Autophagic cell death during *Drosophila* embryogenesis

by

Olga Cormier

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Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis including any required final revisions, as accepted by my examiners.

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Abstract

The amnioserosa (AS) is an extraembryonic tissue that undergoes programmed cell death (PCD) during the normal course of *Drosophila* embryogenesis. AS degeneration involves morphological evidence of autophagy as well as caspase activation, but the relationship between these two processes is not well defined. While the bulk of the AS tissue dies at the conclusion of the morphogenetic process of dorsal closure (DC), approximately 10% of AS cells are actively extruded from the epithelium during DC. Using live imaging confocal microscopy and various fluorescent protein sensors, I have been able to observe caspase activation as well as autophagy upregulation in the context of epithelial extrusion events as well as overall AS degeneration. The data show that epithelial extrusion events are caspase-dependent but are also associated with localized onset of autophagy. Furthermore, extensive characterization of loss of function mutants of the key *Drosophila* regulator Atg1 kinase indicates that autophagy is not required for the normal degeneration of AS, contrary to earlier studies. This thesis also introduces new relationships between caspase activation and autophagic cell death. In addition, new data suggest that the InR/TOR and EGFR/Ras/MAPK signaling pathways interact with the pro-apoptotic protein Head involution defective (Hid) and Atg1 kinase to regulate the progression of programmed cell death in the AS.

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Table of Contents

List of Figures

List of Tables

List of Abbreviation

Akt	Protein Kinase B
AS	Amnioserosa
Atg	Autophagy Related
BIR	Baculovirus IAP Repeat Domain
DC	Dorsal Closure
DER	Drosophila Epidermal Growth Factor Receptor
dib	*disembodied*
DT	Dually Tagged (with GFP and mCherry)
eGFP	Enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
FLP	Flippase
FRT	Flippase Recognition Target
GAL4	Galactose 4 transcription factor from *Saccharomyces cerevisiae*
GBR	Germ Band Retraction
hid	*head involution defective*
IAP	Inhibition of Apoptosis Protein
InR	Insulin Receptor
JACoP	Just Another Colozalization Plug-in
JNK	Jun Kinase
M/Z	maternal/zygotic mutant
MAPK	Mitogen Activated Protein Kinase
NLS	Nuclear Localization Signal
PCD	Programmed Cell Death
PI3K	Phosphoinositide 3-kinase
RFP	Red Fluorescent Protein
RGH	Reaper, Grim, Hid (a group of pro-apoptotic proteins)
spo	*spook*
sSpi	Secreted Spitz
TEM	Transmission Electron Microscopy
TGF-β	Transforming Growth Factor Beta
TOR	Target of Rapamycin
UAS	Upstream Activating Sequence
UBI	Ubiquitin

Chapter 1. Introduction

1.1 Amnioserosa

Three main morphogenetic events occur during *Drosophila* embryogenesis and these are germ band extension (GBE), band retraction (GBR), and dorsal closure (DC). During the process of GBR, the tail moves towards the posterior of the embryo and reveals a dorsal hole covered by a stretched epithelium, the extra embryonic amnioserosa (AS) (1). Following GBR, the amnioserosa pulls the two lateral epidermal sheets over the dorsal hole and these sheets fuse along the dorsal midline to produce a continuous sheet of epidermal cells (1). At the conclusion of DC, the internalized AS degenerates and this degeneration coincides with innervation of the somatic musculature, which can be visualized by the onset of movement in the developing embryo.

The AS is essential for the morphogenetic process of GBR. Experimental evidence indicates that precocious degeneration, malformations or absence of the AS leads to a failure in GBR and this results in embryonic lethality. The amnioserosa is also essential for DC (1). During dorsal closure, the AS generates contractile force, and may also function in cell-cell signaling that coordinates morphogenesis (1-3). The leading edge cells of the lateral epidermis, those epidermal cells that are immediately adjacent to the AS, develop a myosin-actin contractile structure to assist in the closing of the dorsal hole. This "purse-string" like mechanism is thought to be one of the main forces behind dorsal closure (3). During DC a secondary source of contractile force is generated within the AS itself. The maintenance of AS contractile force involved the removal of approximately 10% of AS cells, which undergo epithelial delamination

and are actively extruded from the basal surface of the epithelium. Many extruded AS cells are actively engulfed by the underlying yolk (3). Extrusion events are characterized by the reduction of the extruded cell's apical surface, while the neighboring cells remain largely unchanged. This process leads to a decrease in AS surface area connected to the adjacent epithelia, which consequently brings the two epidermal sheets together.

Although AS plays an important role in coordinating GBR and DC, it appears to have no further purpose following the completion of DC, consequently it undergoes degeneration. This degeneration is coordinated by programmed cell death (PCD), which involves both autophagy and caspase activation (4). PCD can be perturbed by various mutations as well as ectopic overexpression, and depending on whether the effect is one of pro-death or pro-survival, the AS may degenerate prematurely or it may also persist well beyond its normal time of degeneration. In either scenario such disruptions are often associated with failures in GBR or DC. Due to its relatively flat shape, large cells and availability of genetic tools, the AS is ideal for live imaging confocal microscopy; as such it represents a good model system for analysis of PCD and permits detailed *in vivo* characterization of the role of PCD during development.

1.2 Programmed Cell Death

In general, programmed cell death (PCD) encompasses the various ways in which cells can die in an organized and genetically controlled manner. In *Drosophila* there are two well characterized general mechanisms in which PCD can occur, seen in Figures 1 and 2 (5). Type I PCD is associated with apoptosis-type death and requires the activation of the family of proteases known as caspases. Type II PCD is an autophagic cell death, which involves the formation and activity of the subcellular structure known as the autophagosome. Both types of cell death are characterized by the morphology and behavior of cells following a death stimulus, as described below.

1.2.1 Apoptosis

Apoptotic, or Type I, cell death is associated with specific changes in cell morphology, including the contraction of the cytoplasm, chromatin condensation, membrane blebbing and the eventual engulfment by phagocytic cells such as macrophages (Figure 1A) (5). In many systems, the activation of apoptosis involves signals to and from the mitochondria, such as Cytochrome C release, however this has not been observed in *Drosophila* (5). In *Drosophila*, the onset of apoptosis is a direct consequence of the activation of caspases through the action of pro-apoptotic proteins. In order to bring about the apoptotic response, apical (initiator) caspases recruit and promote proteolytic cleavage of the effector caspases, thus activating them. The activated effector caspases cleave substrates that ultimately result in apoptotic morphology. The number of identified caspase substrates is presently in excess of 350 and among these substrates are structural proteins, such as actin, transcription factors, and other kinases, such as Raf-1 (6).

To regulate the onset of apoptosis, Inhibitor of Apoptosis (IAP) proteins ubiquitinate apical caspases as well as directly bind them via BIR domains, thereby preventing pro-apoptotic

activity. When apoptotic signals are present, IAPs are degraded, allowing the apical caspases to initiate the apoptotic response (Figure 1B) (5). In *Drosophila*, three apical caspases (Dronc, Dcp-1, Drice) and two IAPs (Diap-1, Diap-2) have been characterized. During the induction of apoptosis, three proteins, Reaper, Hid, and Grim (RHG) act as pro-apoptotic signals. When apoptosis is induced, Reaper, Hid, and Grim protein expression is increased, and RHG ubiquitinate the IAPs targeting them for degradation (5). RHG can also compete with caspases for IAP's BIR domains thus releasing bound caspases. Since IAPs can no longer function, Dronc, Dcp-1 and Drice, undergo Dark-dependent activation and activate the effector caspases, leading to the manifestation of apoptotic morphology (Figure 1 A,B). Due to the direct role that caspases play in apoptotic cell death in *Drosophila*, experimentally attenuating their activation, i.e. by overexpressing a baculoviral p35 protein, can prevent the onset of apoptosis (5).

1.2.2 Autophagy

Autophagic, or Type II, cell death is characterized primarily by the presence of the autophagosome or autophagolysosome in the cell (Figure 2B)(7). Generally, autophagy is thought of as a cell survival mechanism, as it promotes recycling and clearance of old organelles and large protein aggregates. A growing body of evidence indicates that autophagy can also be used as a cell death mechanism, both independent and in conjunction with apoptotic cell death. A feature of exclusive autophagic cell death is tissue degeneration in the absence of caspase activation, such as when viral p35 protein is expressed (8).

The genetic analysis of autophagy regulation in *Saccharomyces cerevisiae* has identified about 20 autophagy-related (Atg) proteins (9). However a recent review describes over 35 genes encoding specifically autophagy-related proteins (10). Ultimately, the activity of Atg gene products results in the formation of the autophagolysosome (Figure 2 A,B). Autophagolysosome

formation requires the Atg12-Atg5 complex, the Atg8-phosphatidylethanolamine (PE) conjugate, Atg1 kinase, Atg4 and the lysosome. Formation of the complex between Atg12 and Atg5 requires Atg7, Atg10 and ATP, whereas the formation of the Atg8-PE conjugate requires Atg 4, Atg7 and Atg3 (7). Atg4 is responsible for both processing Atg8 for conjugation, as well as cleavage of Atg8-PE to release Atg8 from the autophagosome membrane. Notably, the association of Atg12 with Atg5 is so rapid that free Atg12 protein can only be detected in trace amounts. The steps required for the autophagolysosome to form are shown in Figure 2A. Both Atg12-Atg5 and Atg8-PE complexes are required in order to form the isolation membrane around the material that is to be degraded. Atg1 kinase is also required in order to activate and promote the formation of the autophagosome, and is generally thought to be the key regulator of autophagy (7). Prior to the completion of autophagosome formation, membrane-associated Atg8-PE is cleaved by Atg4, and the Atg12-Atg5 complex dissociates from the membrane. Some unknown proportion of Atg8a remains within the autophagosome. The mature autophagosome fuses with a lysosome in order to complete its transformation into an autophagolysosome. In contrast to apoptotic cell death, cells undergoing autophagy are generally not engulfed by phagocytic cells, but are degraded within their own lysosomal compartments (7).

Autophagic regulation is known to be controlled by the Insulin Receptor (InR)/Target of Rapamycin (TOR) pathway since autophagy is involved in the starvation response (Figure 2C) (11-13). Activation of InR leads to the activation of PI3K and subsequent activation of Akt. Akt is able to directly activate TOR leading to an inhibition of Atg1 and Atg8, and consequently autophagy. Although there is some evidence that there may be a TOR-independent regulation of Atg1, so far no conclusive evidence has been presented, and Atg1 and Atg8 remain as key regulators of autophagosome formation. Limited research in other *Drosophila* tissues suggests a

possibility of autophagy regulation via Hid or TGF-β (Figure 2C) (14, 15). Additionally, the InR pathway is thought to regulate apoptotic activation via transcription factor, Foxo, although this connection is not well described (16). The interplay between JNK signaling involving JNKK, JNK and AP-1, and InR pathways may also be possible (as seen in Figure 2C) however this regulation still requires further study. In addition, the overexpression of Ras may activate PI3K although whether this occurs endogenously is still unclear (16, 17). Overall, the regulation of autophagy and autophagic cell death is a complicated network of pro-death and pro-survival signals whose feedback mechanisms are, at present, not entirely clear.

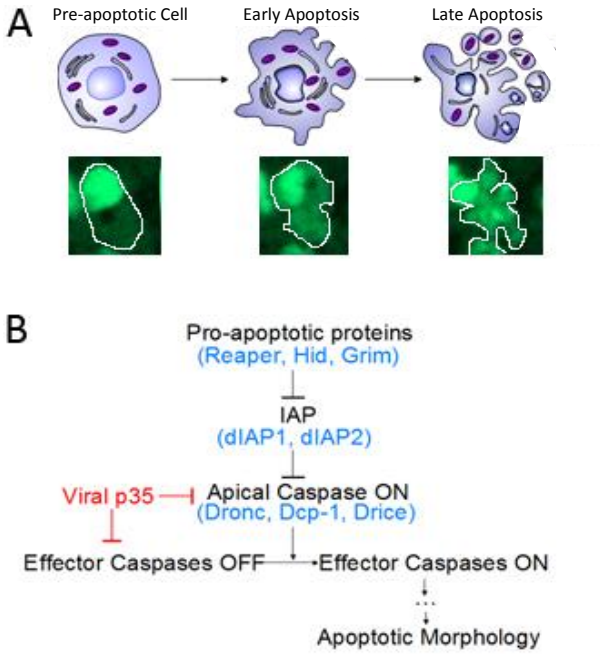

Figure 1: Mechanisms and regulation of apoptotic cell death. Apoptotic cellular morphology (A) as seen by nuclear GFP, is the consequence of caspase activation (B). Adapted from O'Day, 2006.

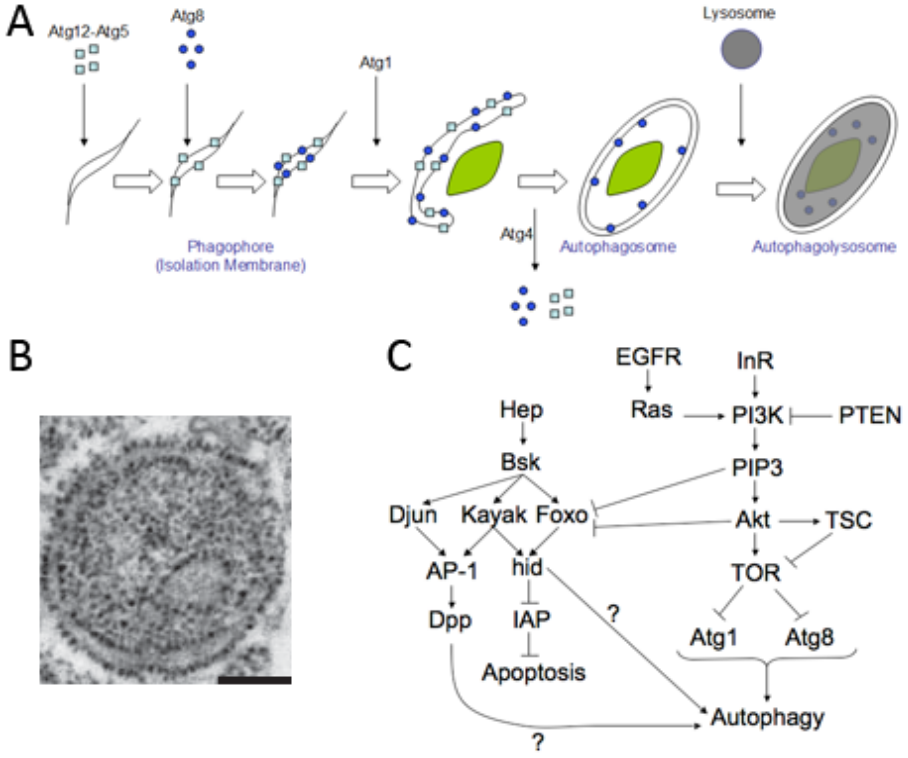

Figure 2: Mechanisms and regulation of autophagic cell death. Autophagic cell death is the consequence of autophagolysosome (B) formation (A) and is regulated by InR/TOR and other pathways (C). Adapted from Pattingre et al., 2008 and Cormier et al., 2012.

1.3 EGFR signaling

Epidermal growth factor receptor (EGFR) is implicated in many developmental and cell growth pathways in various species including *Drosophila*. When activated by a ligand, EGFR can activate Ras which activates the MAPK response. Among many consequences of MAPK activation, the inhibition of Hid, an RGH proapoptotic protein, can occur. EGFR has several known ligands in *Drosophila*, including Argos, Sprouty and Spitz (18). Transduction of EGFR signaling is dependent on Drk and SOS. Ultimate activation of Ras, can lead to several fates, one being activation of MAPK. Thus, the activation of EGFR leads to prevention of cell death, which is consistent with its role in cell proliferation (Figure 3) (18). Additionally, some evidence suggests that Ras can directly activate PI3K response which brings about the inhibition of autophagy (Figure 1E) (17). EGFR has also been implicated in interacting with TGF-β (*decapentaplegic*) during cell death and differentiation that occurs during *Drosophila* wing development (19). Overall, this suggests that EGFR signaling may play an important role in PCD during DC.

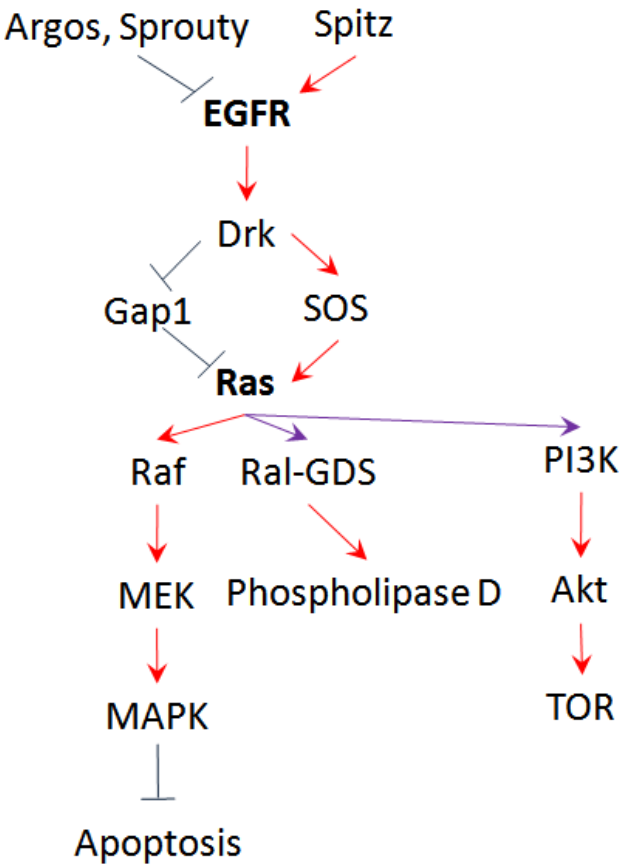

Figure 3: EGFR/Ras signaling pathway in *Drosophila.* Red arrows show known endogenous regulation, purple arrows show activation during ectopic expression of Ras. Adapted from Bergmann et al., 1998.

1.4 Head Involution Defective

Head Involution Defective (Hid) can act as a conventional pro-apoptotic protein, by ubiquitinating IAPs and biding their BIR domains to release apical caspases, leading to caspase activation and apoptotic morphology (Figure 4) (5). RHG pro-apoptotic proteins are thought to be mostly regulated transcriptionally. However recent studies show that Hid, in addition to transcriptional regulation, can be modulated by post-transcriptional modifications. Some of these modification are controlled by miRNAs such as miR-6,2/13 and *bantam*, which is itself under the control of Hpo tumour suppressor, and mRNA binding proteins such as, Nos (20). It is also known that Hid has several phosphorylation sites that regulate its function although the precise mechanism is unknown. The absence of phosphorylation is associated with over activity of Hid, meaning that endogenous phosphorylation likely attenuates Hid activity (18). One possible source of this phosphorylation is MAPK, which can be activated by EGFR/Ras pathway, attenuating cell death.

Although the apoptotic role of Hid is well characterized, literature suggests that it may also be involved in regulation of autophagic cell death (Figure 4). For example, in the larval fat body the overexpression of *hid* leads to an increase in autophagic structures (14). Furthermore in addition to EGFR, *hid* is thought to be regulated by JNK signaling via Fos and Foxo, which has been implicated in PCD regulation, making Hid an interesting candidate for examination (16). Overall, there is some indication that Hid can regulate PCD, both caspase-dependent and autophagic, however the detailed mechanisms by which Hid does so are still unclear.

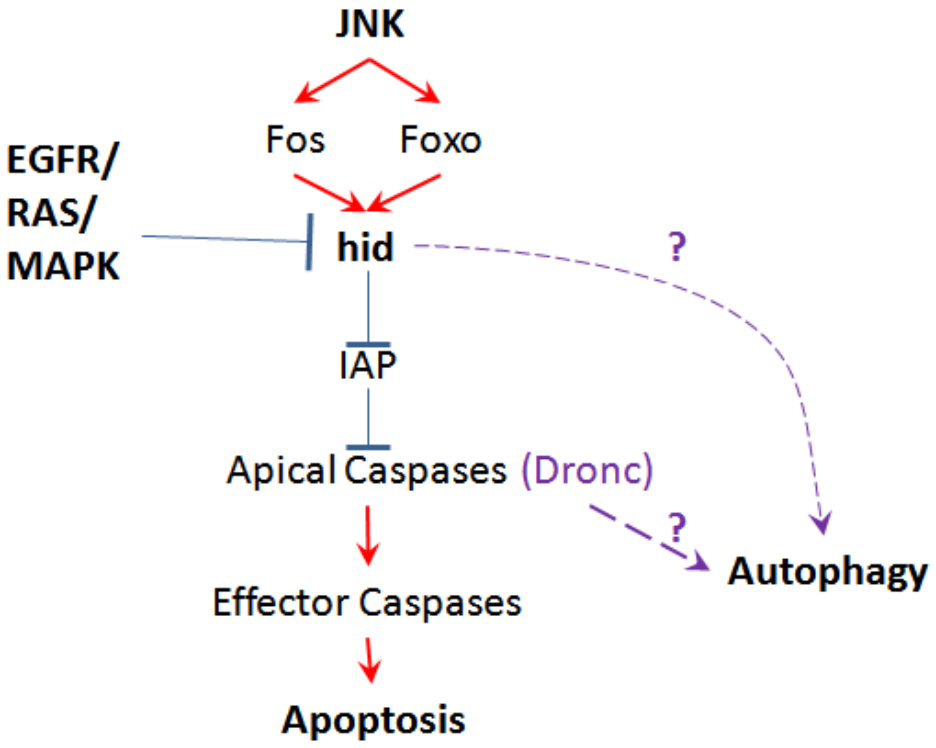

Figure 4: EGFR/Ras and JNK signaling pathways in *Drosophila* regulate the activity of Hid to inhibit or promote apoptotic death respectively. Hid is able to stimulate autophagy although whether the mechanism is direct or indirect is unclear (shown in purple).

Chapter 2. Materials and Methods

2.1 Confocal Live Imaging

Drosophila melanogaster embryos were collected at 25°C on grape juice agar overnight. Embryos were further selected using yolk autofluorescence to establish developmental stages using a Leica MZ 16 FA stereomicroscope. Prior to confocal imaging, the embryos were hand dechorionated as described previously (21). Embryos were placed in halocarbon oil on an inverted cover slip over a humidified imaging chamber. Time-lapse images were captured on Nikon Eclipse 90i fitted with a Nikon D-eclipse C1 scan head using Nikon EZ-C1 software at 20x or 40x objective, where appropriate. All image processing was performed using Nikon EZ-C1 software, as well as NIH ImageJ software where needed.

2.2 GAL4-UAS system

The GAL4-UAS expression system is adapted from yeast studies and is the standard method of inducible gene expression used by *Drosophila* researchers. The GAL4 protein is a transcription factor that specifically binds only to Upstream Activating Sequences (UAS) and thereby promotes transcription from downstream minimal promoters.

In *Drosophila*, GAL4 protein expression can be driven by endogenous enhancer sequences achieved by random generation of so called "enhancer trap" P-element transposons. Alternatively specific upstream enhancer elements have been used to design constructs that express GAL4 with a high degree of tissue and temporal specificity. In both cases, a large variety of GAL4 expressing ‘‘drivers’’ have been generated and are available from *Drosophila* resource

centers. For example, LP1-GAL4 is a driver that expresses the GAL4 factor in the spacial and temporal pattern of the gene *calderón* (*cald*) without disrupting this gene's normal endogenous expression. Gene *cald* is first expressed in the AS during the early germband retraction stage and continues through dorsal closure. An enhancer trap line, NP3312, places the expression of GAL4 transcription factor under the control of the *hindsight* enhancer sequence, which is activated prior to germ band retraction and continues to express until end of DC.

In *Drosophila*, the UAS sequence is only recognized by the exogenous GAL4 factor. Many "UAS-reporter" constructs have been created using the P-element transposon in order to overexpress or misexpress gene products at various times in development using the GAL4 drivers. Generally, the UAS sequence is placed upstream of the target reporter, this is sufficient to promote reporter gene expression by GAL4. Like the GAL4 drivers, thousands of UAS-reporters have been created and are also publicly available from *Drosophila* stock centers. Available UAS-reporters include cloned cDNAs for gene expression, RNAi constructs for knockdowns, and a variety of dominant negative proteins that permit the abrogation of numerous signaling pathways and cellular processes. Although some data indicate that RNAi constructs may not be functional in the presence of cell death (22).

2.2.1 Caspase indicator "Apoliner"

An *in vivo* caspase detection system known as *UAS-Apoliner* has previously been developed using the GAL4-UAS system (23). Apoliner consists of a transmembrane domain fused to an RFP protein, which is linked via a caspase sensitive site to an eGFP protein that contains a nuclear localization sequence (Figure 5A). Without caspase activity the chimeric RFP-GFP protein is maintained in association with membranes and RFP/GFP signals co-localize (seen in Figure 5B, white arrowheads). When caspases are activated, they cleave the caspase sensitive

site leading to the physical separation of the two fluorophores. As a consequence, RFP is maintained in the membranes, while the liberated GFP becomes localized to the nucleus due to its NLS (seen in Figure 5B, white arrows). Overall this indicates that Apoliner is a sensitive *in vivo* method to monitor caspase activation that can be expressed using any GAL4 driver.

2.2.2 GFP-mCherry-DrAtg8a

An *in vivo* detection system of autophagic activity has been developed previously (24). The construct GFP-mCherry-DrAtg8a (referred to as DT-Atg8a for **D**ual **T**agged Atg8a) is a fusion of an enhanced GFP and a red fluorescent protein, mCherry, as well as the *Drosophila* Atg8a protein (Figure 5C). As mentioned previously, Atg8a is an autophagy-related protein that is important for the formation of the isolation membrane and is targeted to this location following activation by proteolytic cleavage and lipidation. Hence, in the absence of autophagic activity, GFP and mCherry are localized to the cytoplasm and appear homogenous (seen in Figure 5D, white arrowheads). In the presence of autophagic activity, both GFP and mCherry signals will be first localized to the isolation membranes and appear punctate. Subsequently as the autophagosome fuses with a lysosome to form the autophagolysosome, the GFP becomes unfolded and loses fluorescence due to the acidity of the compartment, while the mCherry is still active (Figure 5D, white arrow). Technical limitations relating to speed of image acquisition make it difficult to produce colocalized mCherry/GFP puncta due to the rapid movement of these structures, hence often mCherry and GFP puncta will appear close together but not overlaid. Overall, UAS-DT-Atg8a is a sensitive detection system of autophagic activity in cells, as well as the autophagic flux.

2.2.3 ref(2)P-GFP

There are several known proteins that are routinely degraded via the autophagic route. One of these proteins is ref(2)P, the homologue to human p62. It is thought to target aggregated protein for degradation via the autophagic route and becomes degraded itself in the process. Previously described fusion between ref(2)P and GFP,*UAS-ref(2)P-GFP*, is used to assess whether autophagy is ongoing. The accumulation of ref(2)P-GFP protein indicates the lack of autophagy whereas the absence of ref(2)P-GFP indicates autophagic turnover of the protein is occurring normally (24).

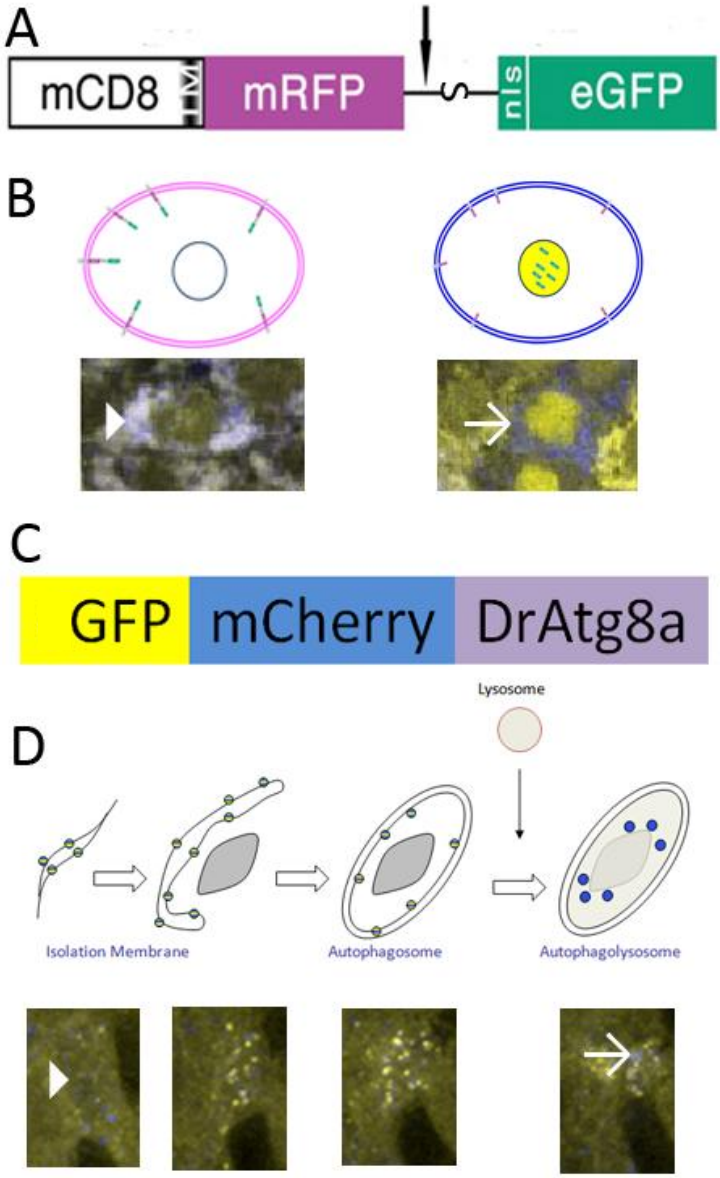

Figure 5: Select reporters used to analyze caspase activation and autophagy. The Apoliner construct (A) when expressed in the absence of caspase activation appears as white (B, white arrowhead) and it appears as a bright yellow nucleus with surrounding blue membranes when caspases are activated (B, white arrow). Dual Tagged (DT) Atg8a construct (C) appears smooth when no autophagy is ongoing (D, white arrowhead) and as white puncta during the isolation membrane/autophagosome stage when autophagy is activated. After the fusion with a lysosome, only the mCherry signal is visible as blue puncta (D, white arrow). Adapted from Bardet et al., 2008.

2.3 Generation of maternal/zygotic mutants

During *Drosophila* egg development, many transcripts necessary for early gene expression will be deposited into the egg by maternal loading. This means that in order to study the effects of such genes during embryogenesis, it is necessary to knock down both the zygotic copies and the maternally contributed transcripts. Atg1, a gene explored in this thesis (see Chapter 3), is a gene that is strongly maternally loaded (25). In order to construct an embryo that is a maternal/zygotic (m/z) mutant, a combination of FLP/FRT system and the ovo^{D} mutation is utilized (26). FLP/FRT is a system of targeted recombination commonly used in *Drosophila* and elsewhere. When flippase (FLP) is expressed it specifically induces recombination between two homologous FRT sites. The dominant mutation in the *ovo* gene, ovo^{D}, in the germ-line leads to sterile females that produce no eggs. The m/z mutant generation process involves generating a female fly that contains a copy of an inducible FLP and is heterozygous for the mutation of interest and the ovo^{D} mutation on the homologous chromosome with homologous FRT sites (Figure 6). Under conditions permissive for FLP expression, recombination between the FRT-bearing chromosomes can occur. If this occurs during mitosis in the germ-line as part of oogenesis, and recombination is successful, three types of germ-line clones may be produced (Figure 6). However only one of them will not contain the ovo^{D} mutation, this will give rise to an egg that is homozygous for the mutation of interest and lacks maternal transcripts of that gene. This female can then be crossed to a mutant male (or a heterozygous male for the mutation) to recover the m/z mutant embryos. Please see Appendix A for full stock descriptions and crossing scheme.

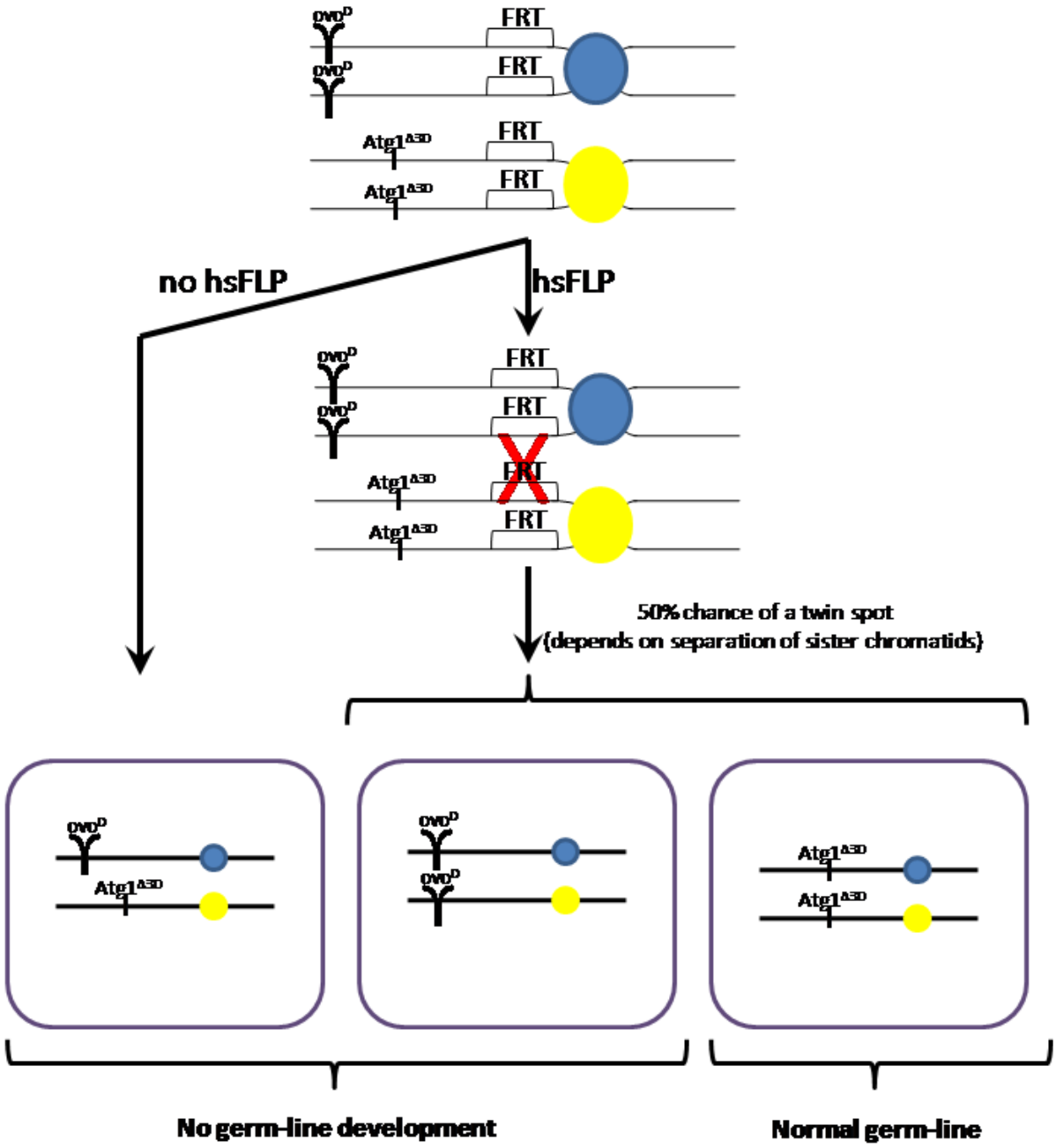

Figure 6: Generation of maternal/zygotic mutants using the FLP/FRT and *ovo*^{D} method. During mitosis in the germ-line FLP induces recombination between homologous FRT sites. If no recombination occurs or recombination occurs only between sister chromatids, then this will give rise to germ-line cells with a copy of *ovo*^{D} which renders that prevents normal germ-line development. If recombination does occur between homologous chromosomes, then there is a 50% chance, depending on the segregation of sister chromatids, that a twin spot will occur. A germ-line containing two copies of *ovo*^{D} will be sterile. Therefore the germ-line that is homozygous for *Atg1*$^{\Delta 3D}$ will be the only fertile germ-line and will produce embryos deficient in both zygotic and maternal copies of Atg1.

Chapter 3. Experimental Results

3.1 Evaluation of PCD in AS

The AS completely degenerates during normal embryonic development. The degeneration occurs partly during the morphogenetic process of dorsal closure, during which about 10 % of AS cells are extruded from the epithelium. At the onset of these studies I wished to determine if the rate of AS cell extrusion could represent a sensitive and useful assay for the onset and execution of PCD. Extrusions were easily scored in embryos carrying the *Ubi-DE-Cadherin-GFP* construct, which expresses *DE-Cadherin-GFP* under the control of the Ubiquitin promoter. This construct results in robust GFP localization to the apical surface of all epidermal cells, and the AS cells have particularly strong expression and localization of DE-Cadherin-GFP. An extrusion event was recognized by the rapid reduction of apical surface of the extruding cell with respect to its neighbors, typically forming a distinctive rosette pattern (Figure 7A). To control for staging and variation in the total AS cell number, the number of extrusions was expressed as a rate by calculating the number of extrusion events per minute. Control embryos were found to have a mean extrusion rate of 0.133 events/min.

As discussed in Chapter 1, caspase activation is a key step in the onset of apoptosis. To complement the extrusion assay, caspase activity was also evaluated in embryos using Apoliner, an *in vivo* caspase biosensor. A typical example of Apoliner expression and subcellular localization is shown in Figure 7B, where a cell (white arrowhead) in timelapse progresses from being Apoliner negative, indicative of no caspase activation, to Apoliner positive, where caspases are active. Caspase activation, and consequently the cleavage of Apoliner and $eGFP^{nls}$ entry into the nucleus occurred between the 60 min and 80 min (Figure 7B) from the onset of

dorsal closure (0 min). Following caspase activation, the cell was extruded from the AS at the 140 min mark. In this example, the cell which was typical for most extrusions, was Apoliner positive for a period of approximately 60 minutes before extrusion was executed. In general it was found that extrusions and caspase activation, as visualized by Apoliner, were correlated, and that only caspase positive cells were observed to undergo extrusion. As such, extrusions represent a reliable readout of PCD in the AS during DC.

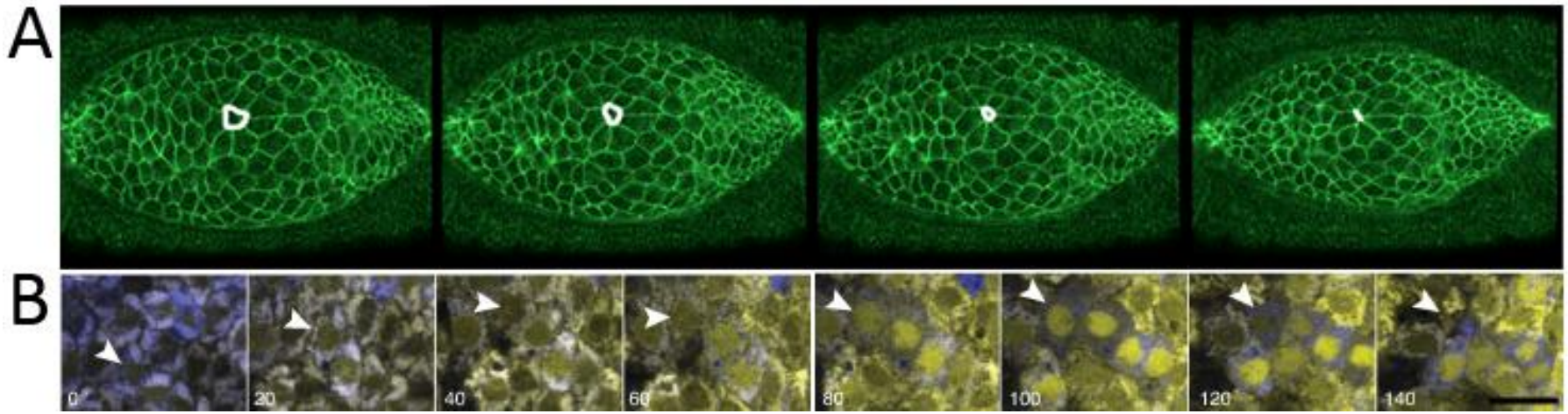

Figure 7: An epithelial extrusion event as visualized by Ubi-DE-Cadherin-GFP over a ten minute interval (A), and an extrusion event as visualized by LP1-GAL4 driven *UAS-Apoliner* (B) over 140 min after DC initiation. White arrowhead indicates the cell destined for extrusion. All times are indicated in minutes. Scale bar represents 20 µm.

3.2 Quantifying Apoliner Activity

In order to assess the caspase activation more accurately, it was desirable to acquire quantitative data with respect to Apoliner subcellular localization. The approach used involved the analysis of images of Apoliner expressing embryos and determination of the degree of eGFP and RFP colocalization. This colocalization can be expressed as the Pearson's correlation coefficient (PCC) using the JACoP plug-in for Image J (NIH), and this permits a quantitation of RFP/eGFP colocalization. An increased coefficient indicates a greater extent of RFP and eGFP colocalization and is indicative of less caspase activity. The corresponding loss of colocalization and a reduced coefficient occurs when eGFP is allowed to enter the nucleus following caspase activation. In order to verify that the coefficient data correlate with observed qualitative Apoliner activation, control embryos expressing Apoliner under the control of LP1-GAL4 were imaged and the resulting timelapse image sets were analyzed using the Pearson's coefficient method. Relative caspase activation was determined as (1-PCC). As seen in Figure 8, relative caspase activation was found to initially decline and then increase through the process of DC (Figure 8A). The initial decrease is likely due to the low levels of Apoliner expression during early DC when the LP1-GAL4 driver is just starting to show expression. At this stage autofluorescence is likely interfering with determination of colocalization. Additionally, RFP and eGFP have different rates of maturation, meaning that the time elapsed from initiation of translation to a fully functional fluorophores varies. As Apoliner expression increased during early DC, and the eGFP and RFP fluorophores were assumed to be fully mature and active, a strong colocalization (= low caspase activity) was observed (Figure 8B). Caspase activity was observed to become more and more prominent during DC progression, and loss of colocalization continued until the end of DC and the onset of AS tissue degeneration (Figure 8C). At the point of tissue degeneration colocalization was found to be constant and this is likely due to the fact that RFP

and eGFP signals overlap as they are engulfed by the underlying yolk (Figure 8D). Overall, this quantitative analysis supports the qualitative observations that caspases are activated throughout the AS, during the process of dorsal closure. A summary of key genotypes examined using the Pearson's coefficient of colocalization analysis can be found in the Appendix B.3.

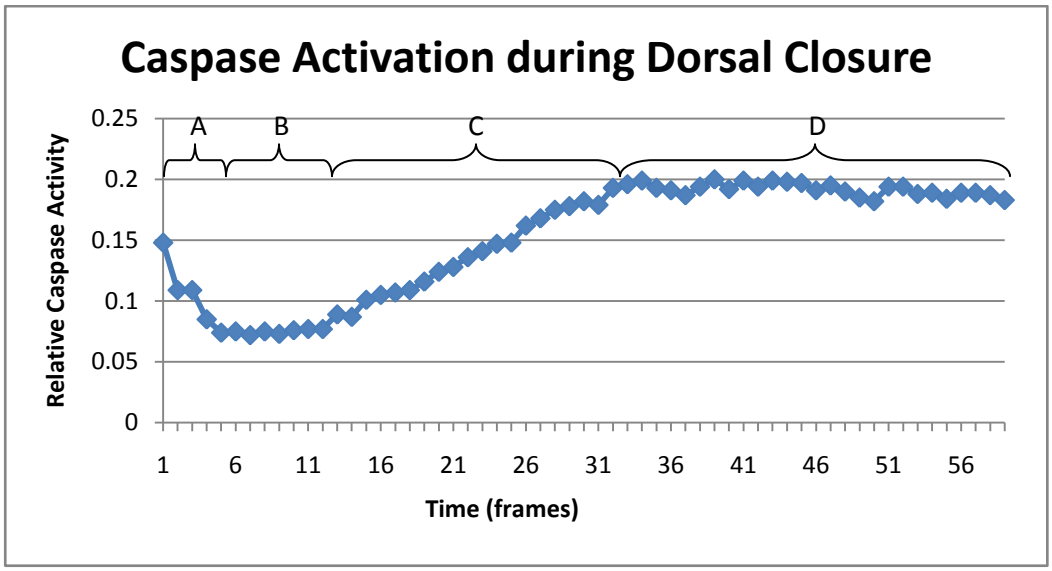

Figure 8: Relative caspase activity as determined by (1 - Pearson's correlation coefficient) between RFP and GFP channels, during progression of DC. Each frame represents approximately 3.5 minutes. Region A is consistent with end of GBR and early DC, region B represents early DC, region C shows the progress of DC, and region D is indicative of the end of DC and degeneration of AS.

3.3 Examining canonical caspase activation in AS

As discussed in Chapter 1, the characteristic apoptotic morphology is the consequence of caspase activation. This process relies on activation of pro-apoptotic proteins, such as Grim, Reaper and Hid, so that they may inhibit IAPs allowing apical caspases to activate effector caspases. In order to perturb this pathway using the GAL4-UAS system in the AS, several tools are available such as overexpression of pro-apoptotic proteins *UAS-reaper*, and *UAS-hid*. In order to diminish caspases activity it is also possible to use the viral caspase inhibitor *UAS-p35*, as well as activated Ras (*UAS-Ras85D*^{V12}) which is also known to inhibit caspase activation via MAPK activity.

Overexpression of pro-apoptotic proteins in the AS using LP1-GAL4 was found to lead to a severe precocious death phenotype. Overexpression of Hid gave a very strong premature AS death, to an extent that it does not allow embryos to complete GBR (Figure 9A). Overexpression of Rpr was carried out using two different transgenic constructs. The overexpression of the stronger *UAS-reaper.C27* insertion resulted in phenotype similar to Hid overexpression (not shown). No intact AS tissue was observed soon after LP1-GAL4 expression initiated during GBR, therefore it was not possible to assess extrusion rates or Apoliner activity in this background. The overexpression of the weaker *UAS-reaper.C14*, however, did result in a detectable AS tissue in which there was an increase in extrusion rate to 0.283 events/min, as well as an increase in caspase activation by 22 % (Appendix B.3) as evaluate via Apoliner subcellular localization (Figure 9D).

As described in Chapter 1, Hid is proposed to be regulated by phosphorylation, where phosphorylation inhibits Hid activity. In order to evaluate whether this mechanism may be active in the AS to suppress death, the unphosphorylatable form of Hid, where all potential phosphorylation sites are replaced by alanine, was overexpressed using LP1-GAL4.

Interestingly, overexpression of *UAS-hid*^{ALA5} resulted in a phenotype identical to the overexpression of wild type Hid and the AS was completely degenerated prior to the completion of GBR (Figure 9B). The strong similarity between the phenotypes of embryos expressing *UAS-hid* and *UAS-hid*^{ALA5} supports the interpretation that endogenous Hid phosphorylation is not sufficient to prevent or delay the activity of wildtype Hid overexpression.

Expression of *UAS-p35* leads to inhibition of caspase activation (Figure 9E). As expected, the live imaging analysis of embryos expression p35 showed that extrusions were largely undetectable (rate of 0.001 event/min). Embryos expressing p35, also failed to degenerate the AS completely and were associated with a persistent tissue at the innervation stage. These observations support the view that caspase activation is necessary for proper tissue death. Similar to p35, overexpression of activated *Drosophila* Ras (*UAS-Ras85D*^{V12}) was found to result in the failure of caspase activation (Figure 9F) and the absence of extrusions. Ras85D^{V12} expression was also found to be associated with a persistent AS phenotype.

Overall the results support the view that AS PCD is dependent on caspase activation and responds to it in a sensitive manner. To complement this knowledge, the contribution of autophagic cell death to the overall PCD of AS was also examined (see following section 3.4).

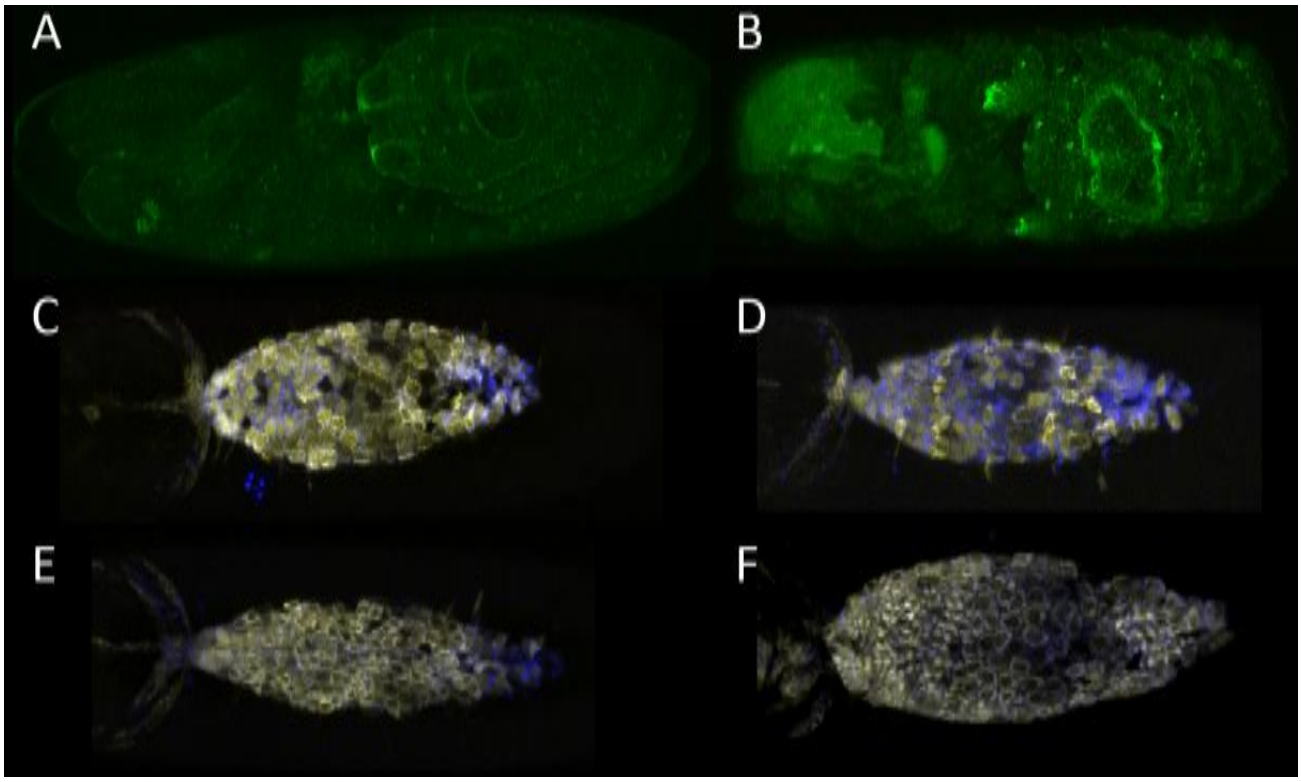

Figure 9: Hid overexpression using LP1-GAL4 (A) leads to the death of AS and incomplete GBR. The unphosphorylatable mutant of Hid (ALA5) leads to the same phenotype as wildtype Hid overexpression (B). Caspase activation, as visualized with LP1-GAL4 driven *UAS-Apoliner* occurs in control embryos during DC (C), is upregulated in Reaper overexpression embryos (D), and is completely absent from embryos expressing either *UAS-p35* (E) or *UAS-Ras85D*^{V12}(F).

3.4 Examining the effect of autophagic cell death on PCD in AS

Autophagy is known to be active in the AS during DC, as shown by TEM and expansion of the lysosomal compartment by lysotracker staining (4). Here, the effect of autophagy on the overall character of PCD was examined. Two tools utilizing the GAL4-UAS system are available to perturb autophagy. First, it is possible to overexpress Atg1, a key autophagy regulator kinase that upregulates autophagy. In addition, the overexpression of an activated form of InR, *UAS-ΔαInR*, can be used to down-regulate autophagy. As discussed in Chapter 1, the InR/TOR pathway leads to the activation of TOR, which inhibits both Atg1 and Atg8, therefore inhibiting autophagy. Although there are other autophagy related transgenic tools available, such as gene overexpression constructs, RNAi's, and dominant negative lines, all proved to be ineffective in the AS due to factors such as maternal loading and expression levels (see Appendix B.1).

Overexpression of Atg1 in the AS (LP1-GAL4>UAS-Atg1) was found to increase death, and was associated with an increased extrusion rate of 0.287 events/min. In addition, caspase activity was increased as determined by Apoliner subcellular localization (Figure 10 C,D). This increase was also confirmed by quantitation, which showed an elevation of 6% (see Appendix B.3). Based on the increased extrusions rates and Apoliner subcellular localization, these experiments indicate that an upregulation of autophagy is associated with increased caspase activation and PCD at this stage in development.

Overexpression of activated InR (LP1-GAL4>UAS-ΔαInR) resulted in no observable extrusion rate (0.000 event/min). In this background the AS was found to be highly disorganized (Figure 16B) and the cells seemed to swirl as DC progressed. Surprisingly, this overexpression of InR with LP1, when evaluated with Apoliner, showed virtually no caspase activity (Figure 16F). This was unexpected since there are currently no defined pathways by which insulin

signaling is known to inhibit caspase activation. These observations suggest a novel connection between nutrient sensing/autophagy signaling and caspase activation.

Both overexpression experiments suggest that autophagy can have an impact not only on the overall PCD in the AS, but also caspase activation. This required further characterization of the relationship between autophagy and caspases.

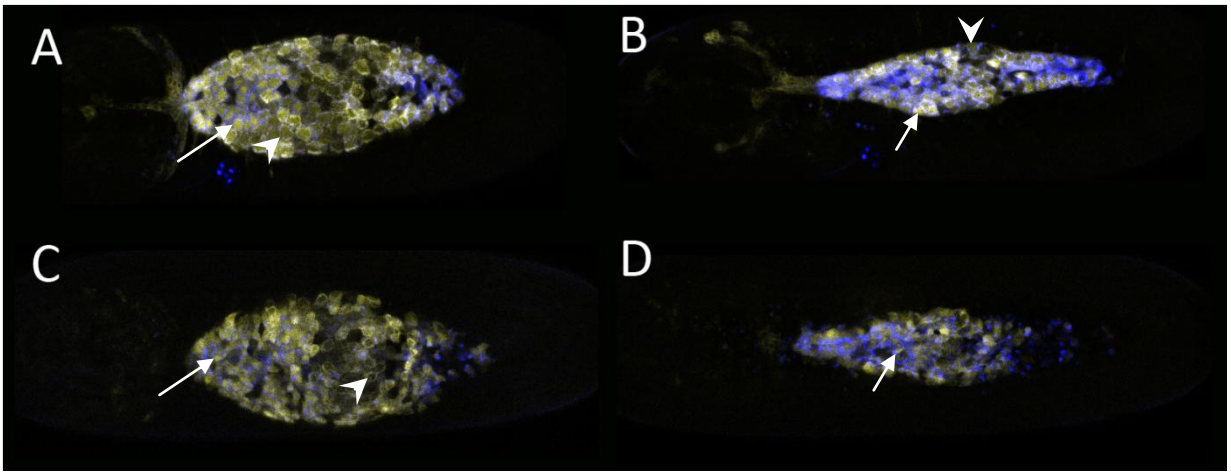

Figure 10: Caspase activation is enhanced during overexpression of Atg1. Atg1 overexpression, LP1-GAL4>UAS-Apoliner, UAS-Atg1$^{6A;6B}$, leads to upregulation of caspase activation (C, D) when compared with control, LP1-GAL4>UAS-Apoliner (A, B), during early (A,C) and late (B,D) dorsal closure. Examples of Apoliner positive cells are denoted by white arrows, and Apoliner negative cells are denoted by white arrowheads.

3.5 Relationship between autophagy and caspase activation

In order to evaluate the connection between apoptotic and autophagic death, it is necessary to be able to evaluate the extent of both processes. As shown above, Apoliner and extrusion rates are effective methods of assaying caspase activity. In order to assay autophagic cell death progression Dual Tagged Atg8a (*UAS-DT-Atg8a*) was used. As described in Chapter 2, the progression through the different puncta states indicates flux through the autophagic pathway. Figure 11 depicts a typical *UAS-DT-Atg8a* expressing cell undergoing cell death activation and extrusion as captured by live imaging. Interestingly the onset of autophagy in cells was correlated to extrusion events, in a manner similar to caspase activation. In general, and being most obvious for extrusion events early in DC, cells showed autophagic activation if and only if they underwent extrusion. This supports the interpretation that autophagy is also connected to PCD in the AS. Interestingly, the autophagic activation observed as Atg8a puncta was visible 30 minutes before extrusion, whereas caspase activation occurs up to an hour before extrusion (see 3.1). This indicates that caspase activation precedes the onset of autophagy in extruding cells.

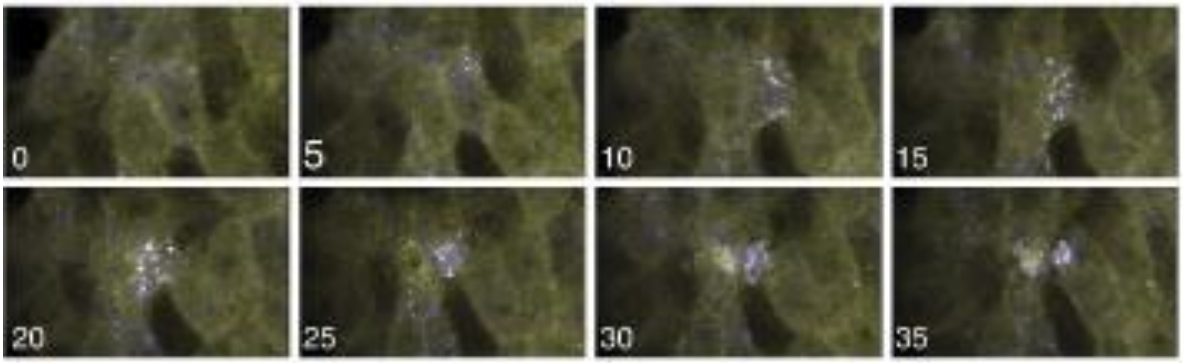

Figure 11: The extrusion of a cell and associated activation of autophagy as visualized by cells expressing NP3312>*UAS-DT-Atg8a*, at 40x. Onset of autophagy is visualized as punctate GFP/ mCherry localization. All times are indicated in minutes.

As discussed in 3.3, overexpression of Atg1 can lead to an increase in Apoliner activity. So it was if interest to evaluate the effect of caspase activation on autophagy either by activating or attenuating caspase activity by expression of Reaper and p35, respectively.

Overexpression of *UAS-reaper.C14* in the presence of *UAS-DT-Atg8a*, was found to be associated with increased autophagy. Overall, there were more colocalized mCherry/GFP-Atg8a structures present. This means that in addition to autophagy upregulating caspase activity, but caspase activation is also able to upregulate autophagy. This interesting observation suggests that these two pathways have some mutual feedback mechanism, perhaps operating to fine-tune the amount of cell death that occurs in the AS. Interestingly, embryos overexpressing *UAS-p35* with *UAS-DT-Atg8a* were found to have normal levels of autophagy. There are sufficient colocalized mCherry and GFP puncta to conclude that autophagy is still occurring. Notably, since there are no extrusions in this background (section 3.4), extrusion associated autophagic structures such as those seen in Figure 11 were not observed, indicating that they may be caspase-dependent. Overall, these observations indicate that autophagy in the AS does not rely on caspases, and is activated in a caspase-independent manner.

Despite the intriguing results, in order to confirm that these observations are indeed representative of what happens in a normal wild type embryo, it was necessary to step away from the overexpression GAL4-UAS model and evaluate loss-of-function mutants.

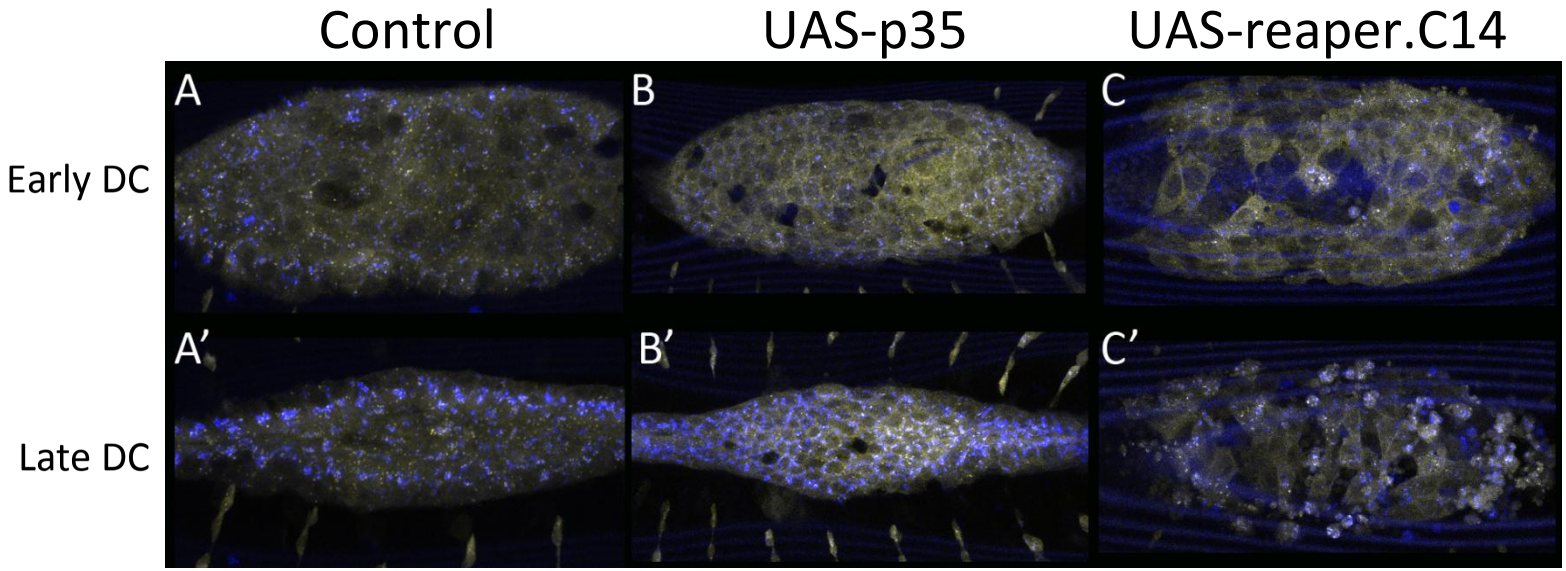

Figure 12: Changes in autophagy as seen using NP3312 driving *UAS-DT-Atg8a* depends on caspase activation. Control embryos are seen in (A, A'). Viral p35, a caspase inhibitor, has no effect on autophagy. in the AS (B, B'). Upregulation of Reaper (C, C') leads to an increase in autophagic structures.

3.6 PCD and the loss of functional autophagy

In order to better understand the process and the role of autophagy in the AS during DC, Atg1 loss-of-function mutants were examined. Since Atg1 is thought to be the key regulator kinase of autophagy, and since autophagy can affect the overall progression of PCD of this tissue, it was expected that mutations of Atg1 kinase would reveal and confirm the role of autophagy in AS death. In addition, analysis of Atg1 mutants would allow me to verify conclusions regarding the relationship between caspase activation and autophagy onset. To do this analysis of Atg1 mutant embryos, it was necessary to construct genetic stocks that would allow the unambiguous identification of mutant embryos lacking all maternally encoded Atg1 kinase as well as being deficient in zygotic Atg1 production (so called maternal/zygotic mutants). Maternal/zygotic loss of function mutants of Atg1 (*Atg1$^{\Delta 3D}$m/z*) were examined for autophagy, caspase activation and extrusions. In addition to Atg1, other components of the autophagic pathway were examined for perturbations (as mentioned in 3.3), however little to no effect was observed (see Appendix B.1), either due to low expression levels of the constructs used, the possible excessive maternal loading of the targeted transcripts, or possible redundancy of the autophagic machinery.

3.6.1 Characterization of Atg1 mutants

The literature reports two alleles of *Atg1* as being complete loss-of-function, or null, alleles. *Atg1^{1}* is reported as an allele via P-element imprecise excision, but has not been used extensively. *Atg1$^{\Delta 3D}$* is a deletion that removes the upstream regulating region as well as the first exon of Atg1 kinase. This is the most commonly used null allele of *Atg1* and is therefore available in combination with other tools, such as FRT sites, which facilitates site specific recombination. For this reason the work was focused on the *Atg1$^{\Delta 3D}$* allele. Interestingly, *Atg1^{1}* homozygous mutants were adult viable, but proved to be male sterile. Also *Atg1^{1}/Atg1$^{\Delta 3D}$*

heterozygous mutants were adult viable, but were female sterile. Although the mutant adults could be recovered, they were generally sick and were not easily manipulated. This suggests that $Atg1^{1}$ is likely a hypomorphic allele, rather than a full null as has been reported previously.

First, homozygous $Atg1^{\Delta 3D}$ embryos generated from heterozygous parents were examined with *DE-Cadherin-GFP* under the constitutive ubiquitin promoter to visualize cells. Such embryos did not have any zygotic *Atg1*, but would still receive maternally contributed Atg1 kinase. Zygotic $Atg1^{\Delta 3D}$ mutant embryos completed embryogenesis normally, but failed to develop to adulthood (Figure 13 B, B', B"). Microarray analysis by others confirms that *Atg1* is maternally contributed (25). To add to this, the initiation of zygotic transcription of *Atg1* was examined by time lapse analysis of embryos expressing nuclear GFP ($UAS\text{-}GFP^{nls}$) under the control of a GAL4 driver (NP5328) in the *Atg1* promoter/enhancer sequences. This confirmed that zygotic expression of *Atg1* does not occur until late in DC (Figure 13 C, C', C") and it is likely that maternal loading of this gene is important for earlier processes. For this reason, $Atg1^{\Delta 3D}$ *m/z* mutant embryos were generated as described in Chapter 2. Again, the embryos were examined with *DE-Cadherin-GFP* under the constitutive ubiquitin promoter. $Atg1^{\Delta 3D}$ *m/z* mutant embryos were found to have a range of phenotypes, and about half underwent normal DC while the other half are extremely perturbed and showed morphogenetic defects (Figure 13 D, E). Regardless of the severity of the observed phenotype, all $Atg1^{\Delta 3D}$ *m/z* mutant embryos were embryonic lethal. In addition to the DC phenotype, $Atg1^{\Delta 3D}$ *m/z* mutant embryos also showed an abnormal midgut development (Figure 14). The midgut did not form typical constrictions or loops in $Atg1^{\Delta 3D}$ *m/z* mutants (Figure 14B). Normal midgut development was observed in siblings that lacked maternal *Atg1* but were heterozygous for $Atg1^{\Delta 3D}$ mutation (Figure 14A).

Overall these observations suggest that Atg1 has an important role in embryogenesis, including a specific role in midgut development.

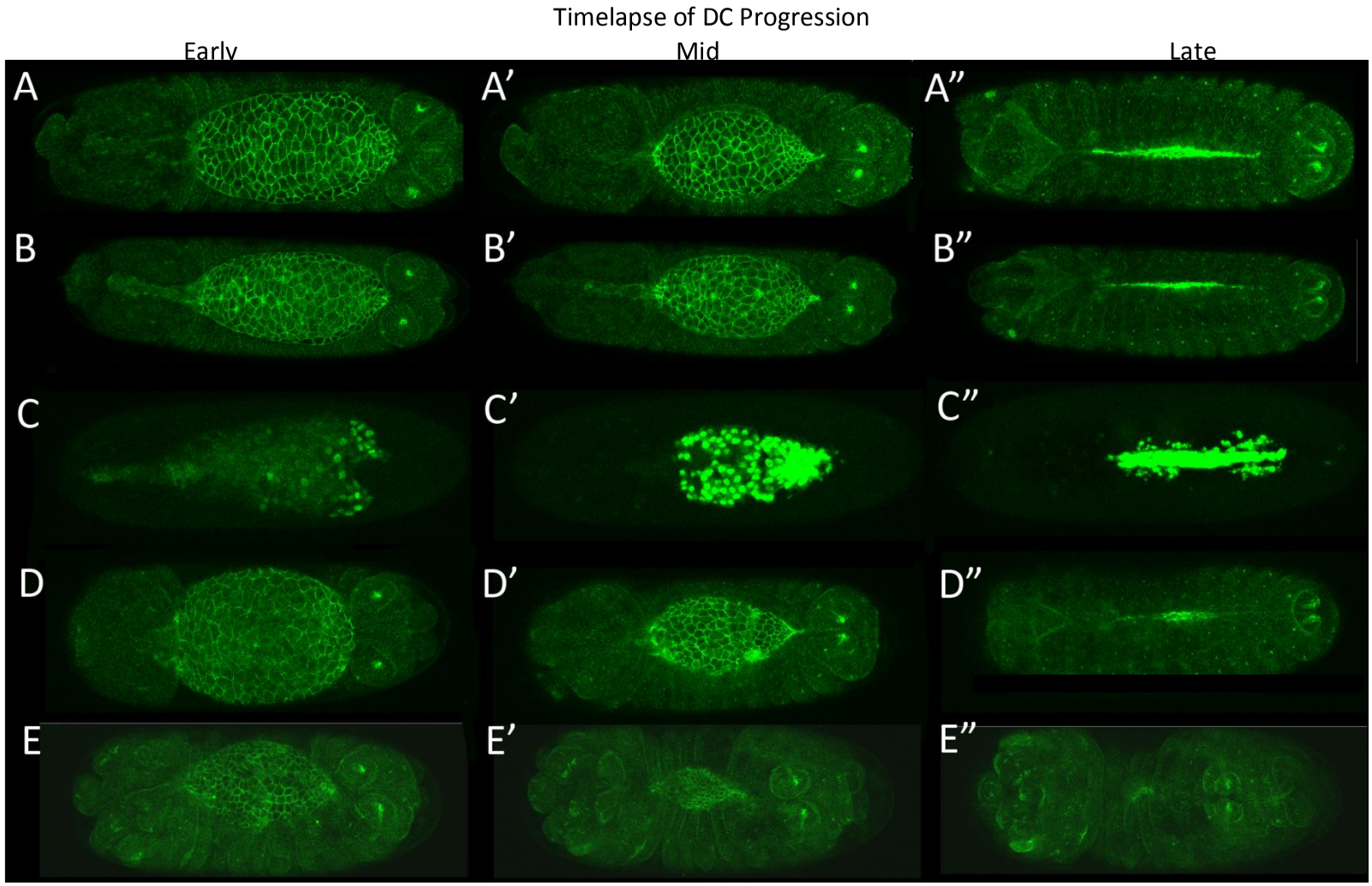

Figure 13: Phenotypic consequences of the deficiency of Atg1 in the AS during DC. Removal of the zygotic copies of Atg1, *Atg1$^{\Delta 3D}$/Atg1$^{\Delta 3D}$*, (B,B',B") has little effect on early, mid and late dorsal closure as compared with control (A,A',A"). Atg1 zygotic expression begins during dorsal closure and increases as dorsal closure proceeds (C, C', C"). Absence of both zygotic and maternally contributed copies of Atg1, *Atg1$^{\Delta 3D}$ m/z*, produces a range of phenotypes from weak (D, D', D") to severe (E, E', E").

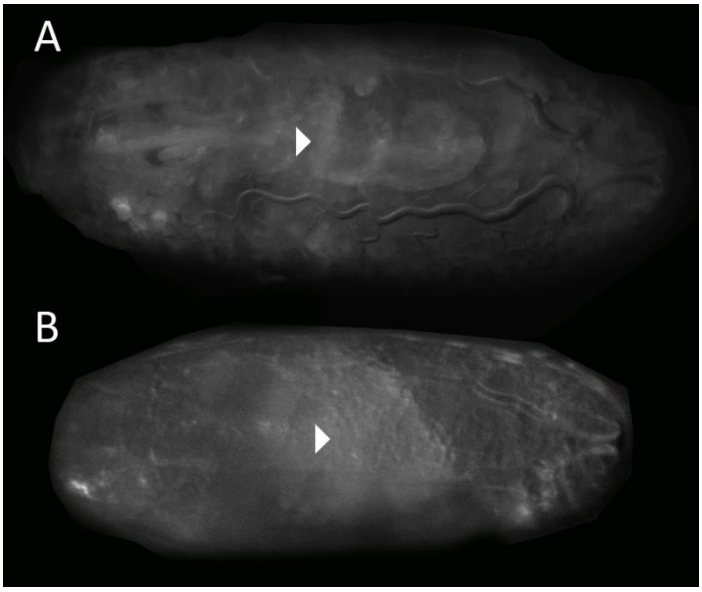

Figure 14: Midgut development of *Atg1*$^{\Delta 3D}$ *m/z* mutant embryos (B) shows no constriction or normal looping of the gut; however, the siblings that lack maternal transcripts and have one zygotic copy of *Atg1* develop normal gut (A). White arrow indicates the location where normal gut structures should appear.

3.6.2 Attenuation of autophagy in Atg1$^{\Delta 3D}$ m/z

In order to determine what effect the absence of *Atg1* has on autophagy in the AS, two autophagy reporters were used. The extent of autophagy was determined using *UAS-DT-Atg8a* and *UAS-ref(2)P-GFP* reporters under the control of LP1-GAL4 in *Atg1$^{\Delta 3D}$ m/z* mutants (Figure 15). Compared to controls, the amount of ref(2)P-GFP protein was found to be increased in *Atg1$^{\Delta 3D}$ maternal/zygotic* mutant embryos. The failure to degrade the autophagy substrate ref(2)P-GFP protein supports the view that autophagy is not active in *Atg1$^{\Delta 3D}$ m/z* mutant embryos. Furthermore, as compared with controls, the character of *UAS-DT-Atg8a* puncta was found to be very different. First, fewer puncta were present, indicating that autophagosome formation is disrupted. This observation is consistent with Atg1's suggested role in promoting the extension of the isolation membrane. Although fewer in number some puncta were observed, which prompted the question as to whether or not autophagy is completely abolished in the absence of all *Atg1*. In viewing time-lapse compilations of the *Atg1$^{\Delta 3D}$ m/z* embryos expressing DT-Atg8a, it was clear that the puncta are extremely long lived, often persisting through the entire process of DC. DT-Atg8a puncta in control embryos puncta are ephemeral, and persist for only a matter of minutes. This indicates that although some punctate structures are be able to assemble, such as the isolation membranes, the autophagic flux, by which I mean the movement of structure through the autophagic pathway, is non-functional. TEM from our lab reveals that when very few autophagic structures are present in the AS of *Atg1$^{\Delta 3D}$ m/z* mutants, but some unusual double-membrane structures were seen within the yolk (27). Overall, these observations confirm that *Atg1* is a functional regulator of autophagy in the AS, and that embryos completely deficient in *Atg1*, both zygotically and maternally, show little or no autophagy.

3.6.3 Caspase activation in Atg1$^{\Delta 3D}$ m/z mutants

In order to examine the effect of autophagy deficiency on caspase activation, the same *Atg1$^{\Delta 3D}$ m/z* mutants were assayed using *UAS-Apoliner* under the control of LP1-GAL4 (Figure 15 F). These embryos appeared to have normal level of caspase activation both qualitatively (Figure 15 F vs E) and quantitatively (see Appendix B.3). No statistical difference between the colocalization of RFP and eGFP of Apoliner between control and *Atg1$^{\Delta 3D}$m/z* embryos was found. *Atg1$^{\Delta 3D}$* maternal/zygotic mutants were also evaluated for extrusion rates; the rates were similar to control embryos and were not statistically different (0.126 events/min and 0.133 events/min, respectively). This is consistent with Apoliner analysis of *Atg1$^{\Delta 3D}$* maternal/zygotic mutants, in that while autophagy is perturbed, AS PCD occurs normally. This also indicates that *Atg1* is not required for normal caspase activation, and this contradicts previous interpretations (4). The experiments and observations described above confirm the notion that caspase activation is independent from the onset of autophagy. Overall the data suggest two independent pathways govern two types of PCD, and that there is some degree of crosstalk between them, which has yet to be fully characterized.

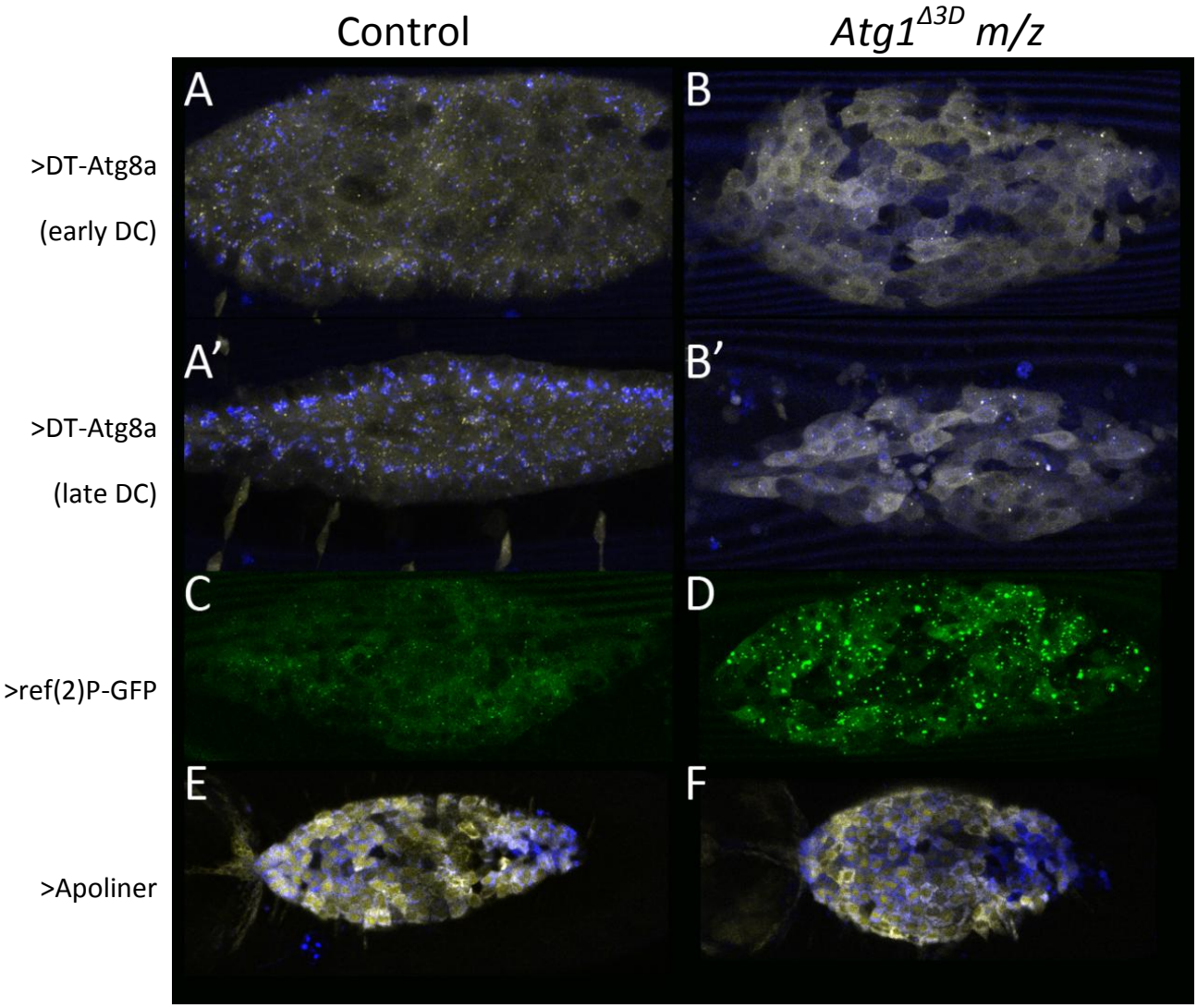

Figure 15: Absence of Atg1 leads to an attenuation of autophagy in the AS during DC. Embryos lacking both maternal and zygotic copies, *Atg1$^{\Delta 3D}$ m/z,* have significantly less *UAS-DT-Atg8a* autophagic puncta (B, B') when compared to control embryos (A, A'). In addition, the ref(2)P-GFP indicator is significantly brighter in the m/z mutant embryos (D) indicating lack of active autophagy, than in control embryos (C). Activation of caspases is not affected by deficiency in Atg1 (F) as compared to the control embryos (E).

3.7 Resolving epistatic relationships between PCD pathways

As mentioned above, Ras activation can attenuate caspase activation, and this was confirmed by Apoliner biosensor data. However, when *UAS-Ras85D^{V12}* was overexpressed with the *UAS-DT-Atg8a*, a striking and somewhat unexpected attenuation of autophagy was observed (Figure 16), *UAS-DT-Atg8a* puncta were completely absent. This suggests that activation of the EGFR/Ras pathway can also attenuate autophagy. Other components of this pathway were therefore examined. These included overexpression of *Drosophila* EGFR *(UAS-DER-GFP*), a secreted form of an EGFR ligand known as Spitz (*UAS-sSpi*), as well as activated EGFR (*UAS-Egfr.λtop*). All phenotypes were similar and showed persistence of AS and little or no epithelial extrusions (data not shown). The most pronounced phenotype was observed using activated Ras (*UAS-Ras85D^{V12}*) which is likely due to the high expression levels of this specific UAS construct.

As described in 3.5, InR signaling, which is known to inhibit autophagy via TOR, was also found to inhibit caspase activation (Figure 16). This means that both signaling pathways (EGFR/Ras and InR/TOR) can inhibit both caspase activation and the onset of autophagy (Figure 16). It is unclear why they should; a mechanism involving crosstalk is likely to exist.

In order to address the relationship between the EGFR/Ras and InR/TOR signaling pathways in the AS, epistasis experiments were designed. In the analysis of regulatory pathways, and given certain assumptions regarding the activity of the expression constructs used, the epistatic gene is interpreted as the downstream component. The epistatic relationships between the autophagy activator Atg1 and both Ras and InR were tested. This was done by coexpressing *UAS-Atg1.6A* with either activated Ras (*UAS-Ras85D^{V12}*) or activated InR (*UAS-ΔαInR*) in the presence of Ubi-DE-Cadherin-GFP, NP3312 driven *UAS-DT-Atg8a*, or LP1-GAL4 driven *UAS-Apoliner*.

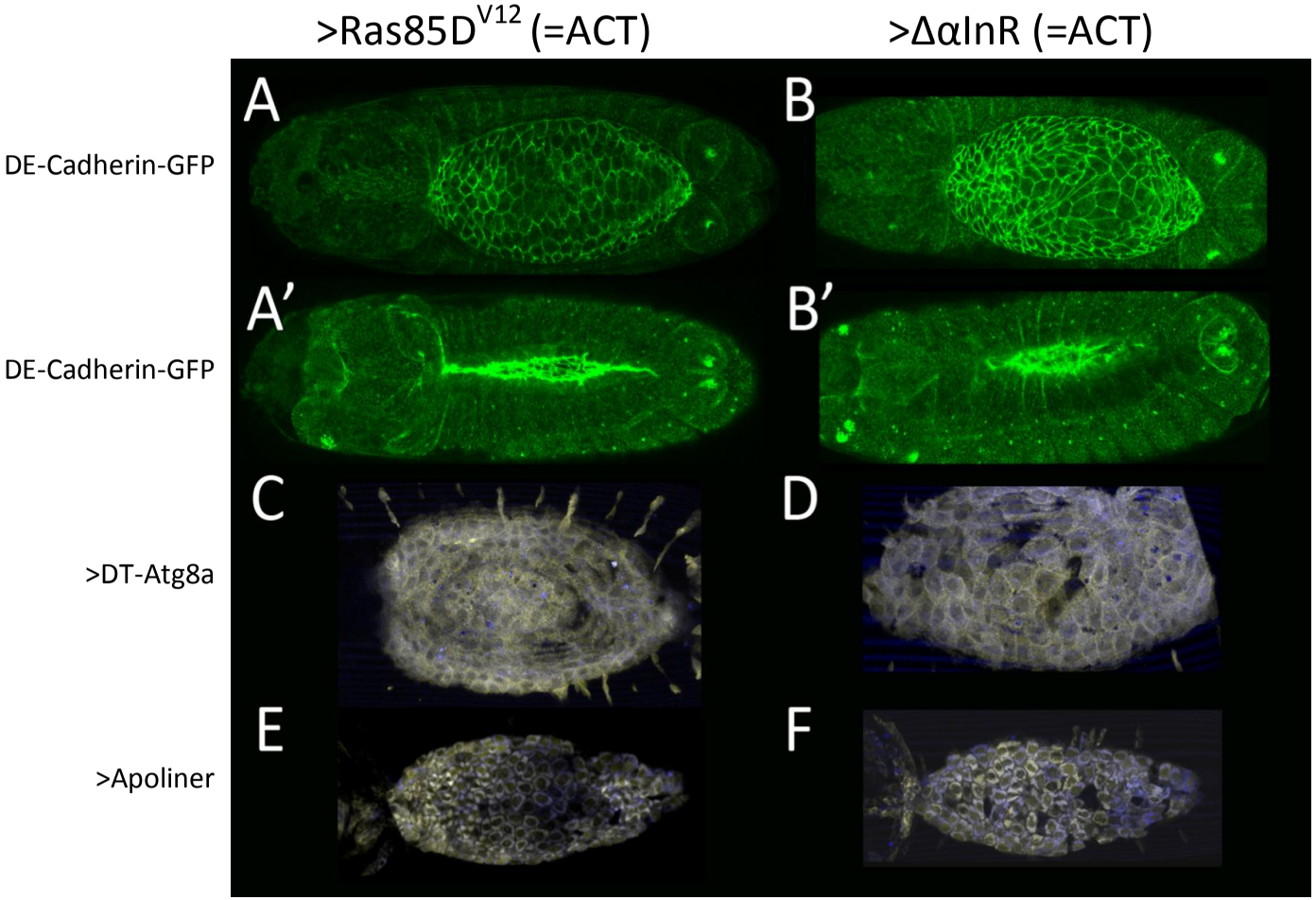

Figure 16: Activation of Ras or InR in the AS leads to reduction in cell death. Overexpression of activated Ras leads to normal closure (A), persistent tissue at innervation (A'), attenuation of autophagy (C) and severely decreased caspase activation (E), when visualized with Ubi-DE-Cadherin-GFP, *UAS-DT-Atg8a*, and Apoliner respectively. Similarly overexpression of activated InR leads to disorganized AS during DC (B), persistent tissue at innervation (B'), attenuation of autophagy (D) and severely decreased caspase activation (F), when visualized with Ubi-DE-Cadherin-GFP, *UAS-DT-Atg8a*, and Apoliner respectively.

The coexpression of Atg1 with InR produced embryos that failed to completely close and degenerate the AS (Figure 17 C,C'). The cells were disorganized and swirled, similar to what was observed in the embryos overexpressing InR alone. However, there were autophagic puncta when observed with *UAS-DT-Atg8a*. These *UAS-DT-Atg8a* puncta were less frequent than in the control, however autophagy has clearly been activated in these coexpression embryos (Figure 17 F, F'). In addition, the characteristic accumulation of RFP in Atg1-only overexpressing embryos (Figure 17 D'), likely indicative of increased autophagic flux, was observed in the embryos expressing *UAS-DT-Atg8a* and quite numerous and bright (Figure 17 F'). This means that autophagy was not inhibited in embryos co-expressing Atg1 and activated InR. In addition, when examined with Apoliner the embryos had attenuated caspase activation during early stages but showed clear nuclear GFP, synonymous with caspase activation, in later stages of DC (Figure 17 I, I'). Given that the Atg1 overexpression was not based on constitutively active protein, but rather was relying on overexpression, a formal interpretation in terms of epistasis is inconclusive. However, these observations are not contradictory to the accepted model in which InR functions upstream of Atg1 kinase. Please see Chapter 4 for further discussion of the regulatory model.

The coexpression of Atg1 with Ras produced embryos that failed to completely close and degenerate the AS (Figure 17 B, B'). In addition, virtually no autophagic DT-Atg8a puncta were observed. Although some Atg1-overexpression RFP accumulations (as described above) were observed, they were very faint and infrequent. This suggests that no autophagy was ongoing in the *UAS-Atg1.6A; UAS-Ras85D*^{V12} co-expressing embryos (Figure 17 E, E'). In addition, when examined with Apoliner, the embryos had attenuated caspase activation during early and late stages (Figure 17 H, H'). Overall, the results were essentially identical to the overexpression of Ras alone. This indicates that all PCD was attenuated in the context of Ras/Atg1 coexpression.

These results suggest that overexpression of Ras can act independently of Atg1, through a parallel pathways or that Ras activation is able to block Atg1 expression or activity. All the phenotypes observed are summarized in Table 1 below.

Table 1: Qualitative Assessment of Autophagy and Caspase Activity in Expression/Coexpression Backgrounds

	Autophagy (DT-Atg8a puncta)	Caspase Activity (Apoliner)
Wildtype	++	++
>Atg1	++++	+++
>ΔαInR (=ACT)	-	-
>Ras85D^{V12} (=ACT)	-	-
>ΔαInR +Atg1	++	+
>Ras85D^{V12}+ Atg1	-/+	-

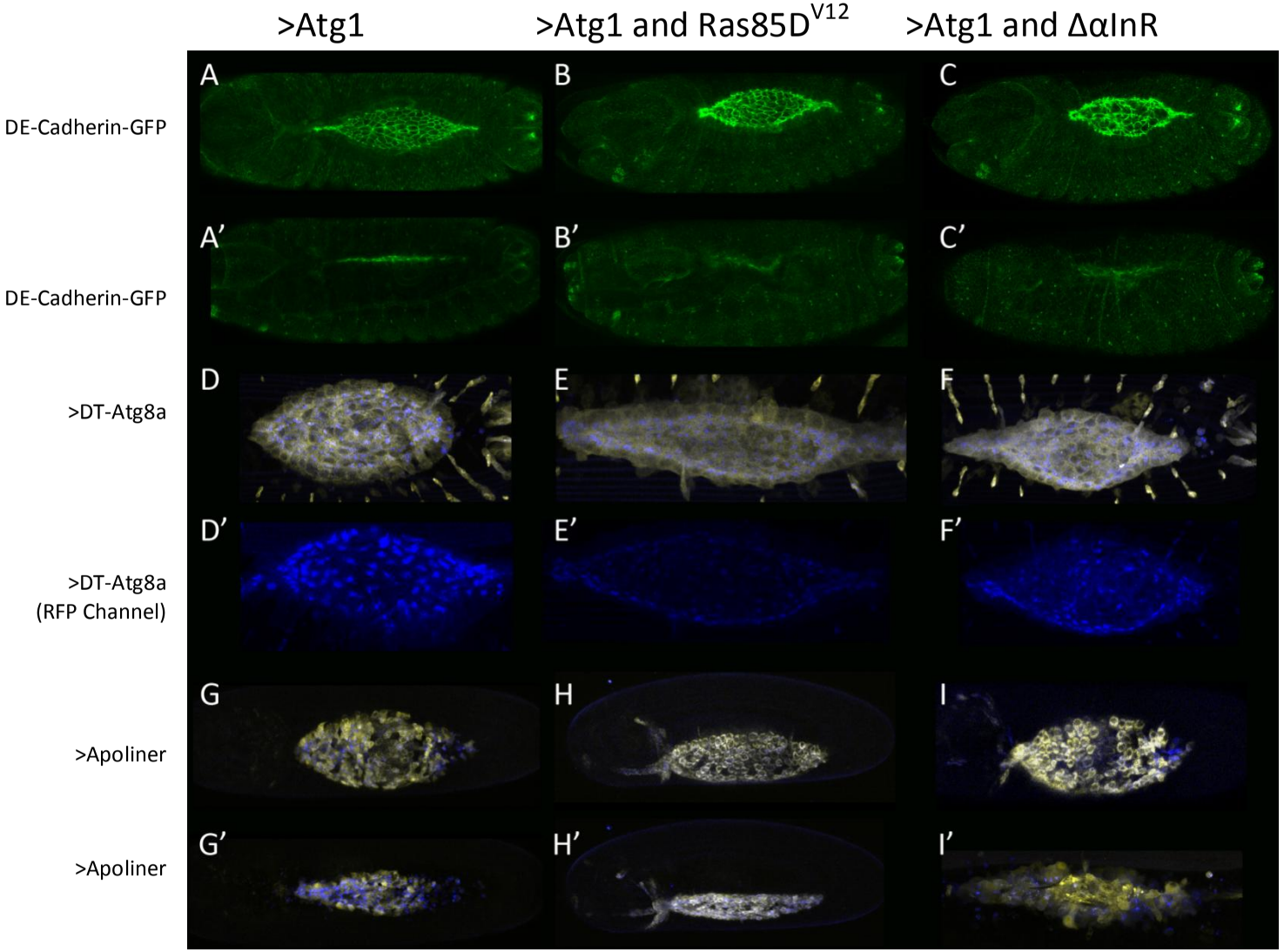

Figure 17: Co-expression of Atg1 and activated Ras rescues the persistent AS phenotype of Ras alone (B, B') but continues to show no autophagy (E) and is able to partially rescue the increased flux seen with Atg1 overexpression alone (D'), when using *UAS-DT-Atg8a* (E'). Co-expression of Atg1 and activated InR does not rescue the AS persistence phenotype of overexpression of InR alone (C, C') and shows limited autophagy (F), and is unable to rescue the increased flux seen with Atg1 overexpression only, evaluate using *UAS-DT-Atg8a* (E'). Caspase activation is diminished when Ras is co-expressed with Atg1 (H, H'). When Atg1 is co-expressed with InR, some early caspase activation is attenuated (I), however at later stages caspase activation is present (I').

To investigate how Atg1 may interact with the EGFR/Ras and InR/TOR pathways, it was necessary to examine the epistatic relationships of these pro-survival pathways with known pro-apoptotic activators. As discussed earlier in 3.3, transgenic constructs for overexpression of *UAS-hid* and *UAS-reaper* are available. In an attempt to establish epistatic relationships several coexpression genotypes were examined including: coexpression of Hid and activated Ras, coexpression of Hid and activated InR, coexpression of Reaper and activated Ras, and coexpression of Reaper and activated InR. The experimental results are outlined in Table 2 below.

Overexpression of *UAS-ΔαInR* with Hid resulted in limited rescue of previously examined Hid-overexpression phenotype seen in Figure 9A. About a third of examined embryos were able to complete germ band retraction and DC, and had an intact AS present (Figure 18 D). Very limited hatching to larvae was observed, with all individuals dying before reaching second instar stage. Similarly, the overexpression of *UAS-ΔαInR* with Reaper had limited rescue of the Reaper overexpression phenotype discussed in 3.3 (not shown).

Overexpression of *UAS-Ras85D^{V12}* with Hid, however, was very effective in rescuing the Hid-overexpression phenotype seen previously. Most embryos were able to hatch to larvae, and limited survival to adulthood was observed. The embryos were perturbed in an observed number of AS cells and elevated cell death was still likely occurring. The coexpression of *UAS-Ras85D^{V12}* with Reaper failed to completely rescue previously observed precocious death. The observed embryos did undergo mild rescue similar to *UAS-reaper;UAS-ΔαInR* embryos; the rescue was very limited and no larvae were observed. This indicates that Ras is able to specifically interact with Hid and not Reaper, which is consistent with the literature. Overall

these finding allow us to propose a model as to what factors control PCD in the AS during dorsal closure.

Table 2: Observed rescue phenotypes of pro-death and pro-survival coexpression experiments

	Degree of Rescue
> ΔαInR +Hid	Mild
> ΔαInR +Rpr	Mild
> $Ras85D^{V12}$+ Hid	Complete
>$Ras85D^{V12}$+ Rpr	Mild

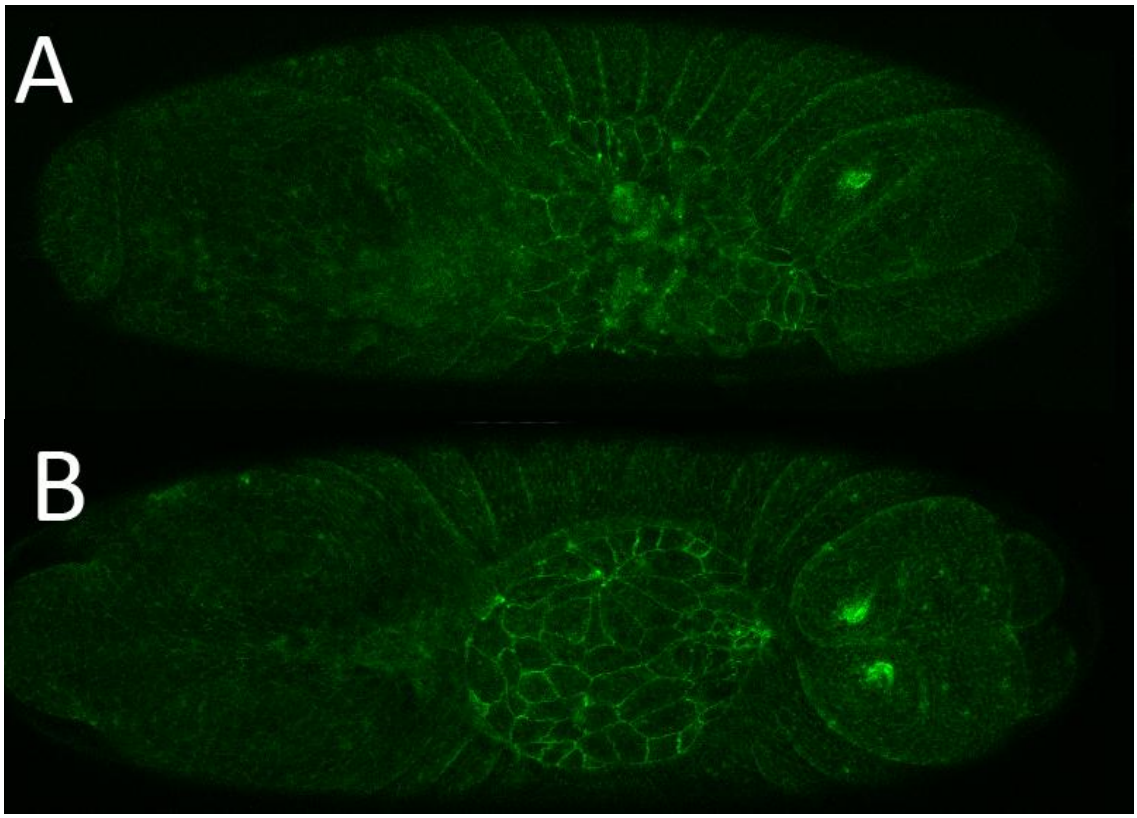

Figure 18: Coexpression of *UAS-Ras85D*^{V12} and *UAS-hid* (A) results in abnormal DC but survival to adulthood. Coexpression of *UAS-ΔαInR* and *UAS-hid* (B) leads to an embryonic and early larval lethality. Embryos were visualized using *Ubi-DE-Cadherin-GFP.*

Chapter 4. Discussion

4.1 Atg1 is required for embryogenesis, but not degeneration of AS

Undoubtedly, Atg1 has a role in autophagy in the AS, since embryos deficient in Atg1 show reduced autophagy within this tissue as examined using DT-Atg8, ref(2)P-GFP, and ultrastructure (27). As shown in Chapter 3, the absence of *Atg1* leads to abnormal embryogenesis and embryonic lethality. In addition, Atg1 appears to be essential for embryonic midgut development. Overall, this demonstrates that Atg1 is necessary for normal embryogenesis. In contrast, Atg1 is dispensable for the degeneration of the amnioserosa, since PCD during DC can occur normally in $Atg1^{\Delta 3D}$ *m/z* mutants. Presently, it is unclear why the absence of *Atg1* leads to embryonic lethality. Also, it is unclear why some $Atg1^{\Delta 3D}$ *m/z* mutant embryos display a severe phenotype while others are close to wildtype. A possible explanation for this variation could be the production of $Atg1^{\Delta 3D}$ clones in the somatic follicle cells during the generation of germ-line clones using the FLP/ovo^{D} technique. Although it is unclear as to why there would be an effect, a recent study has raised the possibility of autophagy's involvement in germ-line/follicle cell interactions (28).

The data indicate that autophagy is not required in the AS for programmed cell death, although it readily occurs in normal embryos. Literature suggests that autophagic cell death is not prevented by the overexpression of viral p35 and leads to normal tissue degeneration in the *Drosophila* larval fat body (14). As shown here, the AS persists when p35 is over-expressed, hence the PCD is not exclusively autophagic and autophagy is not the primary cell-killing mechanism. The role of autophagy in AS is not clear as any attempts to disrupt it in a tissue specific manner using the GAL4-UAS system have been unsuccessful (see Appendix B.1).

Disruption of autophagy by generating m/z mutants for other Atg genes is technically lengthy, and is likely to yield similar results since Atg1 is a key regulator of the process. Perhaps, the autophagic pathway represents a general cellular response to upregulation of caspases during epithelial extrusions, and that it assists caspase activation with reorganizing the cell prior to engulfment, extrusion and death. As shown here, caspase activation precedes autophagy in extruding cells by about 30 min. If true, it would be interesting to investigate what signals, if any, in the caspase activation cascade attribute to the activation of autophagy.

The present study also demonstrated that ecdysone is not the signal for the onset of autophagy (Appendix B.4). This is consistent with the idea that caspase activation occurs prior and independently of autophagy, and may trigger or aid in activating autophagy. Since ecdysone deficient mutants can still activate autophagy, the onset is not ecdysone dependent. Despite ample evidence linking ecdysone signaling to autophagy and PCD in the regression of *Drosophila* larval tissues at pupariation, it is not clear what role ecdysone plays in embryonic development (29).

Although DC is not specifically dependent on autophagy, the AS still remains a useful model for study of PCD. As shown, the amnioserosa provides a cellular context where cells are primed to be able to undergo autophagic and caspase-dependent cell death, and are able to regulate their degeneration using both pro-death and pro-survival signals.

4.2 Current Model of PCD regulation

Although autophagy is not required during dorsal closure as shown by *Atg1*$^{\Delta 3D}$ *m/z* experiments, it occurs in wildtype embryos and correlates with caspase activation. As shown, overexpression of Atg1 leads to increased cell death, and greater caspase activation. Similarly, overexpression of pro-apoptotic protein Reaper leads to an increase in autophagy (Chapter 3.5). This means that there are cellular pathways that allow an increase in autophagy to stimulate the caspase activation cascade, and currently it is not clear what could act as a signal to do so. As described earlier, *hid* is known to stimulate autophagy is certain tissues, such as the larval fat body (14) . It is possible that the death stimuli enhance *hid* expression/activity, which can in turn up-regulate autophagy either through the action of caspases such as Dronc, or by some other currently unknown mechanism. It is unclear, however, how autophagy can up-regulate caspase activation. It is possible that increased autophagy and increased membrane trafficking leads to a faster turn-over rate of signaling receptors leading to increased signaling resulting in caspase activation (30). Overall the feedback mechanism is unclear, but strong evidence suggests that it exists. A model summarizing findings in this work is seen in Figure 19.

The specific suppression of *hid*-induced death with Ras/MAPK confirms that this pro-survival signaling is able to specifically attenuate the activity of *hid* in AS, possibly through phosphorylation (18). The tools to further evaluate the effect of phosphorylated Hid are not available at this time. Additionally, Ras was not able to rescue overexpression of Atg1 as well as InR could, suggesting that Atg1 is likely not regulated directly by the Ras pathways. Based on the degree of rescue, my results are consistent with the accepted placement of Atg1 downstream of InR. However some of the phenotypes associated with overexpression of InR, such as the disorganization of the AS and persistence of tissue, are not related and cannot be rescued by autophagy. In contrast, Ras and Atg1 are likely in parallel pathways but are able to interact to a

limited degree; since Ras was able to inhibit autophagy in the Atg1 overexpressing embryos, it is possible that Ras can act on something downstream of Atg1 to attenuate autophagy. This is contradictory to the idea that Ras activates PI3K. If that was the case, overexpression of Ras would lead to the upregulation of TOR via PI3K. Since Atg1 is downstream of TOR, the coexpressing embryos should appear similar to Atg1/InR coexpression. Given that this is not the case, any activation of PI3K by Ras would be insignificant, if any. Interestingly, both Ras and InR are able to prevent AS degeneration, caspase activation and autophagy in the AS, indicating that there is a degree of cross talk between the two pathways.

In order to explain the observation that activated InR can prevent caspase activation, some signal from the InR/TOR pathway must be able to interact with the EGFR/Ras pathway. As shown in Figure 19 (arrow 1), in mammalian literature insulin signaling is able to upregulate Ras via an intermediate GRB2 which interacts with Sos (31). Although this has not been shown in *Drosophila*, a GRB2 homologue has been identified as Drk. If this crosstalk is occurring in *Drosophila*, the observed attenuation of caspase activation by InR overactivation can be easily explained. Alternatively, it is possible that the observed inhibition of PCD is due to the change in cellular context that make the cells non-permissive for cell death signaling.

Similarly, in order to explain the observation that activated Ras can prevent autophagy onset, some signal must directly interact with autophagy regulating pathway. The data presented in this work indicated Ras is likely acting downstream of Atg1, or directly inhibiting Atg1. There are a few possibilities that would be consistent with this observation. Since Hid has been implicated in promoting autophagy (Figure 19, arrow 3), it is possible that both autophagic and apoptotic cell death in AS are Hid-dependent (14). The observed suppression of autophagy by Ras could then be exampled by the suppression of Hid that is required, perhaps as a permissive

signal, for onset of autophagy. An alternate explanation would involve some intermediate that is being activated by Ras to inhibit a component of the autophagic machinery. It is unclear at this time what that may be.

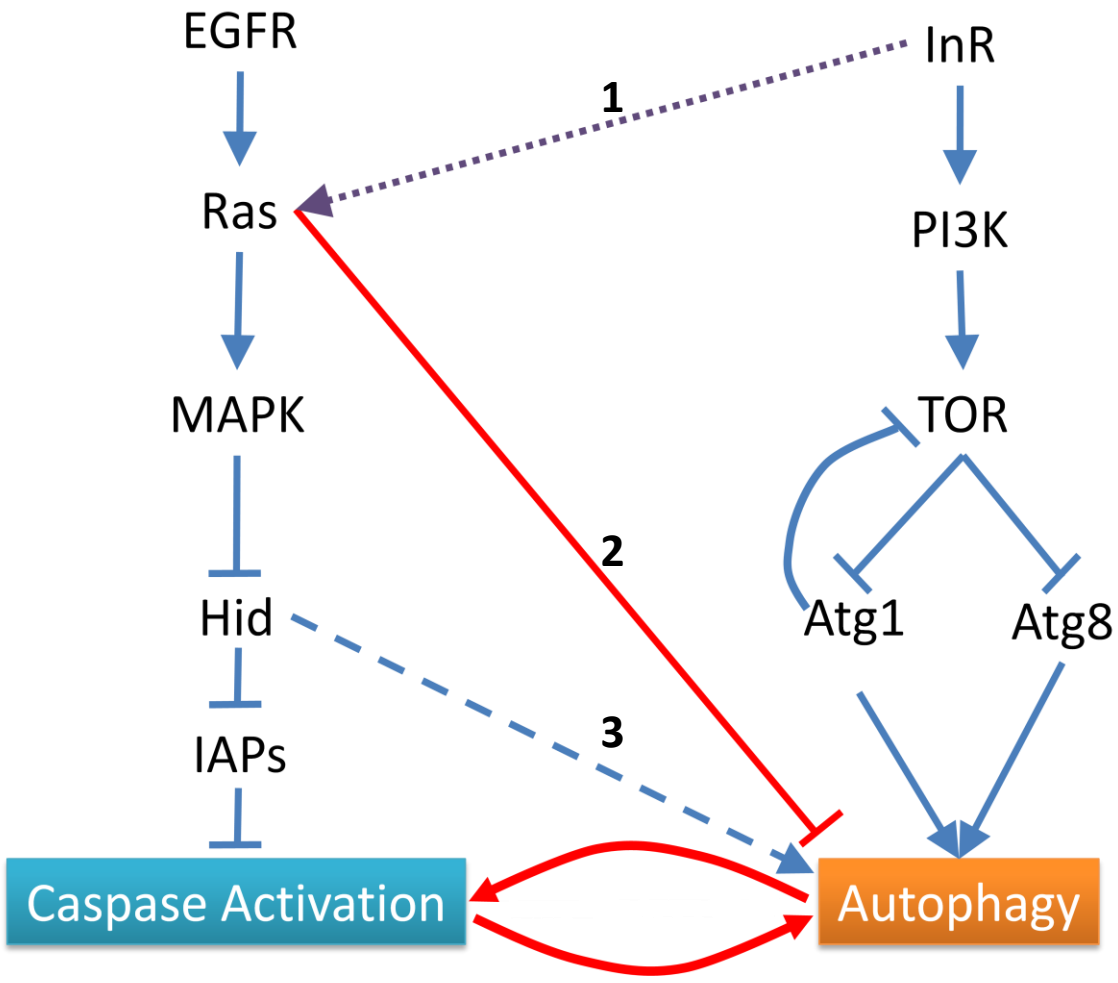

Figure 19: A model demonstrating known and proposed regulation of autophagy and caspase activation. All solid arrows indicate established regulation pathways. Arrow (1) indicates a proposed activation based on mammalian studies (Skolnik et al., 1993), while arrow (2) indicates observations of the current study. Arrow (3) indicates observation in larval tissues (Juhasz and Sass, 2005).

Chapter 5. Future Directions

5.1 Role of Atg1 in embryogenesis

In order to expand on the presented work, it is necessary to determine the specific requirement for Atg1 during embryogenesis. Some work indicates that Atg1 may be involved in the yolk, and as shown it is implicated in midgut development (27). More work is needed to identify the specific stages and tissues where Atg1 is required during embryogenesis, and what role it plays. Although Atg1 is a key autophagy regulator, it is possible that it is involved in other membrane trafficking processes as some other autophagy proteins are. In addition, it would be interesting to resolve the dichotomy of Atg1 m/z mutant phenotypes. If the reason does lie in follicle cell clones, perhaps a novel role of Atg1 can be uncovered. In order to evaluate these potential roles, it would be necessary to find an appropriate combination of available tools and possibly develop new ones. It is important to be able to look at Atg1 deficiency in a tissue specific manner, such as RNAi. Since the use of RNAi is limited in embryos, in part due to ongoing cell death, this may not be a practical approach (22). Alternatively, it may be possible to make cell clones using the FLP/FRT approach, however since the mutant patches are limited to a few cells, it may not be an informative technique. Atg1 regulation is currently an active area of research, since this kinase seems to have indirect regulation. It is known to phosphorylate and become phosphorylated by Atg13, however the specifics of this kinase activity are unknown. Therefore, determining the Atg1 kinase requirement for isolation membrane elongation needs to be elucidated in order to expand our knowledge regarding Atg1 function.

5.2 Mechanism of cross talk between caspase activation/autophagy and pro-survival

The majority of unanswered questions lie in the crosstalk between caspase activation and autophagy. It is clear that these two cellular processes have mechanisms to upregulate one another. It would be interesting to explore whether the upregulation of autophagy is Hid-dependent. In order to resolve whether Hid is the key regulator of autophagy and caspase activation in the AS, it is necessary to develop tools that would allow observation of the effect of Hid deficiency on PCD in AS unambiguous and reliably. Currently, one RNAi and one *hid* mutant allele are available. Unfortunately, the RNAi have limited efficacy during embryogenesis, and the mutant allele is an unreliable construct (22). Therefore, in order to resolve Hid's involvement in autophagy, new loss-of-function alleles of *hid* need to be recovered. Alternatively RNAi efficacy needs to be improved. Furthermore, the role of Hid phosphorylation can be explored if tools, such as constructs with differentially phosphorylated Hid, are developed. Limited studies using a deletion in the Hid region (ED225) have shown a persistent AS and ongoing autophagy, however it has not confirmed if expression of Hid is completely absent in this background (4). Since the deletion only removes enhancer sequences and leaves intact the start of transcription, the coding sequence, and 508bp of upstream sequence, ED225 may not be reliable as a Hid loss-of-function tool (32). Another deletion (Df (3L)H99) that reportedly removes *hid* in its entirety can be used, however technical difficulties exist in recognizing H99 homozygous embryos.

Similarly, the upregulation of caspases due to autophagy needs to be determined. As mentioned, it is unclear which autophagic components or mechanisms can upregulate caspases. Although the possibility that Hid regulated both processes exists, there is likely some other feedback mechanism that leads to caspase activation in response to elevated autophagy. The

interaction between EGFR and InR also needs to be elucidated to determine if Drk, or another factor, can link Ras and TOR, and whether InR can induce Ras. Lastly, it remains unclear what signal initiated caspase activation during DC. From the work presented, it is clear that ecdysone is not the signal for PCD in the AS. There is still potential for TGF-β to play a role, however experimental work does not corroborate this possibility (see Appendix B.2).

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Appendix A. Stocks and Schemes

A.1 Genetic Stocks Used

w; Ubi-DE-Cadherin-GFP; LP1-GAL4

w;; UAS-Apoliner, LP1-GAL4 /TM6, Tb

w; UAS-ref(2)P-GFP; LP1-GAL4

NP3312/Fm7,KrGAL4, UAS-GFP^{nls}; UAS- GFP-mCherry-DrAtg8a

w;; UAS-Ras85D^{V12}

w;; UAS-ΔαInR "G"

w; UAS-p35 [TTR033]

UAS-p35; UAS-p35

UAS-hid on X

ywUAS-hid^{ALA5} / M5

UAS-hid;; UAS-Ras85D^{V12}

UAS-hid;; UAS-ΔαInR "G"

w; UAS-reaper C{14}

UAS-reaper C{27} on X

UAS-reaper C{27};; UAS-Ras85D^{V12}

UAS-reaper C{27};; UAS-ΔαInR "G"

w; UAS-Atg1^{6A}; UAS-Atg1 ^{6B}

w; UAS-Atg1^{6A} ; UAS-ΔαInR "G"

w; UAS-Atg1^{6A} ; UAS-Ras85D^{V12}

yw;; Atg1^{NP5328}/ TM6 UW23-1

w;; $Atg1^{1}$ / TM3, twist-GAL4, $UAS\text{-}GFP^{nls}$

w;; $Atg1^{\Delta 3D}$ / TM3, twist-GAL4, $UAS\text{-}GFP^{nls}$

ry+, hsflp, yw ; Dr^{mio}/ TM3, ry, Sb

yw;; ovo^{D} FRT^{2A} /Dr^{mio}

w;; $Atg1^{\Delta 3D}$ FRT^{2A}/ TM3, Sb

w; Ubi-DE-Cadherin-GFP; $Atg1^{\Delta 3D}$ / TM3. twistGAL4, $UAS\text{-}GFP^{nls}$

w; UAS-Apoliner; $Atg1^{\Delta 3D}$, LP1-GAL4/ TM3. twistGAL4, $UAS\text{-}GFP^{nls}$

w; UAS-GFP-mCherry-DrAtg8a; $Atg1^{\Delta 3D}$, LP1-GAL4/ TM3. twistGAL4, $UAS\text{-}GFP^{nls}$

w; UAS-ref(2)P-GFP; $Atg1^{\Delta 3D}$, LP1-GAL4/ TM3. twistGAL4, $UAS\text{-}GFP^{nls}$

A.2 Generation of Atg1$^{\Delta 3D}$ maternal/zygotic mutants

I ry+, hsflp, yw ;Dr^{mio}/ TM3, ry, Sb x yw;; ovo^{D} FRT^{2A} /Dr^{mio}

II w;; Atg1$^{\Delta 3D}$ FRT ^{2A}/ TM3, Sb x ry+,hsflp,yw;; ovo^{D} FRT^{2A}/ TM3, ry, Sb

III ry+, hsflp, yw / w;; Atg1$^{\Delta 3D}$ FRT ^{2A}/ovo^{D} FRT^{2A} x *♂*

(heat shocked females)

Where *♂* can be:

w; Ubi-DE-Cadherin-GFP; Atg1$^{\Delta 3D}$ / TM3. twistGAL4, UAS-GFP^{nls}

w; UAS-Apoliner; Atg1$^{\Delta 3D}$, LP1-GAL4/ TM3. twistGAL4, UAS-GFP^{nls}

w; UAS-GFP-mCherry-DrAtg8a; Atg1$^{\Delta 3D}$, LP1-GAL4/ TM3. twistGAL4, UAS-GFP^{nls}

w; UAS-ref(2)P-GFP; Atg1$^{\Delta 3D}$, LP1-GAL4/ TM3. twistGAL4, UAS-GFP^{nls}

Appendix B. Other experimental data

B.1 Other examined autophagy related proteins

The following genetic tools were used to examine alternate autophagy related genes. The UAS constructs were over-expressed using LP1-GAL4 in the presence of Ubi-DE-Cadherin-GFP and, in a case of abnormal extrusion rate, using UAS-mCherry-DrAtg8a, an earlier progenitor of the DT-Atg8a tool. Overall, the following constructs were not useful in attenuating autophagy in the AS during DC.

UAS-Atg3 IR (UAS-DrAut1 IR)	Lower extrusion rate but normal mCherry-Atg8a expression
UAS-Atg7-2C IR	No phenotype
UAS-Atg6-2D IR	No phenotype
UAS-Atg12-3 IR	No phenotype
UAS-Vps34-KDm8-II	No phenotype
UAS-Vps34-KDm4-III	No phenotype
UAS-Atg5 IR	Lower extrusion rate but normal mCherry-Atg8a expression
Atg1Δ3D/Atg1[1]	No embryonic phenotype, Adult female sterile
Aut1[EY08396]/ Aut1[EY08396]	No phenotype
Atg7Δ77/Atg7Δ14	No phenotype
Atg1Δ3D, Atg13Δ74	No phenotype

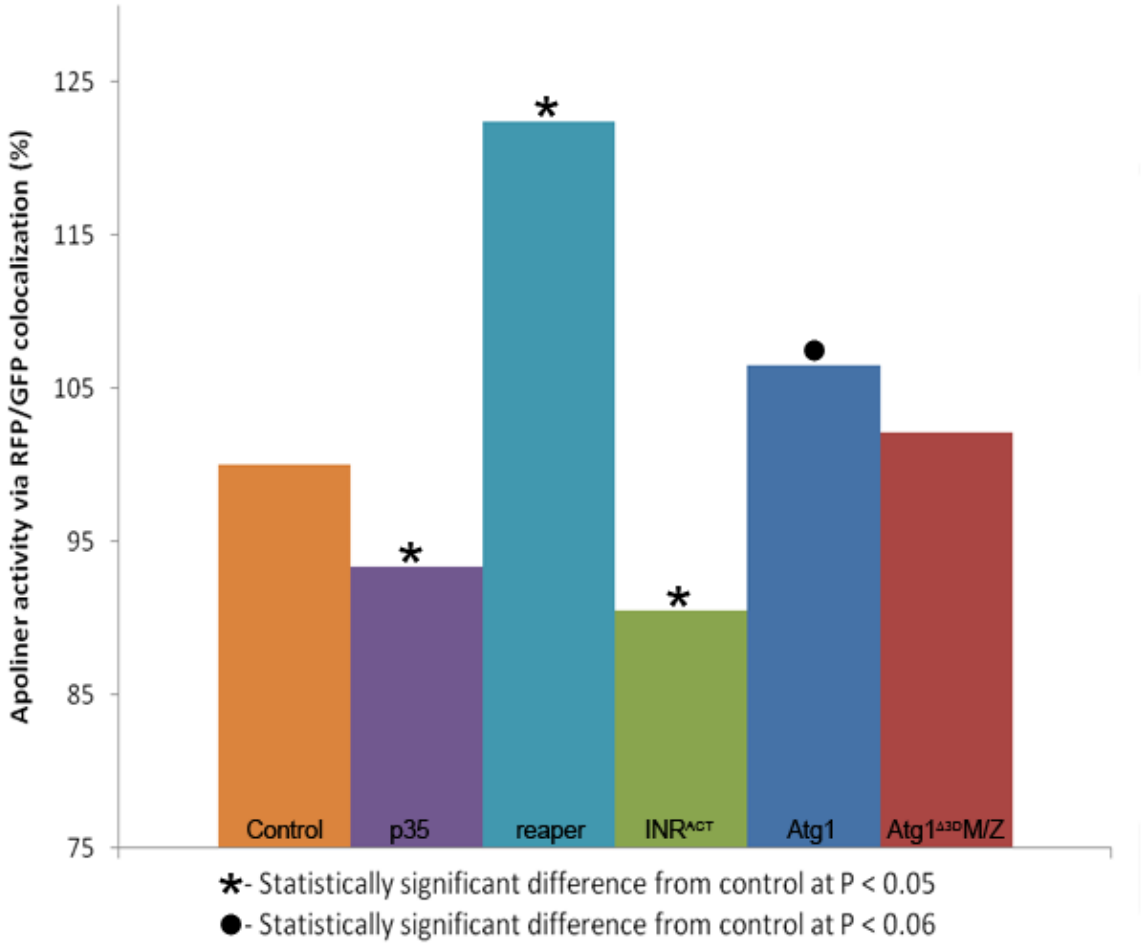
B.2 Examined TGF-β signaling components

The following genetic background were examined with respect to autophagy and cell death in the AS using the extrusion rate assay to evaluate select TGF-β (*dpp*) backgrounds. Although the 4x copy *dpp* genotype lead to an increased rate of extrusions, simply overexpressing *dpp* using the GAL4-UAS system did not confirm the results. It is possible that the increased rate was due to the expansion of the AS in the 4x *dpp* genotype. Other known or proposed components of the *dpp* pathway were either not effective (i.e. tkv^{QD}) or their effects could be attributed to other pathways (i.e. EP(1)1179). Overall, no conclusions regarding TGF-β signaling and AS PCD could be made from the observations.

Construct	**Extrusion Rate**
CONTROL (LP1;Ubi-DE-Cadherin-GFP)	0.148
UAS-Foxo[TM-m3-16] x LP1	0
LP1,UAS-tkv[QD]	0.11
w; dpplacZ(10638) + Ubi DE-Cadherin-GFP	0.139
w; dpp10638 + Ubi	0.199
LP1,UAS-hntRNAi	0.227
LP1, EP(1)1179	0.322
4xDPP Ubi-DE-Cadherin-GFP	0.583

B.3 Quantified Apoliner Activity

As described in Chapter 2, quantification of Apoliner is possible through colocalization analysis of timelapse images. In the figure below, the relative Apoliner activity of examined backgrounds is shown. As seen below overexpression of p35 and activated InR had decreased caspase activation. Both Reaper and Atg1 overexpression increased Apoliner activity. Lastly, Atg1 m/z mutants did not have a change in Apoliner activity.



B.4 Ecdysone and autophagy

Ecdysone is a steroid hormone found in a variety of invertebrate species and sometimes is referred to as the molting hormone. Ecdysone is responsible for many morphogenetic changes during *Drosophila* development, including the programmed death of polyploidy larval tissues, and the extensive rearrangement and shape changes in the mitotically active diploid cells of the imaginal disks (29). Evidence linking ecdysone to autophagy shows that within the *Drosophila* fat body, treatment with ecdysone or ecdysone agonists leads to an increase in autophagy in structures where autophagy in not normally upregulated. Similarly, the expression of dominant negative ecdysone receptor leads to a decrease in autophagosomes, albeit not the early structures involved in autophagy. It is hypothesized that ecdysone is able to regulate autophagy via the PI3K pathway in the *Drosophila* fat body (29). During *Drosophila* embryogenesis there are increases in the ecdysone titre that occur before or during important developmental events. One such event is germ band retraction, when a distinct ecdysone titre peak has been reported (33). This increase in titre is followed by PCD during dorsal closure. Also, previous findings show that AS in active in ecdysone signaling via ligand trap assays. Overall this suggests that ecdysone may be one of the signaling molecules involved in the induction of autophagy and overall PCD in the AS (34).

Ecdysone is synthesized by consecutive modifications of cholesterol by at least 7 enzymes including, *shade* (20-Hydroxylase), *shadow* (2-hydroxylase), *disembodied* (22-hydroxylase), *phantom* (25-Hydroxylase), and *spook* (Cytochrome P450) (35). Deficiency in any of these genes results in a severely decreased ecdysone titre and embryonic lethality at the DC stages. This suggests that ecdysone may participate in the PCD at that time in development.

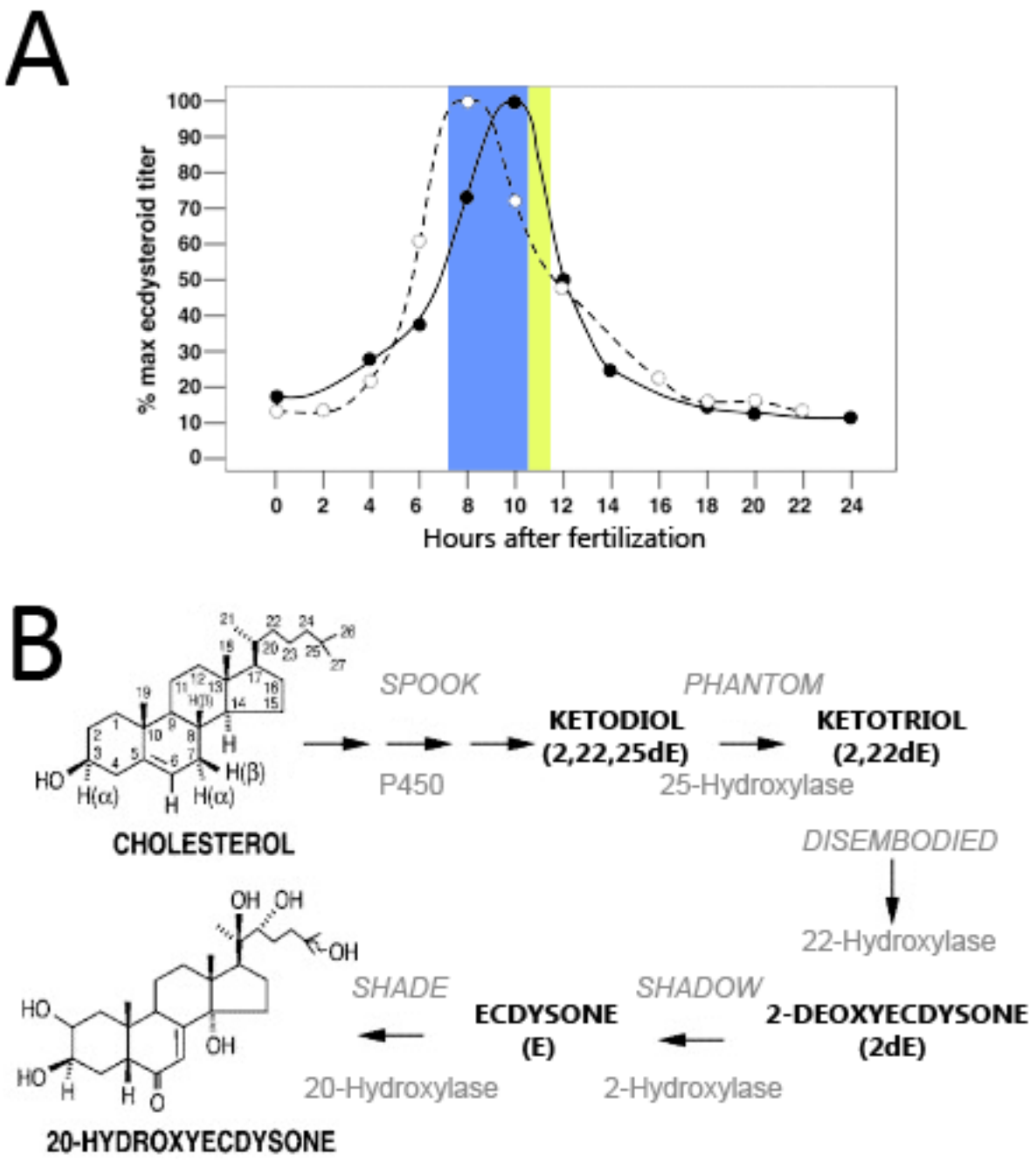


Figure B.4.1: Ecdysone peak prior to AS degeneration (A) occurs during germ band retraction (in blue) and through DC (in yellow). The two curves represent two different experiments by Maroy et al (dotted line) and by Kraminsky et al (solid line). The biosynthesis of biologically active 20-hydroxyecdysone (B) involves *spook* and *disembodied*. Adapted from Sullivan and Thummel, 2003 and Ono et al., 2006.

In order to study whether ecdysone is responsible for the onset of autophagy in the amnioserosa during dorsal closure, mutant embryos deficient in ecdysone biosynthetic enzymes were examined. Both *disembodied* (*dib*) and *spook* (*spo*) are part of the pathway. Embryos homozygous for the null alleles of either *dib* or *spo* are known to have little or no ecdysone titre. These embryos were analyzed using DE-Cadherin-GFP under the ubiquitin promoter and showed developmental delays, contractile abnormalities during dorsal closure, and were left with a dorsal hole at the end of DC (Figure B.4.2). Examination of these embryos using mCherry-Atg8a and DT-Atg8a, labeling autophagic structures, revealed that autophagy is not aberrant in these embryos. Figure B.4.2, shows that as in control (D), embryos homozygous for a mutation in *dib* (F) or *spo* (E) show normal autophagy despite having severe morphological defects. These findings indicate that ecdysone, although important in proper dorsal closure, is not a regulator for autophagy in the AS.

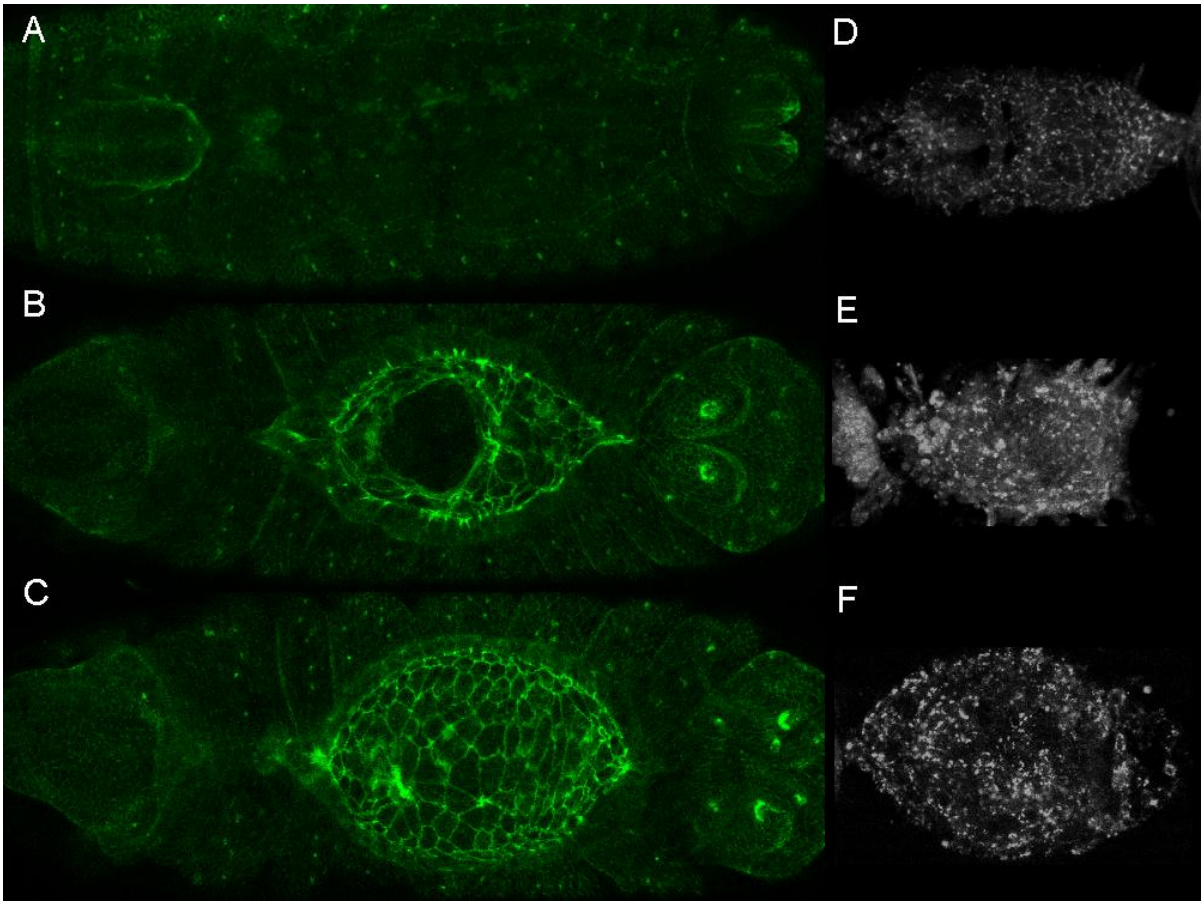


Figure B.4.2: Ecdysone deficient mutants show abnormal morphology but normal autophagy. Spook mutant (B) shows a dorsal hole phenotype but normal mCherry-Atg8a localization (E). Disembodied mutant (C) shows a persistent tissue and incomplete DC but normal autophagy (F). (A,D) Control embryo at same stage.