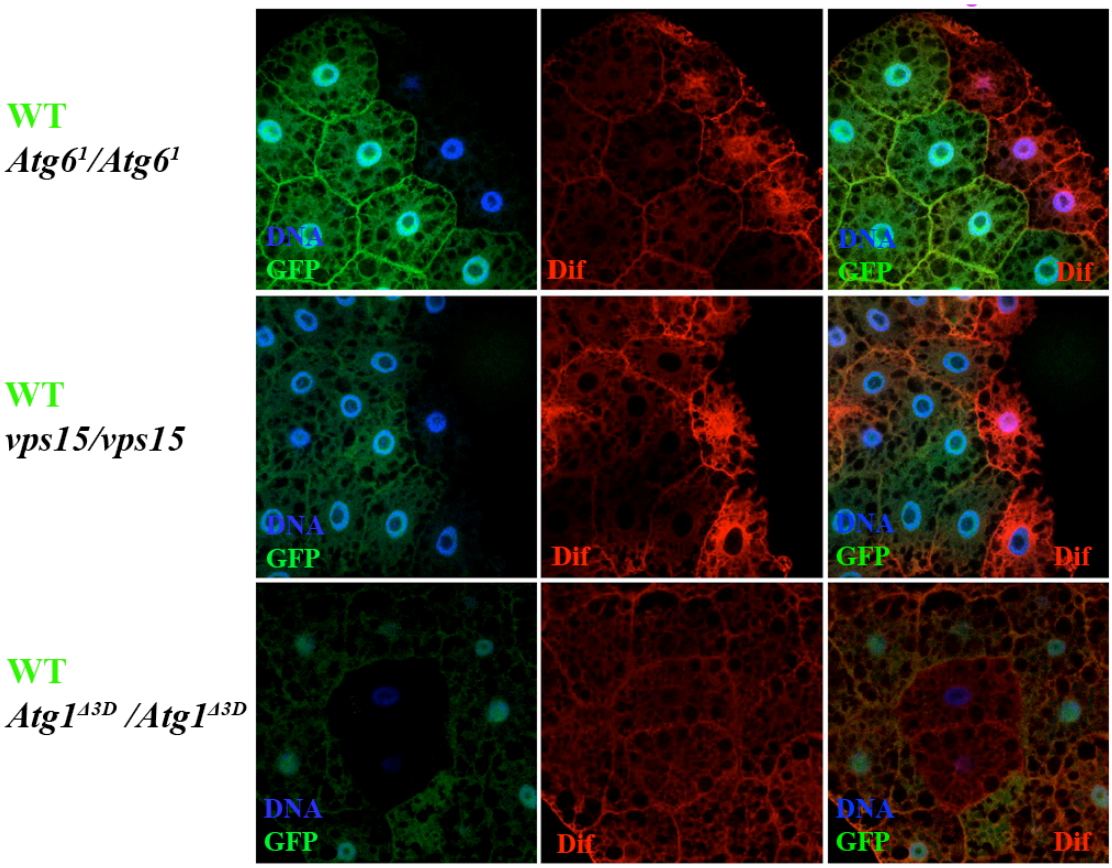

Figure 3-7. The NFκB protein Dif localizes to the nucleus of *Atg6* mutant cells. Staining of mosaic third instar larval fat body with rabbit α-Dif antibody and DAPI. Mitotic clones were induced with hs-FLP. Mutant cells were marked by the absence of GFP. **Genotypes:** (A) *y,w, hs-FLP;+; FRT82B Atg6^{1}/FRT82B Ubiquitin-GFP*, (B) *y,w hs-FLP;+; FRT82B vps15/FRT82B Ubiquitin-GFP*, (C) *y,w hs-FLP; +; Atg1$^{\Delta 3D}$ FRT80B/Ubiquitin-GFP FRT80B*.

The current model for Beclin 1 function during tumor progression suggests that ectopic p62 signaling and down-regulation of tumor necrosis factor alpha (TNFα)-induced NFκB activation promote cell over-growth through activation of non-canonical NFκB signaling (Mathew et al., 2009). To determine whether Ref(2)P and NFκB proteins are required for blood cell tumor/melanotic mass formation in homozygous *Atg6* mutant larvae, mutant alleles of *relish*, *Dif*, and *ref(2)p* were combined with $Atg6^{1}$ for double mutant analysis. Due to an inability to generate animals that possess mutations in both *Atg6* and *dorsal (dl)*, the third *Drosophila* NFκB, we ubiquitously expressed a *dl* inverted repeat (IR) to knock down expression via RNA interference (RNAi). Double mutant analyses showed that despite Ref(2)P accumulation and NFκB activation in *Atg6* mutant cells, these proteins are not required for melanotic mass formation in *Atg6* mutant larvae (Fig. 3-8A-E). These results suggest that while Ref(2)P accumulation and nuclear NFκB expression in the fat body are consequences of loss of *Atg6*, these events are not the cause of melanotic mass formation.

To determine how *Atg6* deficiency might contribute to melanotic mass formation, we tested several additional pathways for their ability to suppress melanotic mass formation in $Atg6^{1}$ larvae. Using an RNAi-mediated approach to knockdown potential regulators of cell proliferation, we expressed gene specific inverted repeats (IR) either ubiquitously (Actin-Gal4) or in a tissue-specific manner, in the hemocytes (*Croquemort (Crq)*-Gal4, *HmlΔ*-Gal4), lymph gland (*HmlΔ*-Gal4), or fat body (*Cg*-Gal4) (Table 3-1). Many of these genes are essential for embryonic development, so ubiquitous Actin-Gal4 driven knockdown led to embryonic lethality in many cases. We knocked down expression of the JAK/Stat pathway transcription factor Stat92E, the cell cycle regulators

Myc and Cyclin E, the growth factor signaling proteins EGFR and Pvf1, and the apoptosis inhibitor IAP2. RNAi-mediated knockdown of these proteins in the fat body, embryonic hemocytes, larval hemocytes, or larval lymph gland did not suppress melanotic mass formation or lethality of $Atg6^{1}$ (Table 3-1). Furthermore, we overexpressed the potent cell death inducer Rpr in the larval hemocytes and lymph gland of $Atg6^{1}$ larvae, but did not observe suppression of melanotic mass formation. Thus, we were unable to pinpoint the pathways required for hemocyte overproliferation and melanotic tumor formation in *Atg6* mutant larvae.

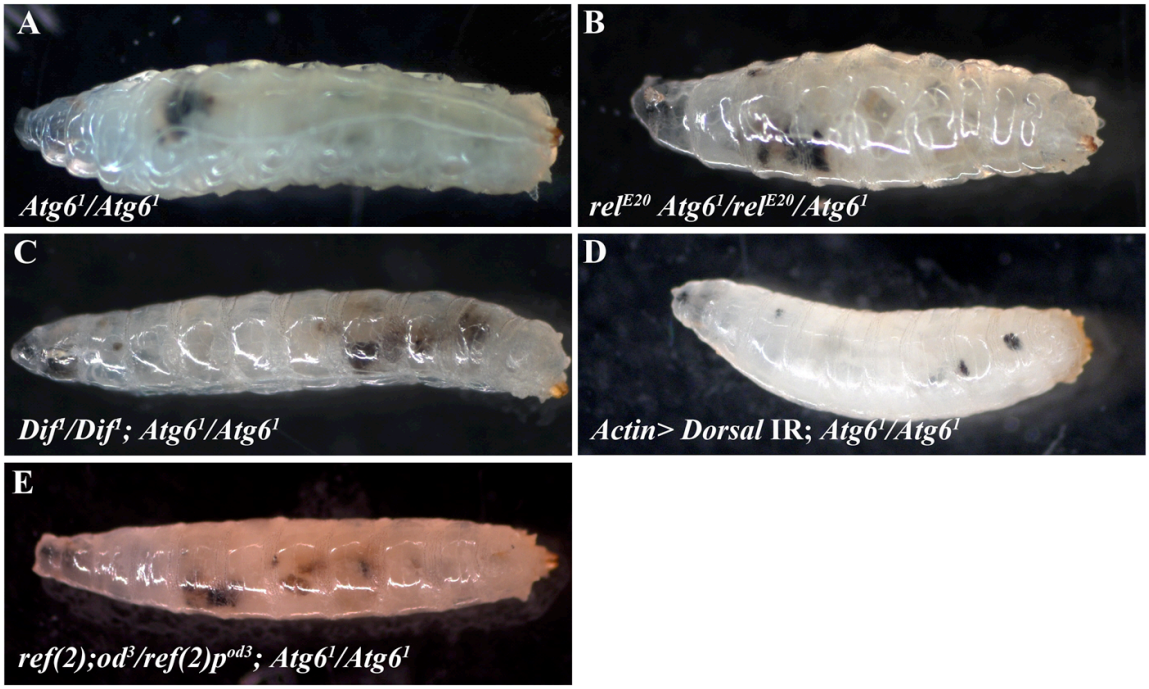

Figure 3-8. Ref(2)p and NFκB proteins are not required for melanotic mass formation in *Atg6* mutant larvae. NFκB mutations were combined with *Atg6*^{1} animals and *ref(2)p*; *Atg6* double mutant animals were generated. *Dorsal* expression was knocked down ubiquitously by RNAi. Wandering third instar larvae were observed for the presence of melanotic masses. **Genotypes:** (A) *w; Sp/Cyo; Atg6*^{1}*/Atg6*^{1}, (B) *w; Sp/Cyo; rel*^{E20}*Atg6*^{1}*/ rel*^{E20}*Atg6*^{1}, (C) *w; Dif*^{1}*/Dif*^{1}*;Atg6*^{1}*/Atg6*^{1}, (D) *w; Actin-Gal4{25F01}/UAS-Dorsal-IR; Atg6*^{1}*/Atg6*^{1}, (E) *w; ref(2)p*^{od3}*/ref(2)p*^{od3}*; Atg6*^{1}*/Atg6*^{1}.

Transgene expressed	Human homolog	Cellular function	Gal4 Driver
Dif-IR		NFκB transcription factor	*Actin*
Stat92E-IR	Stat5A	Transcription factor, differentiation, cell division	*Cg* *HmlΔ* *Crq*
dm-IR	c-Myc	Transcription factor, cell cycle regulator	*Cg* *HmlΔ* *Crq*
CycE-IR	Cyclin E	Cell cycle regulator	*Cg* *HmlΔ* *Crq*
Egfr-IR	EGF receptor	Tyrosine kinase, growth factor signaling	*Cg* *HmlΔ* *Crq*
Pvf1-IR	PDGF, VEGF	Growth factor signaling	*Cg* *Crq*
Iap2-IR	BIRC3 (Baculoviral IAP repeat-containing 3)	Inhibitor of apoptosis	*Cg* *HmlΔ* *Crq*
Hep^{Act}	MAP2K7	Kinase, activator of c-Jun N-terminal kinase (JNK)	*Cg* *HmlΔ* *Crq*
Rpr		Inducer of apoptosis	*HmlΔ*

Table 3-1. Transgenes tested for suppression of melanotic mass formation in *$Atg6^1$* larvae. The UAS/Gal4 system was used to drive transgene expression ubiquitously or in the larval fat body (*Cg*-Gal4), larval lymph gland and hemocytes (*HmlΔ*-Gal4), or embryonic hemocytes (*Crq*-Gal4). "IR" denotes inverted repeat, used to knockdown expression of the endogenous gene via RNAi.

Loss of *Atg6*, but not autophagy, leads to tissue overgrowth

The *Drosophila* eye is an excellent model for studying tissue growth, as mosaic clonal analysis can be used to study mutations that lead to differences in eye cell growth without killing the adult (Tapon et al., 2001). Mutant cells can be identified by the presence or absence of eye color markers, and cell size/number and overall tissue size can be easily observed. In order to determine how loss of *Atg6* may lead to altered growth and/or proliferation, homozygous mutant cell clones were generated in the adult eye. Induction of wild type clones, marked by the red eye color marker *w+*, during eye development resulted in an adult eye with an equal distribution of red and white eye subunits (called ommatidia) (Fig. 3-9A). When *Atg6* mutant cell clones, marked with *w+* (red) (Fig. 3-1), were induced during development, the adult eye was made up almost entirely of red cells (Fig. 3-9E). No wild type cells, which would have been white due to the absence of *w+* red pigment, were observed. Because of the strength of expression of the *w+* transgene carried with the $Atg6^{1}$ allele, we were unable to distinguish whether the eye was made entirely of homozygous *Atg6* mutant cells, or heterozygous cells that did not undergo mitotic recombination. The mosaic *Atg6* adult eye also contained dark patches that we assume are dead cells.

To determine whether this phenotype could be attributed to the function of the Atg6/Vps34/Vps15 complex, *vps34* and *vps15* clones were induced in the eye. Neither *vps34* nor *vps15* mutant ommatidia were over-represented compared to control ommatidia (Fig. 3-9F, G). While loss of *vps34* did lead to a slightly rough eye phenotype (Fig. 3-9F), there was an even distribution of wild type and *vps34* mutant cells. Loss of

vps15 in the eye during development was lethal to the animal. A small number of adults eclosed, and those animals had small eyes with equal portions of control and mutant ommatidia (Fig. 3-9G). Finally, *Atg1* mosaic eyes were normal with no morphological defects and an equal distribution of mutant and control ommatidia (Fig. 3-9H). Together, these results suggest that loss of *Atg6* in the eye leads to a reduction in the number of wild type ommatidia that compose the adult eye. Significantly, the *Atg6* phenotype is distinct from mutations in other core components of the Vps34 complex (Vps34 and Vps15). Furthermore, mutations in the essential autophagy gene *Atg1* fail to exhibit an abnormal phenotype that is similar to *Atg6.* Therefore, *Atg6* appears to function in a Vps34 complex and autophagy-independent manner in the regulation of growth.

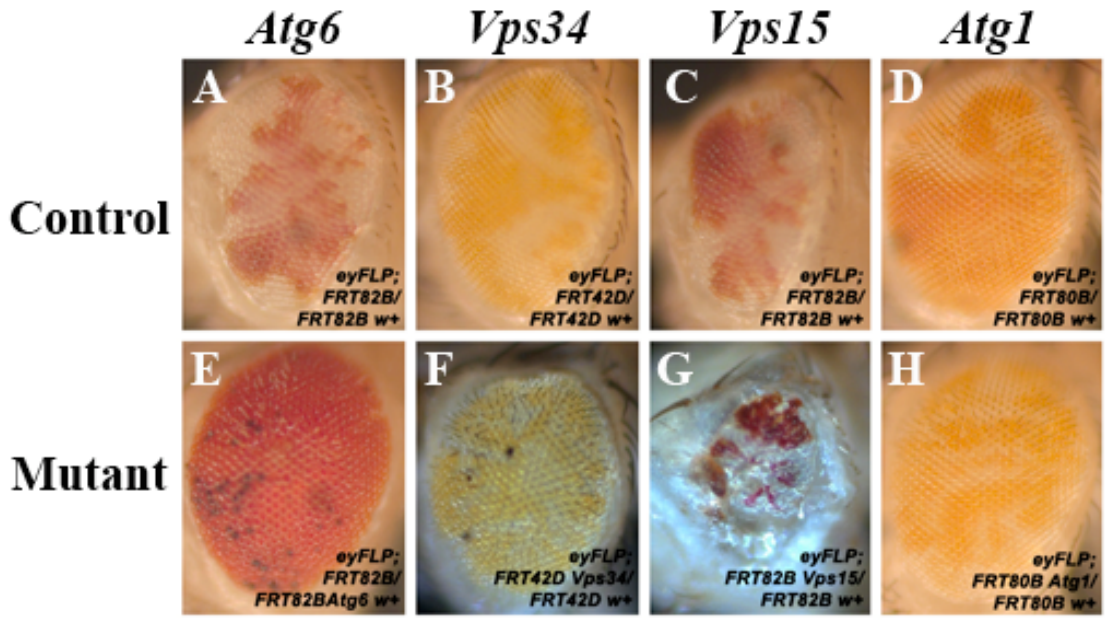

Figure 3-9. *Atg6* mutant cells outcompete wild type cells in the mosaic developing eye. *Eyeless*-FLP was expressed to induce mitotic recombination in the developing eye, generating eyes mosaic for *Atg6*, *Vps34, Vps15, or Atg1* mutations. **(A and C)** Wild type (*w+*) clones induced in *w-* background using the third chromosome FRT site, FRT82B. **(B)** Wild type (*w-*) clones induced in a *w+* background using the second chromsome FRT site, FRT42D. **(D)** Wild type (*w+*) clones induced in *w-* background using the third chromosome FRT site, FRT80B. **(E)** $Atg6^{1}$ clones, which carry a *w+* transgene and are red in color, were induced in a *w-* background. **(F)** $vps34^{\Delta m22}$ clones (*w-*) induced in a *w+* background. *vps34* mutant cells are white, and wild type cells are orange. **(G)** *vps15/vps15* clones, which are *w-*, induced in a *w+* genetic background. *Vps15* mutant cells are white, wild type cells are red. **(H)** *Atg1* clones (*w-*) induced in a *w+*background. *Atg1* mutant cells are white and wild type cells are orange. **Genotypes:** (A and C) *y,w ey-FLP GMR-lacZ; +; FRT82B w+/FRT82B*, (B) *yw ey-FLP GMR-lacZ; FRT42D/FRT42D lac25 {w+}51D*, (D) *yw ey-FLP GMR-lacZ; +; Ubi-GFP {w+}61EF FRT80B/FRT80B*, (E) *ey-FLP GMR-lacZ; +; FRT82B* $Atg6^{1}${w+}/*FRT82B*, (F) *yw ey-FLP GMR-lacZ; FRT42D* $vps34^{\Delta m22}$*/FRT42D lac25 {w+}51D*, (G) *yw ey-FLP GMR-lacZ; +: FRT82B w+/FRT82B vps15*, (H) *yw ey-FLP; +;* $Atg1^{\Delta 3D}$ *FRT80B/Ubi-GFP {w+}61EF FRT80B*.

Discussion

Since Beclin 1 was identified as a tumor suppressor, several studies have sought to determine how autophagy dysfunction can lead to tumorigenesis. The most recent model suggests that loss of Beclin 1 promotes tumorigenesis by preventing autophagy, leading to accumulation of ROS, protein stress, failure in degradation of the adaptor protein p62, and accumulation of DNA damage (Mathew et al., 2009). Furthermore, the model suggests that down-regulation of the canonical NFκB pathway, caused by p62 accumulation, triggers oncogenesis via activation of the non-canocial NFκB pathway and down-regulation of tumor necrosis factor alpha (TNFα) signaling (Mathew et al., 2009).

In order to understand the physiological functions of Beclin 1, we generated a null allele of the *Drosophila* ortholog, *Atg6*. This approach allowed us to observe the *in vivo* consequences of *Atg6* deficiency and to investigate genetic interactions with other pathways at the cellular level. Strikingly, loss of *Atg6* resulted in larvae with an excess of hemocytes, the *Drosophila* equivalent of macrophages, and formation of melanotic blood cell tumors prior to death (Fig. 3-3). The fact that these phenotypes could only be rescued by ubiquitous expression of *Atg6* (Fig. 3-3D), and not by expression in the fat body or hemocytes (data not shown), suggests that hemocyte over-production could either be the result of a tissue non-autonomous effect of *Atg6* deficiency, or a defect in the cell lineage before the activation of the promoters that were used to attempt rescue of this phenotype. Melanotic tumor masses are thought to be caused by at least 2 possible mechanisms: (1) tissue damage that recruits blood cells to encapsulate the unhealthy tissue and potentially protect the organism, or (2) over-proliferation of the blood cell lineage due to a defect in the hematopoietic stem cell niche (Minakhina and Steward,

2006). In support of the latter possibility, a recent study showed that hemocytes with decreased autophagy have an impaired ability to be recruited to epidermal wounds because of impaired cortical remodeling of blood cells (Kadandale et al., 2010). RNAi-mediated knockdown of autophagy genes, including *Atg6*, impaired the remodeling of blood cells. Though hemocytes clearly surround the melanotic masses in *Atg6* mutant larvae, it is undetermined whether the masses themselves are composed strictly of hemocytes and whether the masses result from hemocyte over-proliferation, or if hemocytes are induced to proliferate by the presence of melanotic masses. The lack of overgrowth in other larval tissues suggests a tissue specific tumor suppressor function for Atg6 in blood cells. It is possible that an Atg6-regulated pathway controls hemocyte number prior to pupal development, when hemocytes are mobilized for tissue remodeling. Further studies are needed to empirically test if loss of *Atg6* causes defects in blood cell progenitors because of possible defects in the hematopoietic stem cell niche.

While heterozygous $Atg6^{1}$ larvae also had significantly more hemocytes than controls, heterozygous adult flies did not display any obvious morphological defects or melanotic tumors. It is possible that *Drosophila Atg6* is not haploinsufficient as its mammalian homolog *beclin 1*. This could be due to differences in function between the fly and mammalian proteins. Beclin 1 interacts with anti-apoptotic Bcl-2 proteins in mammals, via an N-terminal BH3 domain. Atg6 and Beclin 1 shares less than 40% sequence identity in this domain, and it is not yet known whether the *Drosophila* Bcl-2 homologs, Buffy and/or Debcl, interact with Atg6. This functional difference in oncogene interaction could potentially account for the lack of haploinsufficiency in *Atg6* mutants. Finally, it is possible that subtle hemocyte-associated phenotypes may have been

overlooked in adult flies. A more thorough histological study of heterozygous *Atg6* adult flies may reveal additional phenotypes.

At the cellular level, *Atg6* mutant larvae had a defect in starvation-induced autophagy in the larval fat body (Fig. 3-2B and C), which resulted in accumulation of Ref(2)P, the *Drosophila* homolog of p62 (Fig. 3-5A). Ref(2)P accumulation also occurred in fat body cells lacking either Vps15, Vps34, or Atg1 (Fig. 3-6). Accumulation of Ref(2)P correlated with nuclear localization of the NFκB proteins Relish and Dif in $Atg6^{1}$ and *vps15* mutant fat body clones, but not in *Atg1* fat body clones (Fig. 3-7, 3-8), suggesting that modulation of NFκB activity in cells lacking Vps34 complex components is not dependent on p62 accumulation. Interestingly, we saw nuclear localization that is indicative of activation of NFκB proteins in *Atg6* mutant cells, whereas Mathew et al. observed down-regulation of NFκB signaling in *beclin* $1^{+/-}$ cells overexpressing p62 (Mathew et al., 2009). One explanation for this difference is that accumulated Ref(2)P in *Atg6* mutant fat body cells may be inactive due to protein aggregation, while transgenically over-expressed p62 in *beclin* $1^{+/-}$ cells might retain function that aggregated p62 does not. Alternatively, Mathew et al. (2009) did all of their experiments in the context of over-expression of the strong oncogene Bcl-2, and this may lead to differences in NFkB signaling from the otherwise wild type genetic background where all of our studies were conducted. Finally, one cannot exclude the possibility that fly cells may be different from those of mammals, but the conservation of the Vps34 complex and Atg6 from yeast to humans may be considered evidence against this possibility.

Our data suggest that defects in cell quality control that cause protein and organelle stress are not the sole reason for over-proliferation of *Atg6* mutant cells.

Although we observed accumulation of Ref(2)P (p62) and the ER stress protein Hsc3, a loss of function allele of *ref(2)p* did not suppress melanotic mass formation in *Atg6* mutant larvae (Fig. 3-8E). These data suggest that Ref(2)P accumulation is not required for over-proliferation of cells lacking *Atg6*.

Due to the difficulty of studying cell-cell interactions in circulating hemocytes, we further investigated $Atg6^{1}$-associated over-growth in two adult tissues, the eye and the ovarian follicle cells. *Atg6* mutant cells were over-represented compared to neighboring control cells in the mosaic eye. Experiments in the third larval instar eye imaginal disc, the precursor tissue to the adult eye, revealed the presence of both wild type and *Atg6* mutant cells (data not shown). Whether *Atg6* mutant cells simply over-proliferate enough to outcompete wild type cells or they "win" by inducing apoptosis of wild type cells is undetermined.

Experiments in the ovarian follicular epithelium have revealed a novel defect in *Atg6* mutant cells. When homozygous $Atg6^{1}$ mutant cell clones are induced in follicle cells, they form multilayered epithelia and invade neighboring cells (Shravage, Hill, Baehrecke, data not shown). This invasiveness is associated with decreased levels of the adherens junction protein E-cadherin and septate junction protein Discs Large (Dlg). Altered E-cadherin and Discs Large have been implicated in invasive cells including the epithelial to mesenchymal transition that has been associated with cancer metastasis and poor clinical outcome (Pagliarini and Xu, 2003; Thiery, 2002). Significantly, Atg6 has not previously been shown to regulate epithelial cell polarity. Whether this disruption of polarity is either a cause of or a secondary effect of *Atg6* mutant cell invasion is unknown. Loss of function mutations in other autophagy genes, such as *Vps34* or *Atg1*,

do not cause similar phenotypes in the follicular epithelium, but homozygous *vps15* mutant cell clones do display reduced levels of both E-cadherin and Dlg, as well as invasion of neighboring cells. The fact that *vps15* follicle cell clones exhibit a phenotype distinct from *Vps34* follicle cell clones is surprising, as Vps15 activity regulates Vps34 activity. Furthermore, Vps15 interacts directly with Vps34 in immunoprecipitation experiments, but not with Atg6. It is possible that Vps15 and Atg6 function in a Vps34 independent pathway to regulate cell architecture. Vps15 encodes an understudied protein kinase; it is possible that it could have numerous substrates and that Atg6 could influence Vps15 activity in a Vps34-independent manner. Both *Atg6* and *vps15* mutant cells exhibit nuclear localization of the NFκB proteins Relish (Fig. 3-6) and Dif (Fig. 3-7) in the fat body, and this phenotype seems to be independent of autophagy. We hypothesize that NFκB activation does not contribute to overgrowth phenotypes associated with loss of *Atg6*, as *vps15* mutant cells are not over-represented compared to control cells in the eye. In fact, *vps15* mosaic eyes are very small.

We have presented several results which suggest that autophagy dysfunction in *Atg6* mutant cells is not responsible for over-proliferation phenotypes that we observe. We also showed that *Atg6* is required for endocytosis (Fig. 3-2I). Multiple *Drosophila* tumor suppressor genes function as endocytic regulators. Rab5, Avalanche (Avl), Erupted (Ept), and Vps25 function at different steps of endocytic trafficking, but mutations in the genes encoding any of these proteins results in disruption of cell polarity and overgrowth of epithelial cells (Herz et al., 2006; Herz et al., 2009; Lu and Bilder, 2005; Moberg et al., 2005; Vaccari and Bilder, 2005). These phenotypes were attributed to ectopic Notch signaling due to a defect in endosomal maturation of internalized Notch to the lysosome.

It is possible that Atg6 similarly regulates growth and/or proliferation signaling through its function in endocytosis, and *Atg6* mutant proliferative phenotypes result from a defect in endocytosis of signaling receptors. Consistent with this possibility, Notch signaling is important in ovarian follicle cells at the stage where *Atg6* mutants have defects (Xu et al., 1992). However, it should be noted that *Atg6* mutant fat body cells had little if any endocytosis (Fig. 3-2I). These data suggest that the *Atg6* mutant defect in Notch signaling may be different from *vps25* mutants, which exhibit partial endocytosis and traffic Notch to multivesicular bodies where they signal and fail to be degraded by never reaching the lysosome (Herz et al., 2006).

This study has shown that loss of *Atg6* results in dysregulation of growth in three different tissues. In a physiological context, Atg6 regulates hemocyte number. The increase in hemocyte number in *Atg6* mutant larvae does not appear to be associated with overgrowth of other larval tissues, and the cellular signaling pathways that induce hemocyte over-production have not been identified. Mosaic analyses in the eye allowed us to follow *Atg6* mutant cells through development, and we observed that *Atg6* mutant cells are overrepresented relative to wild type cells in the adult eye. Though the cellular pathways which regulate this phenomenon are yet to be determined, we propose that the function of Atg6 in cell competition is independent of its function in autophagy. Finally, clonal experiments in the follicular epithelium revealed a novel function for *Atg6* in regulating epithelial cell architecture. Future studies will focus on investigating the cellular pathways involved in E-cadherin and Dlg down-regulation in *Atg6* mutant cells, and determining whether similar pathways contribute to overproliferation of blood cells in *Atg6* mutant larvae.

Author contributions

Bhupendra Shravage (University of Massachusetts Medical School) completed ovarian follicle cell experiments referred to in the discussion.

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Materials and methods

Generation of Atg6 targeting construct

Following the "ends-out" gene disruption approach (Rong and Golic, 2000), a targeting construct containing genomic sequence flanking the open reading frame of *Atg6*, separated by a white mini-gene, was created. This construct, engineered from pW35 (DGRC) also contained recognition sites for the yeast enzyme I-SceI and FRT sites to enable FLP-induced recombination. Restriction endonuclease recognition sites for BsiWI and KpnI were engineered into the upstream polylinker of pW35 between BamHI and AvrII sites. This linker was created using the oligonucleotide primers 5'GATCCACGTACGAGGTACCAC 3' and 5'CTAGGTGGTACCTCGTACGTG 3', which were annealed in equal amounts (500ng) using T4 DNA ligase. The double stranded linker was then ligated into the existing polylinkers of pW35. 3288bp of genomic sequence flanking the 5' end of the *Atg6* ORF was cloned into the TOPO-TA

vector using the PCR primers 5'GTACGTACGTACCAGAGTTG 3', which contains a BsiWI recognition site, and 5'GGGGTACCCCTGATAAGTTCAACGAACA 3', which contains a KpnI recognition site. This sequence was subcloned from TOPO-TA into the pW35 upstream polylinker using the BsiWI and KpnI restriction sites, to generate pW35-Atg6up.

3267bp of genomic sequence flanking the 3' end of the open reading frame of *Atg6* was cloned into the TOPO-TA vector (Invitrogen) using the PCR primers 5' ACATGCATGCATGTTAACCAACCTGAAATGGGGAT3', includes an SphI recognition site, and 5'ATAAGAATGCGGCCGCTAAACTATTTGCTTAACGCTGAGG 3', which includes a NotI recognition site. This sequence was subcloned, using the SphI and NotI restriction sites, from the TOPO-TA vector into the downstream polylinker of pW35-Atg6up. The resultant construct, pW35-Atg6, was sent to Best Gene, Inc. for injection and P-element mediated transformation of *w1118* flies.

Ends-out targeting of Atg6

Isogenic *w1118* animals were transformed with the targeting construct following standard procedures (Rubin and Spradling, 1982), resulting in integration into the genome. The resultant strain is known as the "donor" line. Donor flies, carrying the targeting construct on the second chromosome, were mated with flies containing heat shock-inducible FLP (hs-FLP) recombinase and I-SceI endonuclease, which induce double-stranded break followed by homologous recombination. FLP and I-SceI expression were induced in progeny of this cross by subjecting larvae to a one-hour heat shock in a 37°C circulating water bath. Recombination occurred between the targeting construct and the endogenous

genomic DNA sequence flanking the *Atg6* open reading frame, and the white mini-gene was inserted into the chromosome within the ORF of *Atg6*. Female recombinants were selected based on mosaic eye color, to eliminate solid white or red-eyed flies in which targeting did not occur, and were crossed with males carrying hs-FLP. These crosses were set up in several vials containing 3 females and 5 males. This step enabled selection of potential mutants based on eye color, as FLP recombinase only excised DNA flanked by FRT sites. Therefore, targeting events, which lack FRT sites after recombination, had solid red eyes, while flies in which the donor construct did not excise contained mosaic eyes at this step. Approximately 86,000 flies were screened, and 48 individuals had solid red eyes. Each individual was out-crossed to flies containing phenotypic markers of known genetic location in order to ensure that the mini-white gene mapped to the third chromosome. Two out of 5 lines mapped to the third chromosome, and PCR was used to confirm deletion of *Atg6* and insertion of mini-white.

Molecular confirmation of Atg6 targeting

Genomic DNA was collected from 7-15 wandering third instar larvae of the following genotypes: *w1118*, $Atg6^{1}/TM6B$, $Atg6^{1}/Atg6^{1}$. The standard Berkeley Drosophila Genome Project (BDGP) protocol for genomic DNA isolation from flies was followed. PCR was used to confirm targeting of the *Atg6* genomic locus. The PCR primers 5'ACTCTGAGATTGACCATCCG 3' and 5'GTTTGTCCAGCTGCTGTTTC 3' which amplify a 408bp region of *Atg6*, were used to confirm the absence of *Atg6* in targeted lines. The primers 5' GAGTAGCCGACATATATCCG 3', within *w*+, and 5'GCAATCAAATCGGTTACCATG 3', upstream of *Atg6*, were used to verify the

presence of the *w+* at the *Atg6* locus. The primers 5' CTTACTCTCGGCTTGGCTTG 3', which is downstream of *Atg6*, and 5' CACATGTACTACTCACATTG 3', within *w+*, were also used in PCR to confirm insertion of *w+* at the correct genomic location.

Reverse Transcription (RT)-PCR

RNA was collected from third instar larvae (n=10) using Trizol Reagent (Invitrogen) and was treated with DNase to remove genomic DNA contamination. cDNA was generated from 1μg of RNA, using Superscript II Reverse Transcriptase (Invitrogen), following standard protocols. cDNA was used as PCR template, using the following primers to amplify *rp49* (control), *Atg6*, and flanking gene sequence: *rp49*: 5'AAGATCGTGAAGAAGCGCAC 3' and 5'ATCTCCTTGCTTCTTGGAGG 3', *Atg6*: 5'CGAGCAGCTGGAGAAGATTAG3' and 5'GCGTTGATCTCTGACCAGTC3' 5', CG5991-RA: 5'CATTGCCTAATTGTGTCCGC 3' and 5'GGAGAATTGGCGCAAGTGAC 3', CG5991-RB: 5'GCACAGCGATACGGAAGCAA 3' and 5'GGAGAATTGGCGCAAGTGAC 3', CG5991-RC: 5'GCCTCTTCGCATTTGACGAC 3' and 5'GGAGAATTGGCGCAAGTGAC 3', CG5986: 5' GGCGATAACGCTTGCATCAC 3' and 5'CGTTGATATCCCGCAAACGG3'.

GFP-Atg8 Quantification

Quantification of GFP-Atg8 puncta was done as described previously (Juhász et al., 2008).

Endocytosis assay

Fat body was dissected from third instar larvae and incubated *ex vivo* with Texas Red-avidin (Invitrogen), diluted in Schneider's media to a concentration of 80μg/mL, for 20 minutes, then chased with cold PBS +0.5% BSA for 10 minutes prior to overnight fixation in 4% formaldehyde. The tissue was washed 3x10 min in PBS + 0.1% Tween-20 and mounted in Slow Fade (Invitrogen). Images were collected on a Zeiss LSM 510 confocal microscope.

Immunohistochemistry

Third instar larvae were fixed and dehydrated for histology according to published methods (Muro et al., 2006). Histological sections were de-waxed with a series of Xylene washes, then rehydrated through a series of decreasing percentage ethanol washes. Following rehydration, antigen retrieval was performed by heating slides in 10mM sodium citrate, pH 6.0. Specimens were blocked in 5% non-fat dry milk + 1% bovine serum albumin (BSA) + horse serum (Vector Laboratories). Rabbit α-GFP (Novus) antibody was used at a 1:500 dilution. The Vectastain Elite ABC kit (Vector Laboratories) was used for immunohistochemical detection, and the signal was visualized by diaminobenzadine staining. Tissue was counterstained with Weigert's hematoxylin and Permount mounting media was applied. Images were collected on a Zeiss Axiophot microscope.

Hemocyte quantification

Individual third instar larvae of similar age (+/- 3h) were bled into 20μL of 1X PBS.

10μL was loaded onto a standard hemocytometer and the average number of cells per milliliter was calculated for 20 animals per genotype. A one tailed student's t-test was used to determine statistical significance.

Antibody staining of tissues

Tissues were dissected in 1X PBS and fixed 30 minutes in 4% formaldehyde. Following fixation, tissues were washed with PBS + 0.1% Tween-20 (PBST), then blocked in PBST + 0.5% BSA (PBSBT) for 1.5-2h. Antibody incubations were done overnight at 4°C, then followed by washes in PBSBT. Secondary antibody was added at a dilution of 1:200, and tissues were incubated for 2h at room temperature. Following a series of short PBSBT washes, tissues were mounted in Slowfade (Invitrogen) or Prolong + DAPI (Invitrogen). The following primary antibodies were used: rabbit α-Relish (T. Ip), 1:1000; rabbit α-Dif (D. Ferrandon) 1:1000, rabbit α-Ref(2)p, 1:1000, Guinea pig α-Hsc3 (H. Ryoo), 1:50. The following secondary antibodies from Invitrogen were used: Goat α-rabbit Oregon Green 488, Goat α-rabbit Alexa Fluor 546. Goat α-guinea pig Alexa Fluor 546.

Chapter 4

Summary and Future Directions

Beclin 1 gene function is essential for development of animals that are as diverse as nematodes, fruit flies, and mice (Qu et al., 2003; Takacs-Vellai et al., 2005; Yue et al., 2003). As a central regulator of autophagy in the Vps34 protein complex, Beclin 1 is required for cell survival during nutrient restriction and oxidative stress. Unmitigated genome damage, brought on by a failure to remove misfolded proteins and damaged organelles, is thought to contribute to tumorigenesis in autophagy defective *beclin 1*$^{+/-}$ tissues (Degenhardt et al., 2006; Mathew et al., 2007). These studies also suggest that defective autophagy is tumorigenic due to a failure to degrade p62, an adaptor protein that regulates growth related signaling pathways (Mathew et al., 2009). While attributing tumor suppressor activity of Beclin 1 to its function in autophagy, this model does not account for the fact that other autophagy gene mutants, particularly in the Vps34 complex, do not display overgrowth/proliferation phenotypes. We sought to characterize the function of the Vps34 complex in *Drosophila melanogaster*, from a physiological context in the developing larva, and using a loss of function approach in adult epithelial tissues. We generated a null allele of the *beclin 1* homolog, *Atg6*, and found that it is required for autophagy and endocytosis. Further, we demonstrated that Beclin 1 also controls blood cell proliferation, and discovered a novel function for Beclin 1 in regulation of epithelial cell polarity. As cancer cells often display loss of epithelial cell

polarity, this finding represents an important contribution to the field of Beclin 1 research.

Regulation of autophagy by the Vps34 complex in *Drosophila*

I investigated whether the Vps34 complex functions in autophagy induction in *Drosophila*, using a cell biological approach to monitor autophagy induction in the larval fat body and a biochemical approach to investigate the physical interactions between Vps34, Vps15, and Atg6. Towards this goal, I generated several transgenic fly lines that enabled ectopic expression of Vps34, Atg6, and Vps15 (gift of L. Wu) (Fig. 2-1) via the UAS/Gal4 system (Brand and Perrimon, 1993). I found that co-expression of either Atg6 and Vps34 or Vps15 and Vps34 was sufficient for autophagy induction in the fat body of fed larvae (Fig. 2-2 and 2-3). However, expression of individual components of the Vps34 complex was not sufficient for autophagy induction in this tissue.

The Vps34 complex is known to act at early steps in the autophagy pathway to regulate formation of autophagosomes. These overexpression results suggest that a stoichiometric ratio of Vps34, Vps15, and Atg6 is required for ectopic autophagosome formation. It is possible that PI(3)P, generated by Vps34 PI3-kinase activity, is the limiting factor at this step of autophagy and thus overexpressed Atg6 is unable to induce autophagosome formation without additional PI(3)P. The only autophagy protein that is known to induce autophagy ectopically in *Drosophila* is Atg1, a kinase that regulates autophagy induction upstream of autophagosome formation via phosphorylation of TOR (Scott et al., 2007). In fact, ectopic Atg1 expression in the fat body induces cell death via a caspase-dependent pathway (Scott et al., 2007). As co-expression of Vps34 complex

proteins can also induce autophagy, future experiments could determine whether the Vps34 complex is required for Atg1-induced autophagy and cell death. Though these pathways have been genetically ordered in yeast, these experiments have not been done in flies and would significantly contribute to our understanding of autophagy regulation within an animal. Further, it is essential to demonstrate colocalization of Vps34, Vps15, and Atg6 inside the cell. Our results show that transgenically expressed Atg6-GFP forms intracellular puncta in the fat body during starvation (Fig. 2-4). For the most part, these puncta are distinct from lysosomes. Due to a lack of antibodies for immunofluorescence experiments, we were unable to assess sub-cellular localization of Vps34 and Vps15. Future efforts should focus on generating *Drosophila* specific antibodies, which will enable characterization of sub-cellular localization of these proteins in wild type animals. As *Vps34*, *vps15,* and *Atg6* mutant lines have now been constructed, the requirement of each for intracellular complex assembly under variable nutritional conditions could be assessed.

To study biochemical interactions between Vps34, Atg6, and Vps15, we generated flies that ectopically express epitope-tagged Vps34 and Atg6 and acquired flies that ectopically express tagged Vps15 (L. Wu). Biochemical analysis of the Vps34 complex confirmed that Vps34 and Atg6 physically interact *in vivo*. This interaction was independent of nutrient availability, as Vps34 and Atg6 co-immunoprecipitated in both fed and starved animals (Fig. 2-5A). Interestingly, Vps34 and Vps15 did not co-immunoprecipitate in fed animals and only weakly interacted in starved animals (Fig. 2-5B). Vps15 is thought to be the catalytic cofactor for Vps34, and the absence of Vps34-Vps15 interaction in fed animals suggests that Vps34 and Atg6 may interact to regulate

an alternative cellular process in the absence of Vps15. Vps15 is required for starvation-induced autophagy in the fat body (Lindmo et al., 2008). However, given these results, it would be interesting to test whether Vps15 is required for Vps34-Atg6 induced autophagy in fed larvae.

Vps34 was tagged with a tandem affinity purification (TAP) tag with the long-term goal of identifying novel interacting proteins via mass spectrometry analysis (Veraksa et al., 2005). TAP consists of two affinity purification steps in order to enrich true interactors and eliminate artifacts of immunoprecipitation. The UAS-Vps34 N- and C-terminal TAP-tagged flies can be utilized for a number of experiments. By utilizing tissue-specific Gal4, one could potentially identify different Vps34 complexes and determine which binding partners dictate specificity between autophagic and endocytic pathways. This approach could launch a multitude of functional studies, where mutant analyses could be used to functionally characterize the interactions between Vps34 and binding partners. These analyses would be a major contribution to the intracellular trafficking field, as we are only beginning to understand how the autophagic and endocytic machinery merge.

Atg6 function in autophagy and development

Due to the lack of an *Atg6* null mutant, we used an ends-out gene targeting approach to generate *Atg6* mutant flies. We found that *Atg6* is required for development, as homozygous $Atg6^{1}$ animals die during the third larval instar. This lethality was rescued by ubiquitous expression of an *Atg6* transgene. *Atg6* is likely required in multiple tissues, as transgenic expression in either the fat body, embryonic hemocytes, or larval hemocytes

did not rescue lethality associated with *Atg6*[1]. The cause of lethality in *Atg6* mutant larvae is unclear. It is possible that it is related to the function of *Atg6* in autophagy. In addition to starvation-induced autophagy, *Drosophila* also undergoes steroid hormone-induced autophagy in the fat body, salivary glands, and midgut during larval and pupal development (Rusten et al., 2004; Lee and Baehrecke, 2001; Lee et al., 2002). We observed a defect in autophagosome formation in the fat body of *Atg6* mutant wandering larvae (data not shown), the stage at which steroid-triggered autophagy occurs (Rusten et al., 2004). It is possible that a failure to remove larval tissues via autophagic degradation contributes to lethality associated with loss of *Atg6*. Due to the larval lethality of *Atg6*^{1}, we are unable to study pupal development of these animals. However, mosaic analysis would allow the simultaneous observation of mutant and wild type cells during pupal development. Future studies could focus on determining whether developmental autophagy in the midgut and salivary glands depends on Atg6 function in these tissues. Specifically, we could generate *Atg6*^{1} clones in the salivary gland and use histological techniques to monitor histolysis. RNAi could also be used to knockdown *Atg6* expression tissue specifically to determine where its function is required for viability. This approach would also allow simultaneous tissue specific knockdown of *Vps34* and *vps15*, enabling investigation of the role of the Vps34 complex in developmental autophagy.

Alternatively, *Atg6*^{1}-associated lethality could be due to the defect in endocytosis. Endocytosis function is important in regulation of growth and differentiation signaling pathways, as internalized receptors can signal from within an endosome, or alternatively be down-regulated by maturation through the endolysosomal pathway (Zwang and Yarden, 2009). One example is the receptor Notch, which, along with its ligand Delta, is

endocytosed and trafficked to different cellular compartments for activation of downstream growth signaling pathways, including TGFβ. The exclusion of TR-avidin from *Atg6* mutant fat body cells (Fig. 3-2) suggests that the endocytic defect occurs at internalization. It is possible therefore that receptors essential for developmental signaling, such as Notch and EGFR, are not internalized by *Atg6* mutant cells. In turn, defects in endosomal Notch activation could lead to developmental defects that prevent the animal from developing into an adult. To test this possibility in the future, antibodies for these receptors and their ligands could be utilized in immunofluorescence studies of mosaic *Atg6* tissues.

Finally, it is also possible that Atg6 regulates an essential Vps34-independent process. A recent proteomic study identified a number human Vps34 complex-interacting proteins, 20 of which share some sequence identity with *Drosophila* proteins (Table 4-1) (Behrends et al., 2010). The functions of these proteins include chromosome maintenance, fatty acid transport, binding to damaged DNA, and growth factor signaling. Available mutant lines could be collected in order to test genetic interactions with *Atg6*. Specifically, mutants could be screened for suppression of $Atg6^{1}$-associated lethality and/or melanotic tumor formation. This approach could possibly lead to the discovery of a new cellular function for Atg6.

Human Protein	Function	Drosophila homolog (putative)	% amino acid identity
PTOV1		CG13609	30%
C13orf18			
GSPT1	Eukaryotic peptide chain release factor GTP binding (translation termination)	Elf (CG6382)	75%
ZWINT			
TBC1D87	Rab GTPase activator?	CG6182	32%
AFG3L2	ATPase, metalloendopeptidase	CG6512	63%
SMC1A	DNA binding, structural maintanence of chromosomes	SMC1	49%
TRABD			
SLC27A4	Fatty acid transport protein	Fatp CG30194	48% 46%
I1L	Nuclearporin (cell size, Pvf pathway)	Nup44a	58%
CPVL	Serine carboxypeptidase activity	CG4572	44%
TP53BP2	Tumor protein, p53 binding		
NRBF2	Nuclear receptor binding factor		
KIAA0831	Atg14/Barkor	CG11877	28%
PTPRA	Protein tyrosine phosphatase		
TGFBRAP1	TGF-beta receptor associated		
VPS33A		Carnation	43%
SMC3	Chromosome associated protein	Cap	53%
VPS18		Deep orange (dor)	34%
UVRAG			
DDA1			
DDB1	Damaged DNA binding protein	piccolo	60%
ATG4B			
KIAA0226/Rubicon			
PIK3R4/p150	Kinase, immune response	Vps15/Ird1	40%
PIK3C3/Vps34	Class III PI3K	Vps34	63%

AMBRA1			
ATG3		Aut1	63%
MAP1B	Microtubule associated protein	futsch	31%
CLPTM1L		CG4332	47%
USP11	Ubiquitin specific peptidase		
TSC1	Growth inhibition	Tsc1	32%
DZIP3	DAZ interaction protein, zinc finger		

Table 4-1. Human proteins that interact with Beclin 1 and their putative *Drosophila* homologs.

Behrends et al., identified Beclin 1-interacting proteins using affinity purification of epitope tagged-Beclin 1 and mass spectrometry (Behrends et al., 2010). The putative functions of these genes, as assigned by NCBI, are listed in the second column. Putative *Drosophila* homologs were identified by Homologene (NCBI) search and BLASTp analyses.

Growth phenotypes associated with *Atg6* deficiency

Increased hemocyte numbers

Our results showed that *Atg6* mutants possess excess blood cells in the developing larva. $Atg6^{1}$ larvae contained nearly 10 times as many hemocytes as wild type larvae (Fig. 3-4D). This is consistent with knockout mouse studies, which showed that mono-allelic loss of *beclin 1* resulted in increased incidence of lymphoma compared to $beclin\ 1^{+/+}$ mice (Yue et al., 2003; Qu et al., 2003). $Atg6^{1}$ larvae also contained hemocyte-associated melanotic tumors (Fig. 3-3C). Mutations in genes that regulate the cell cycle, chromatin structure, cell death, and immunity pathways have been associated with melanotic mass formation (Minakhina and Steward, 2006). These include loss of function mutations in the inhibitor of kappa B (IκB), *cactus*, and activating mutations in the Janus kinase (JAK) gene *hopscotch* (*hop)* and in the gene that encodes the transmembrane receptor Toll (Hanratty and Dearolf, 1993; Qiu et al., 1998; Zettervall et al., 2004). These signaling pathways can contribute to melanotic mass formation by inducing precocious differentiation of blood cell precursors in the lymph gland (Avet-Rochex et al., 2010).

As we have yet to determine which signaling pathway(s) is involved in overproliferation of hemocytes and melanotic mass formation in $Atg6^{1}$ larvae, future studies should focus on that goal. Specifically, a genetic modifier screen could be initiated to identify mutants that suppress melanotic mass formation in an *Atg6* mutant background. We could utilize two approaches: either a classic dominant or recessive modifier screen of second or third chromosome mutations, or a tissue specific RNAi screen, using UAS/Gal4 driven inverted repeat sequences to knockdown gene expression

in the hemocytes. The benefit of a classic mutant screen is that it would enable isolation of mutations that may have a tissue non-autonomous effect on melanotic mass formation. However, we might also miss genes that are lethal prior to the third larval instar, when melanotic tumor formation occurs in *Atg6* mutant animals. On the other hand, an RNAi screen would be inclusive of genes required for embryonic and early larval development, as knockdown would only be targeted to hemocytes. However, it is possible that the melanotic tumor phenotype is caused by a defect in a non-hematopoietic tissue.

In addition to screening for modifiers of the melanotic tumor phenotype in *Atg6* mutant larvae, future studies should also focus on determining the impact of *Atg6* deficiency on the hematopoietic stem cell niche. Hemocytes originate from two distinct lineages. One pool of hemocytes originates in the embryo and persists through larval stages, while the other pool originates in the larval lymph gland (Holz et al., 2003; Tepass et al., 1994). In the absence of infection, hemocytes made by the larval lymph gland are released into circulation during pupal development (Holz et al., 2003). Therefore, the overproliferation of hemocytes in *Atg6* mutant larvae could occur as early as embryogenesis, or it could be the result of ectopic early release of hemocytes from the lymph gland. Hemocyte quantification at earlier developmental stages may shed light on the kinetics of hemocyte overproliferation. RNAi could be used to knock down *Atg6* in embryonic hemocytes, followed by quantification of larval hemocytes. These studies would provide valuable information about the timing of hemocyte proliferation in *Atg6* mutants.

In addition to determining the kinetics of hemocyte overproliferation, cell biological characterization of the lymph gland is essential. Interestingly, E-cadherin is

highly expressed in the immature, undifferentiated cells of the secondary lobe of the lymph gland, and is down-regulated in cells that express markers of mature hemocytes (Jung et al., 2005). Given our data showing reduced E-cadherin in *Atg6* epithelial cell clones, it is possible that down-regulation of E-cadherin induces precocious differentiation of hemocyte precursors in the lymph gland of *Atg6* mutant animals. We could test this hypothesis by generating $Atg6^{1}$ lymph gland clones and staining the mosaic tissue with an antibody against E-cadherin and the mature hemocyte markers hemolectin or lozenge.

Ectopic overgrowth induced by mosaic loss of Atg6

Consistent with whole animal mutant analysis, our mosaic adult eye model suggests that loss of *Atg6* confers a growth advantage to cells. We showed that FLP mediated induction of $Atg6^{1}$ clones in the eye resulted in an adult eye composed almost entirely of mutant cells. We have so far been unable to determine when $Atg6^{1}$ cells become over-represented in the developing eye. Our studies have focused on the third instar imaginal disc, the larval precursor of the adult eye. Future experiments will examine $Atg6^{1}$ mosaic discs during pupal development, utilizing GFP to mark wild type cells, which will allow us to determine the precise stage where $Atg6^{1}$ mutant cells dominate the eye. After determining that time point, cell proliferation and cell death assays can be used to explore the possibilities of $Atg6^{1}$ cell over-proliferation or wild type cell death. It is possible that both occur. In addition, these experiments should allow us to distinguish if either *Atg6* homozygous or heterozygous cells are responsible for displacing wild type cells in the developing eye. Further, as the adult eye phenotype is

easily distinguishable, a genetic screen for modifiers of this phenotype could be utilized to find mutants that enhance or suppress the phenotype. A screen of this nature would identify genes that potentially interact with *Atg6* in growth control and the screen could be followed by functional characterization of modifiers.

Follicle cell clone experiments have provided a novel potential mechanism for $Atg6^{1}$-associated growth phenotypes. Down-regulation of E-cadherin and Dlg contribute to invasiveness of $Atg6^{1}$ cells in the follicle cell egg chamber (Shravage, Hill, Baehrecke, data not shown). Current studies are investigating whether these epithelial cell polarity defects are associated with defects in endocytosis of activated signaling proteins, such as Notch. Notch signaling is required for differentiation and migration of follicle cells during oogenesis (Deng et al., 2001; González-Reyes and St Johnston, 1998). At oogenesis stage 10, where the aforementioned experiments were conducted, Notch signaling is required for adherens junction remodeling (Grammont, 2007). The implication of Atg6 in endocytosis regulation may indicate that a potential non-autophagic process is responsible for overgrowth and proliferation phenotypes seen in *beclin* $1^{+/-}$ tissues. In the long term, a genetic screen for modifiers of the $Atg6^{1}$ follicle cell invasion phenotype may provide more clues about how Atg6 regulates cell polarity.

In conclusion, our studies highlight the complexity of Atg6 function in the cell. We have shown that like its mammalian homolog Beclin 1, Atg6 is required for development and autophagy induction. Using *Drosophila* to study the consequences of *Atg6* loss in a whole animal, we generated a null allele of *Atg6*, and showed that *Atg6* is required to restrict blood cell over-production. Mosaic mutant cell analyses in the larval fat body allowed us to investigate pathways previously implicated in *beclin* $1^{+/-}$-

associated tumorigenesis, and we found that *Atg6* mutant cells accumulated cell stress markers and display nuclear localization of NFκB proteins. However, we do not attribute blood cell overproduction and melanotic tumor formation to activation of these pathways, as loss of function mutations in NFκB genes do not suppress melanotic tumor formation in *Atg6* mutant larvae. Mosaic analysis in the adult eye and ovarian follicle cells provided additional evidence that *Atg6* mutant cells might have a proliferative advantage over wild type cells, as they entirely compose the adult eye and form multi-layered invasive growths in the follicular epithelium. As yet, we have not identified the signaling pathways involved in $Atg6^{1}$-associated phenotypes. However, we identified a novel role for Atg6 in regulation of epithelial cell polarity. This function seems to be independent of the function of Atg6 with Vps34, and it would be interesting to test if Vps34 activity is required for disruption of cell polarity in *Atg6* mutant follicle cells. Collectively, these data suggest a novel mechanism by which loss of *Atg6* might contribute to mis-regulation of cell proliferation pathways.

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