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Isoform-Specific Effects of the DOA Kinase on Autophagic Cell Death of the Larval Salivary Glands in *Drosophila melanogaster*

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DEDICATION

As John F. Kennedy famously said, “We do these things not because they are easy, but because they are hard.” This thesis is dedicated to everyone that told me it would all be worth it in the end, and who pushed me to get to this point in my academic career.

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ABSTRACT

Families of protein kinases have been conserved over millions of years, and they play important roles in many cellular processes such as the cell cycle, differentiation, and signal transduction (Neiman, 1993). *Doa*, or *Darkener of apricot*, is a locus found in *Drosophila melanogaster* that encodes a protein kinase belonging to the LAMMER kinase family. There are seven known protein isoforms of the DOA kinase, which differ by molecular weight and developmental specificity (Kpebe and Rabinow, 2008). While the isoforms contain identical C-terminal catalytic domains, the N-terminal noncatalytic domains are different due to the use of alternative promoters (Kpebe and Rabinow, 2008). Given the number of different structures of DOA isoforms and their respective developmental specificity, it is possible that the isoforms are performing different molecular functions (Kpebe and Rabinow, 2008).

Macroautophagy, referred to here as autophagy, is a crucial process that aids in the regulation of cellular homeostasis (Lockshin and Zakeri, 2004). Autophagy is lysosomally-mediated, and is an important process involved in the removal and degradation of bulk cytoplasm and long-lived proteins and organelles (Berry and Baehrecke, 2007). The salivary glands in *Drosophila melanogaster* offer an ideal model for studying developmental autophagic cell death *in vivo*, since the bulk of the cytoplasm in the larval salivary glands are removed via autophagic cell death during metamorphosis (Lockshin and Zakeri, 2004).

In this project, interfering RNA (RNAi) constructs encoding short hairpin RNAs (shRNAs) were used to silence the expression of specific *Doa* isoforms through the degradation of homologous mRNA. The constructs each target a different *Doa* isoform mRNA, because the N-termini are all different from one another, and are being expressed from alternative promoters. This approach could hypothetically reveal different functions of the Doa isoforms. The segregation of markers (*Tb* on the balancer chromosome *TM6* or *TSTL*) is used to follow genotypes of overexpression/RNAi for *Doa*.

Since RNAi in *Drosophila* is cell-autonomous, the *GAL4-UAS* system can be used to target the expression of the RNAi constructs. By crossing female flies with a *UAS-GFP* responder to males with a Salivary Gland (SG) GAL4 driver, it is possible to express GFP in the salivary glands of the progeny. The salivary glands can be visualized via fluorescence microscopy, and images taken at 6 hour intervals should reveal a construct's effects on autophagic cell death. Differences in levels of autophagy of the salivary glands between flies with the modifier construct and the wild-type could be indicative of an isoform's involvement in the promotion or inhibition of cell death.

INTRODUCTION

LAMMER Kinases

Families of protein kinases have been conserved over millions of years, and play important roles in nearly all cellular processes such as the cell cycle, differentiation, and signal transduction (Crews and Erikson, 1993; Meyerson *et al*., 1992; Neiman, 1993; Pelech and Sanghera, 1992). Homologs of protein kinases have been found in organisms ranging from the yeast *Saccharomyces cerevisiae* to humans (Kosako *et al*., 1993; Neiman, 1993). By examining complementation of certain mutations in yeast, many kinases with high levels of conservation among their amino acid sequences were shown to possess both structural and functional homology (Leopold and O'Farrell, 1991).

Protein kinases can be grouped into separate phylogenetically related families based on their biochemical activities: ones that phosphorylate on tyrosine residues, and ones that phosphorylate on serine/threonine residues (Hanks *et al*., 1988; Hanks and Quinn, 1991). A small number of "dual-specific" LAMMER kinases autophosphorylate with dual specificity (Ben-David *et al*., 1991; Howell *et al*., 1991; Lee *et al*., 1996; Sessa *et al*., 1996), such as CLK1, which is a mammalian paralogue of DOA (Yun *et al*., 1994, 2000; Hartman and Federov, 2002). However, the phosphorylation of known exogenous substrates for the LAMMER/CLK kinases, such as SR and SR-like proteins, only occurs on Ser/Thr and not on Tyr residues (Nikolakaki, 2002; Lee, 1996). *Doa*'s intrinsic protein

kinase activity was demonstrated after showing it could autophosphorylate when expressed as a fusion protein in *Escherichia coli* (Yun *et al*., 1994).

The catalytic domains in LAMMER kinases are highly conserved, with about a 75% similarity between the amino acid sequence between DOA in *Drosophila* and CLK2 in humans (Kpebe and Rabinow, 2008; Yun *et al*., 1994). Motifs within the catalytic subdomains that are essential for phosphotransfer and substrate interaction are nearly 100% identical, suggesting that they could perform similar functions among widely diverged organisms (Yun *et al*., 1994). The "EHLAMMERILG" motif is contained within subdomain X, which is not required for binding to substrates *in vitro*, but could be crucial for LAMMER kinase activity (Savaldi-Goldstein *et al*., 2000).

While the noncatalytic domains do not show similar conservation, across shorter phylogenetic distances, there is still about 96% similarity between species like *D. melanogaster* and *D. virilis*, despite having diverged from one another over 60 million years ago (Kpebe and Rabinow, 2008). LAMMER kinases contain extensive N-terminal noncatalytic domains, with little to no sequence similarity to one another, but are not cyclin dependent (Yun *et al*., 2000). Although the noncatalytic sequences are likely regulatory and may be less important for overall LAMMER kinase activity, differences in the N-termini may be important, or even necessary, to their function (Kpebe and Rabinow, 2008).

Most noncatalytic domains contain short motifs of Ser-Arg residues in unique or double repeats, but not in triple repeats, which could be reminiscent of a

class of substrates for LAMMER kinases called SR proteins (Rabinow, 2012). However, it would be wrong to place these motifs in the same category as the RS domains found in SR and SR-like proteins, as the latter contain more numerous, more extensive tri-peptide RS repeats.

SR and SR-like proteins are among the only identified substrates *in vivo* of LAMMER kinases to date (Nikolakaki *et al*., 2002). SR proteins are best known for their roles in pre-mRNA splicing, as they effect mRNA localization and stability, and aspects of RNA metabolism, such as transcript elongation (Manley and Tacke, 1996; Yun *et al*., 2000). The proteins are phosphorylated both *in vivo* and *in vitro* on Ser-Arg (SR) rich domains, which affect the specificity of both their RNA-protein and protein-protein interactions (Cao *et al*., 1997; Tacke *et al*., 1997; Xiao and Manley, 1997; Stojdl and Bell, 1999).

Introduction to DOA as a Kinase

In *Drosophila,* the *Darkener of apricot (Doa)* locus encodes a protein kinase, belonging to the LAMMER kinase family, which is expressed ubiquitously throughout the organism (Kpebe and Rabinow, 2008; Yun *et al*., 2000). The insertion of a copia retrotransposon into the second intron of the *white* gene resulted in the $white^{apricot}$ allele (Bingham and Judd, 1981). While screening

for dosage-sensitive modifiers of the *white*^{apricot} allele, many loci, such as *Doa*, were identified (Rabinow and Birchler, 1989; 1990; Rabinow *et al*., 1993).

Doa alleles are unique given their dominant suppression of copia-induced mutations, including *white*^{apricot}, which results in a darker eye-color phenotype (Rabinow and Birchler, 1989; 1990; Rabinow *et al*., 1993). Increased rates of copia-specific transcription would help explain a two- to fourfold increase of the copia transcript (Rabinow *et a*l., 1993). This could also be explained by decreased rates of degradation, which could be linked to nonsense-mediated decay (Chapin *et al*., 2014). *Doa* alleles have the ability to alter the accumulation of mRNAs, independent from the directionality of the transposon insertion into the host-locus (Rabinow *et al*., 1993). This suggests that the suppression of phenotypes induced by *copia* is strand-independent, and it is possible that the suppression of *white*^{apricot} by *Doa* is indirect, depending on DNA rather than the transcript itself (Yun *et al*., 2000). It also suggests that LAMMER kinases could have an effect on cellular processes other than splicing (Rabinow *et al*., 1993).

Molecular cloning techniques were used to uncover developmentally-regulated patterns of *Doa* expression (Yun *et al*., 1994; 2000). *Doa* alleles are pleiotropic in nature, and are almost always recessive lethal, suggesting that *Doa's* product plays an important role within the fly (Kpebe and Rabinow, 2008; Rabinow *et al*., 1993). Although homozygous larvae from heterozygous mothers can hatch normally, they do not usually survive past the early larval stages (Rabinow and Birchler, 1989). Homozygotes from heteroallelic mutant mothers

show severe disruption of differentiation in the ventral nervous system, as well as a loss of cuticular segmentation (Yun *et al*., 1994). This finding supports the hypothesis that there is a maternal contribution of *Doa* function to the oocyte which can rescue homozygous mutants during early developmental stages, and suggests that LAMMER kinases might play a role in differentiation (Kpebe and Rabinow, 2008; Yun *et al*., 2000).

While it is rare that heteroallelic adults or homozygous mutants manage to escape lethality, the ones that do show interesting phenotypes, such as smaller imaginal discs, aberrant wing venation, and retinal photoreceptor degeneration (Yun *et al*., 1994). The degeneration of the photoreceptors suggests that constant *Doa*, or LAMMER kinase, activity is required for cellular viability (Yun *et al*., 2000). *Doa* plays a role in the development of bristles on the scutellum and head capsule, as well as eye morphology, given that trans-heterozygotes often display phenotypes in these areas (Rabinow *et al*., 1993). It also suggests that *Doa* activity is required in the nervous system (Yun *et al*., 2000). Its function is also necessary in the germ line, since cells homozygous for null alleles of *Doa* from both the soma and germline are inviable (Yun *et a*l., 2000; Morris *et al*., 2003).

Doa has been found to play a role in many different cellular, differentiative, and behavioral processes (Figure 1). Screens done in S2 cells have linked *Doa* to cell cycle progression, as it invokes the DNA damage checkpoint from both the G1 to S phase (Bettencourt-Dias *et al*., 2004) and the G2 to M phase (Bjorklund *et al.,* 2006), as well as to protein secretion (Bard *et al*., 2006).

Doa very likely plays a role in autophagy (Gorski *et al*., 2003), as analysis of genes expressed differentially during autophagic cell death of the salivary glands throughout pupation indicate that *Doa* transcripts are among one of the four most induced (Gorski *et al.,* 2003). *Doa* also influences alternative splicing via the direct phosphorylation of SR and SR-like proteins (Du *et al*., 1998).

Reduced SR protein phosphorylation and abnormal intranuclear localization found in *Doa* mutants suggest that LAMMER kinase activity plays a role in the regulation of alternative pre-mRNA splicing (Du *et al*., 1998). However, it is unclear whether this is a constitutive or regulated requirement (Du *et al*., 1998). Unusual pre-mRNA splicing of *doublesex (dsx)*, encoding a key sex-regulatory protein, induces mild female-to-male transformations in *Doa* mutants (Du *et al*., 1998; Rabinow and Samson, 2010). Mutations in the DOA kinase can lead to hypophosphorylation of SR-related splicing factors, *TRA* and *TRA2,* which are direct determinants of somatic sex determination (Du *et al*., 1998).

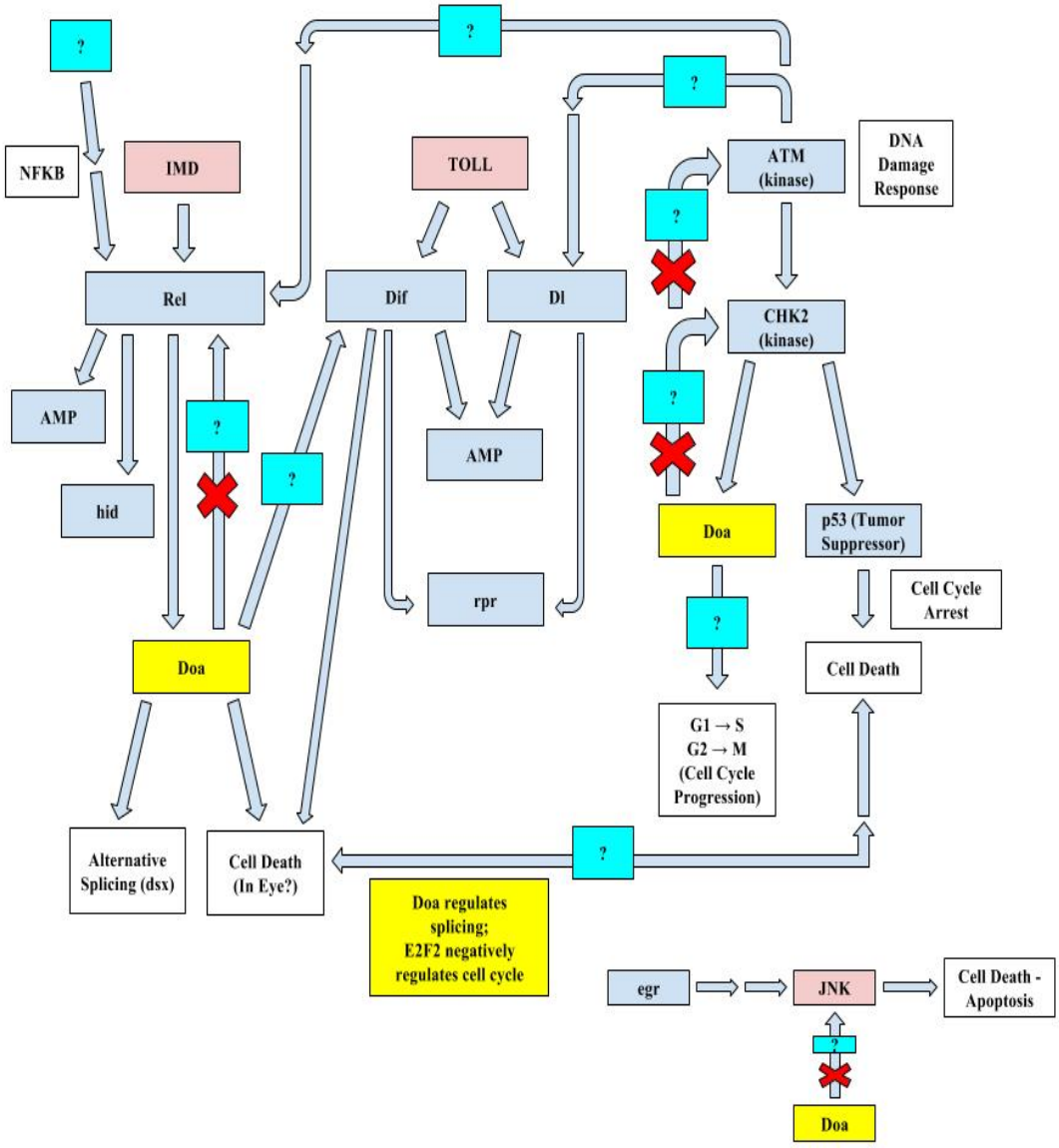


Figure 1. Overview of processes *Doa* is involved in. I put this graph together, to illustrate the number of different cellular, differentiative, and behavioral processes *Doa* has been linked to. This chart only attempts to show the number, and complexity, of the roles *Doa* could be playing. While this image makes a guess as to some of the molecular mechanisms that could be involved in these processes, much of the way *Doa* regulates, and is in turn regulated by, certain genes or pathways is still unknown. This experiment focuses specifically on *Doa*'s involvement in cell death in the salivary glands.

Molecular Structure of the *Doa* Locus

There are seven known protein isoforms of DOA kinase (Figure 2), which differ by molecular weight and developmental specificity (Kpebe and Rabinow, 2008). While the isoforms contain identical C-terminal catalytic domains, the N-terminal noncatalytic domains are different, due to the use of alternative promoters (Kpebe and Rabinow, 2008). Given the number of different structures of *Doa*'s isoforms and their respective developmental specificity, it is possible that the isoforms could be performing different molecular functions (Kpebe and Rabinow, 2008).

Some alleles and functions have previously been linked to specific DOA isoforms. For example, the 55 kD and 69 kD proteins are thought to play a major role in alternative splicing, given their nuclear localization and phenotypes resulting from isoform-specific mutations (Kpebe and Rabinow, 2008). The 227 kD isoform has been implicated in the regulation of organelle transport along microtubules (Serpinskaya *et al*., 2014). However, there is still much research to be done in regards to the potential roles each isoform might be playing, and just how diverse those roles might be. The functions of different isoforms may be independent, overlap, or could even oppose one another.

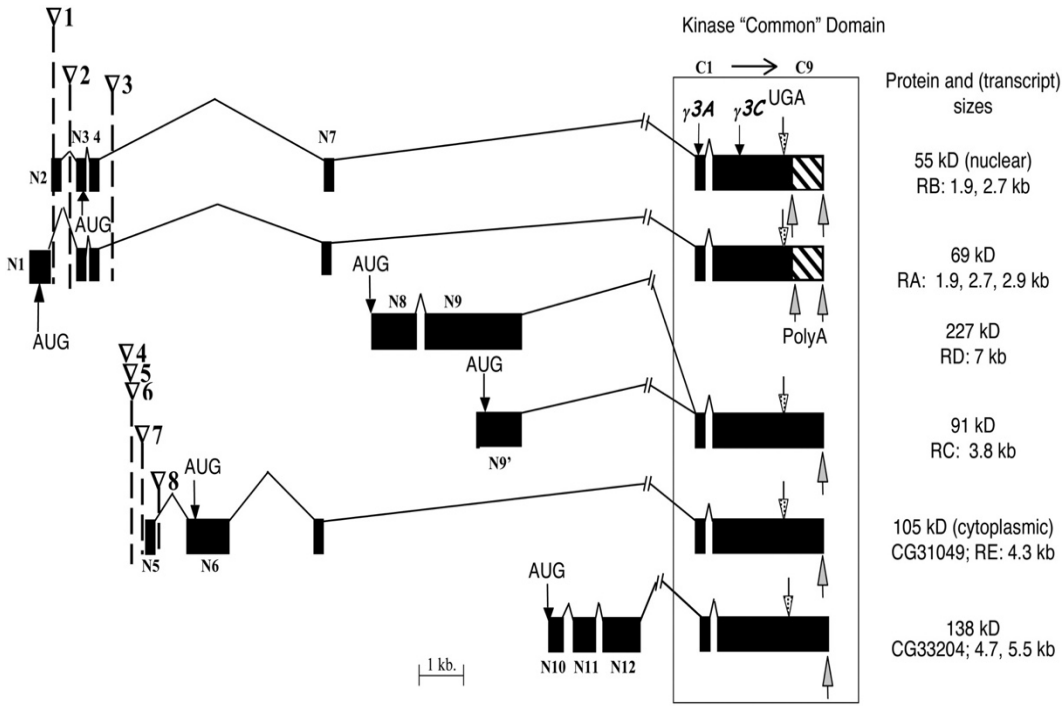


Figure 2. Map of the *Doa* locus. This figure shows the majority of different isoforms of *Doa* (55 kD, 69 kD, 91+227 kD, 105 kD, and 227 kD), along with their associated proteins (Kpebe and Rabinow, 2008). Since the 138 S+L kD and 138 L kD isoforms were identified more recently, there is only one structure for the 138 kD isoform shown in the image above (which happens to be the 138 S+L kD isoform). It is not possible to target just the 91 kD isoform, as it is completely overlapped by the 227 kD isoform. The C-terminal catalytic domains are identical, however, the N-terminal noncatalytic domains are different due to the use of alternative promoters (Kpebe and Rabinow, 2008).

RNAi

The use of interfering RNA (RNAi) in experiments became quite popular following the discovery that double-stranded RNA (dsRNA) could be used to knockdown an individual gene's activity (Fire *et al*., 1998). Unlike previous

genetic screening techniques, RNAi offers a unique reverse genetic screening approach, which became particularly useful after various organisms' whole genomes were sequenced (Adams *et al*., 2000). In *Drosophila*, the creation of various genome-wide RNAi resources allowed for reverse genetic screens to be performed both in tissue culture and *in vivo* (Boutros *et al*., 2004; Perrimon *et al*., 2010). RNAi is a useful tool in characterizing the functions of pleiotropic genes such as *Doa* (Perrimon *et al*., 2010).

There are four types of RNAi reagents that are generally used in vivo: small interfering RNAs (siRNAs), small hairpin RNAs (shRNAs), small hairpin microRNAs (shmiRNAs), and long double-stranded RNAs (dsRNAs) (Echeverri and Perrimon, 2006; Lee and Kumar, 2009). Dicer, a ribonuclease, processes dshRNA or shRNA inside of the cytoplasm, and generates siRNAs from there (Perrimon *et al*., 2010). The siRNAs can be transfected into cells, where one strand from the siRNA duplex is then incorporated into the multi-subunit ribonucleoprotein complex RISC (Perrimon *et al*., 2010). RISC then can complementary base pair to the target sequence, ultimately resulting in the silencing of the target gene via the degradation of homologous mRNA (Figure 3) (Perrimon *et al.,* 2010).

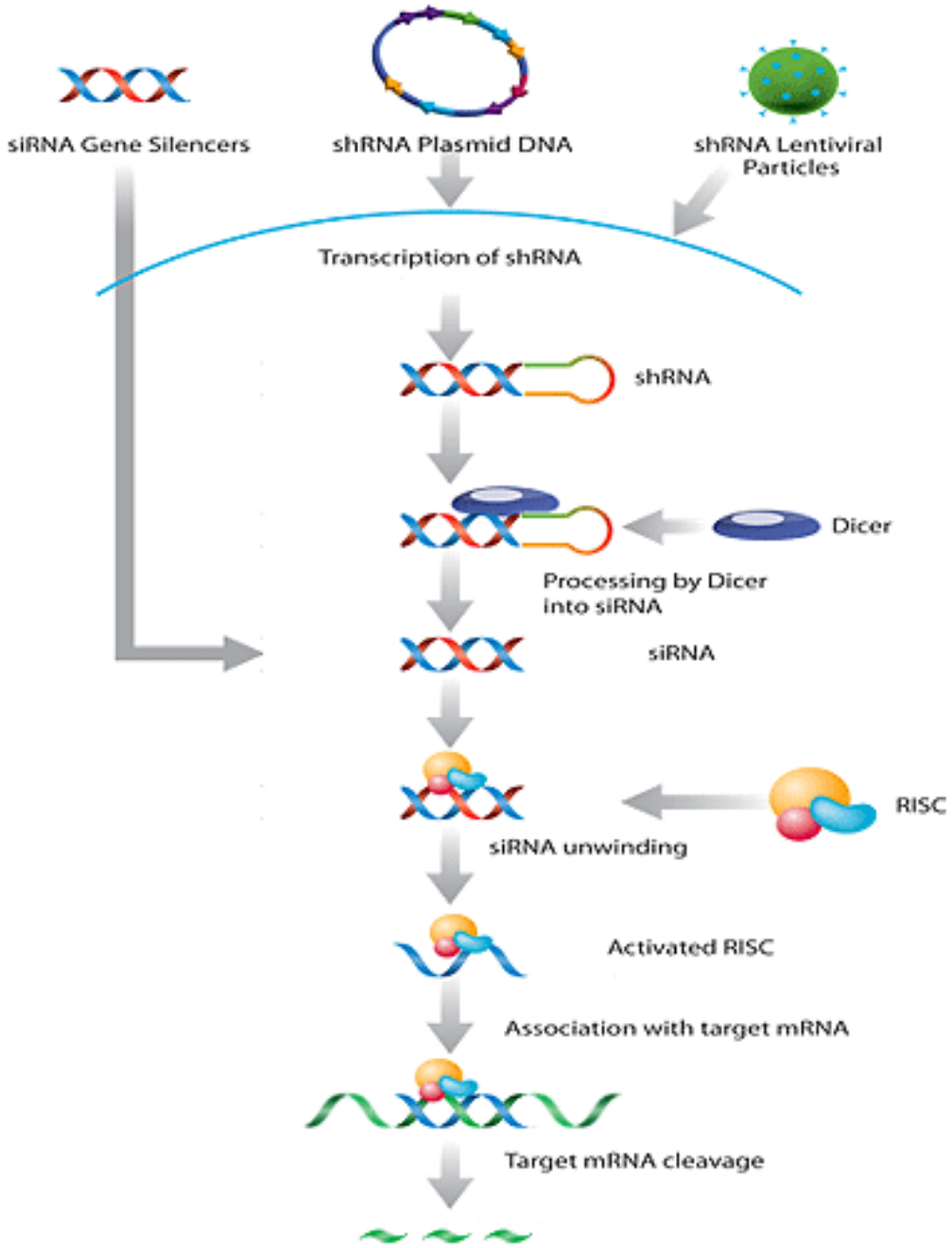


Figure 3. RNAi regulation of gene expression. This figure shows how RNAi can be used to knockdown a gene's activity by silencing a target gene through the degradation of homologous mRNA (Santa Cruz Biotechnology).

The *GAL4-UAS* System

In 1993, Andrea Brand and Norbert Perrimon developed the *GAL4-UAS* system, which can be used to target gene expression in *Drosophila* and allows for the selective activation of any cloned gene in numerous tissue- and cell- specific patterns (Brand and Perrimon, 1993). The ability to control the ectopic expression of a gene of interest is a useful tool for understanding the role a gene plays in vivo (Duffy, 2002).

The GAL4 protein, first identified in the yeast *Saccharomyces cerevisiae*, is induced by galactose, and was demonstrated to regulate the transcription of genes *GAL1* and *GAL10* (Laughton *et al*., 1984; Laughon and Gesteland, 1984; Oshima, 1982). The protein makes a good candidate for a transcriptional activator, given that it has no endogenous targets in *Drosophila*, and should therefore only express the gene of interest (Brand and Perrimon, 1993). GAL4 can be expressed in all embryonic cells and tissues derived from the endoderm, ectoderm, and mesoderm, and thus has the ability to direct expression in a number of different embryonic patterns in *Drosophila* (Brand and Perrimon, 1993).

GAL4 activates transcription by binding directly to four related 17 bp sites, which define an Upstream Activating Sequence (*UAS*) element (Giniger *et al*., 1985; Fischer *et al*., 1988). *UAS* elements are necessary for the transcriptional activation of GAL4-regulated genes (Duffy, 2002), and can control the expression of the gene of interest in a variety of systems (Kakidani and Ptashne, 1988; Ma *et*

al., 1988; Webster *et al.,* 1988). If the cell-type does not express GAL4, then the *UAS*-element will remain almost completely transcriptionally silent (Duffy, 2002). By crossing flies containing the gene of interest linked to a *UAS* sequence, to flies expressing a GAL4 driver, the resulting progeny will then express the gene in a transcriptional pattern specific to the driver used (Figure 4) (Duffy, 2002).

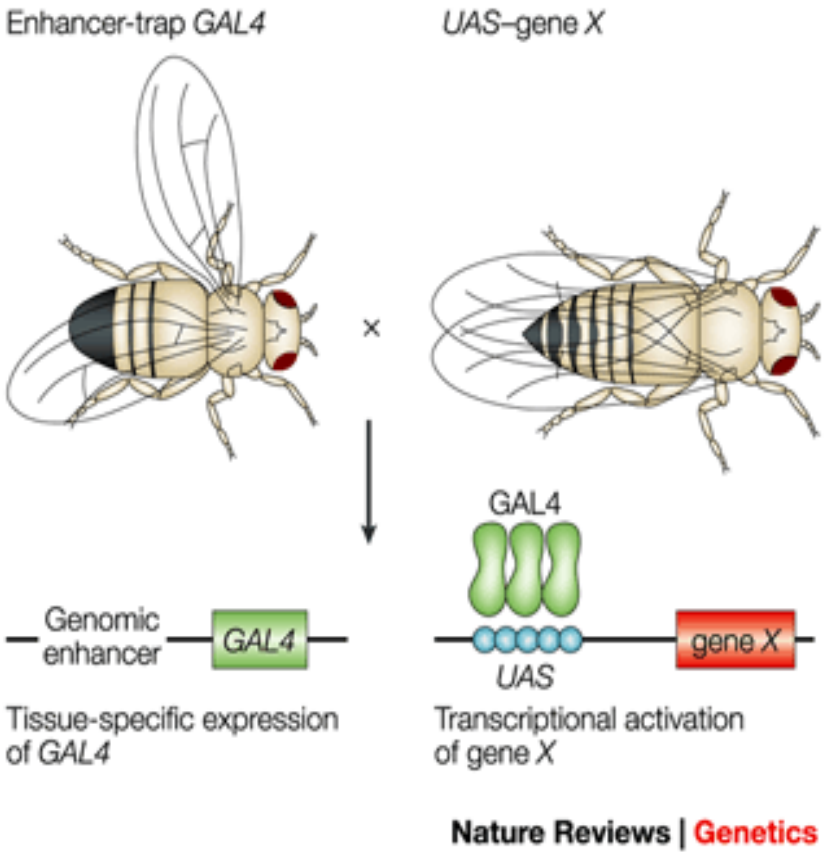


Figure 4. The *GAL4-UAS* system in *Drosophila*. The *GAL4-UAS* system can be used to drive expression of the gene of interest within a specific cell type or tissue in *Drosophila*. Transgenic flies with the target gene are crossed to flies with a specific GAL4 driver. The target gene within the progeny will be transcribed in a pattern specific to the driver used (St Johnston, 2002).

Autophagic Cell Death

Historically, most examples of cell death were categorized as lysosomal prior to the discovery of caspases (Lockshin, 1969). Over time, as the relationship between lysosomes, autophagic vacuoles, and autophagosomes became clearer, the term 'autophagic cell death' was introduced (Lockshin and Zakeri, 2004). Depending on both the location and role of the lysosomes, at least three forms of cell death are commonly known to occur during the development of an organism (Schweichel and Merker, 1973). These forms of cell death are: Apoptosis (type 1), autophagy (type 2), and necrosis (type 3) (Schweichel and Merker, 1973; Clarke, 1990). While additional forms of cell death do exist, such as necroptosis and anoikis, both apoptosis and autophagy are among the most common forms of developmental cell death (Cabon *et al*., 2013).

Macroautophagy, hereafter referred to as autophagy, is an evolutionarily ancient process well-documented in species as simple and diverse as yeast and *Dictyostelium*, and is often observed in metamorphosing insects (Cornillion *et al.,* 1994; Levraud *et al*., 2004; Olie *et al*., 1998). Despite having been around for so long, the mechanistic role of autophagy is still a subject of debate (Levine and Yuan, 2005), and does not always lead to cell death (Lockshin and Zakeri, 2004). Autophagy could also serve as a means of reducing cell mass prior to the induction of apoptosis (Bursch *et al*., 2004; Lockshin and Zakeri, 2001; 2004).

The catabolic process is best known for its role in the removal of bulk cytoplasm and long-lived proteins or organelles (Klionsky and Emr, 2000). Once the cytoplasmic cargo is sequestered into double-membrane vesicles, it can be delivered to the lysosome and targeted for degradation (Berry and Baehrecke, 2008). This normal physiological process plays a crucial role in the maintenance of cellular homeostasis, via organelle turnover, and atrophy (Lockshin and Zakeri, 2004). A disruption of either could affect cell growth, division, and death, and could result in diseases, such as cancer, or abnormal tissue and organ size (Lowe *et al*., 2004; Levine and Kroemer, 2009; Cuervo and Wong, 2014). There are also numerous neurodegenerative diseases associated with the failure of autophagy, most likely due to a cell's inability to maintain cellular homeostasis, and the inhibition of autophagy in mice leads to neurodegeneration and premature death (Gorman, 2008; Wong and Cuervo, 2010; Navone *et al*., 2015).

Autophagy is a process often used for the recycling of materials during times of starvation, and can be provoked by starvation and catabolic hormones (Wang and Klionsky, 2003). It is possible that autophagy functions as a protective mechanism, in which the induction of the process is an attempt by the cell to reduce its metabolic demand (Lockshin and Zakeri, 2004). This survival response could be intended to block cell death, or could allow for the self-digestion of cells in the absence of phagocytes (Berry and Baehrecke, 2008). The induction of autophagy could also be a means of generating maintenance resources, or as a way of sequestering the mitochondria to prevent the release of cytochrome c

(Bauvy *et al*., 2001). Whether or not autophagy acts to protect cells, or is instead a means to their destruction, is not always clear, and could be dependent on the status or history of the cell (Lockshin and Zakeri, 2004)

While apoptotic cell death is caspase-dependent, autophagic programmed cell death is often caspase-independent, does not require engulfing phagocytes, and autophagosomes are present within the dying cells (Kerr *et al*., 1972; Clarke, 1990; Martin and Baehrecke, 2004). Caspases (Cysteine-Aspartic Proteases) are cellular proteases, which require a carefully coordinated release from inhibition to become activated (Lockshin and Zakeri, 2004). This activation event can occur as a result of metabolic changes that have depolarized the mitochondria and caused the subsequent release of cytochrome c and APAF-1, or as a response to the ligation of membrane-bound receptors (Lockshin and Zakeri, 2004). At times when caspase activity is blocked, autophagy could provide an alternative pathway for cell death to occur (Lang-Rollin *et al*., 2003).

Autophagic cell death is preceded by growth arrest, and maintaining growth, via the activation of *Ras* or a positive regulator of the PI3K pathway, can inhibit autophagy of the salivary glands (Berry and Baehrecke, 2008). However, maintaining growth is not sufficient in preventing caspase activation (Berry and Baehrecke, 2008). *Atg* genes are required for autophagy to occur, and developmental degradation is inhibited in *Atg* mutants (Berry and Baehrecke, 2008). The majority of *Atg* and caspase genes in *Drosophila* are induced at the time of salivary gland cell death (Gorski *et al*., 2003; Lee *et al*., 2003). While the

inhibition of caspases alone, via inhibitors of apoptosis such as *p35* (Jiang *et al*., 1997; Lee and Baehrecke, 2001; Lee *et al*., 2002; Martin and Baehrecke, 2004), can prevent DNA fragmentation and nuclear lamin cleavage (Lee and Baehrecke, 2001; Martin and Baehrecke, 2004), it is not sufficient in completely preventing autophagic cell death (Lee and Baehrecke, 2001). Increased suppression of cell death in the salivary glands can be achieved through the combined inhibition of both autophagy and caspases (Berry and Baehrecke, 2008).

Drosophila melanogaster as a Model Organism

The larval salivary glands in *Drosophila* offer an ideal model for studying developmental autophagic cell death *in vivo*. Insects often utilize autophagy in many of their tissues, especially as cells are remodeled during metamorphosis, when the bulk of the cytoplasm in the larval muscles and salivary glands are removed (Lockshin and Zakeri, 2004). The only tissues that do not exhibit cell death during metamorphosis are those in the central nervous system, such as the imaginal discs and cells in histoblast nests. In *Drosophila*, the formation of autophagic vacuoles are most prominent within the salivary glands (Lockshin and Zakeri, 2001). The first 90% of cell death of the salivary glands is autophagic, with no sign of the activation of caspases or other indicators of cell death

(Lockshin and Zakeri, 2004). Blocking autophagy during metamorphosis will result in lethality during pupariation (Juhasz *et al*., 2003).

Following a rise in the steroid hormone 20-hydroxyecdysone (ecdysone) at 10 hours After Puparium Formation (APF) at 25°C, and its peak at 12 hours APF, the synchronous death of the larval salivary glands is activated through a transcriptional regulatory hierarchy (Baehrecke, 1996; Yin and Thummel, 2005; Nicolson *et al*., 2015). By 13 hours APF, the salivary glands have become more round in shape, some fragmentation of cytoplasmic vacuoles has occurred, and new vacuoles associated with the plasma membrane have appeared (Lee and Baehrecke, 2001). At 14 hours APF, following the recent rise in ecdysone and its effects on tubulin and actin within the cytoskeleton, autophagic vacuoles containing cytoplasmic structures can be found within the salivary glands (Lee and Baehrecke, 2001). By 16 hours APF, the salivary glands have been significantly degraded, and cell morphology suggests that the production and movement of vacuoles, cytoplasmic reorganization, as well as the degradation of the cytoplasm by proteases may have played a role in the autophagic cell death of the salivary glands (Lee and Baehrecke, 2001; Baehrecke, 2003).

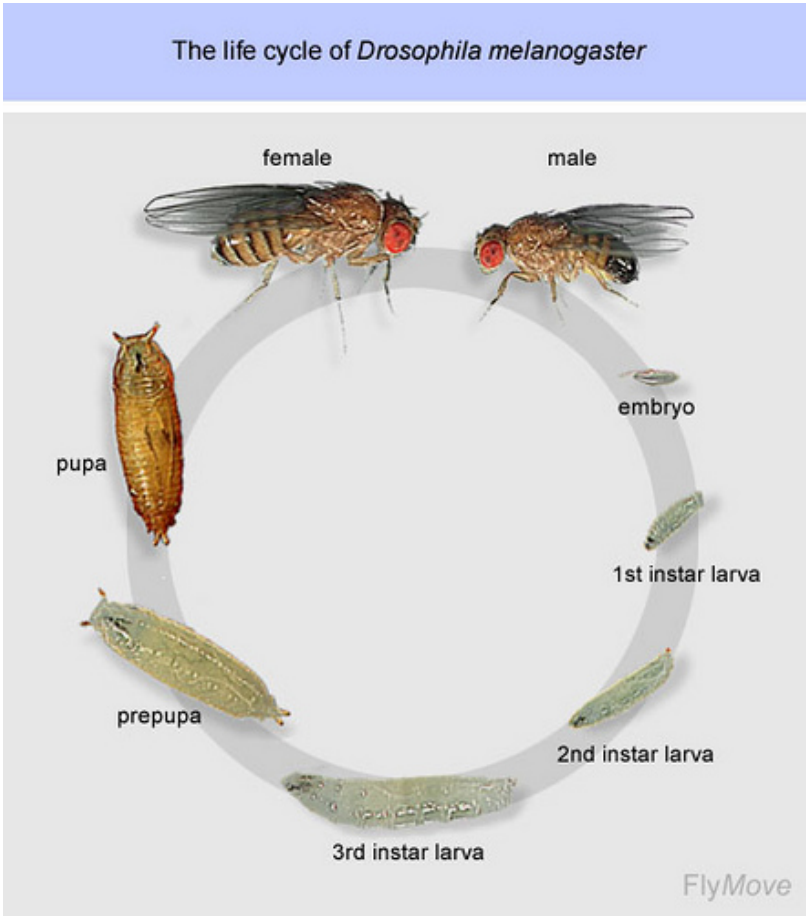


Figure 5. The life cycle of *Drosophila melanogaster*. The average life cycle of *Drosophila melanogaster* takes about 10 days. The organism will continue to grow and feed until about 24 hours prior to pupariation. The larva will then stop feeding and transition into the perusal state, and metamorphosis will begin and continue until eclosion (FlyMove).

Experimental Rationale

Given the number of different structures of DOA isoforms, and their respective developmental specificity, it is possible that these isoforms could be performing separate molecular functions (Kpebe and Rabinow, 2008; Serpinskaya

et al., 2013). These functions may be independent, overlap, or even oppose one another. Within the scope of this experiment, it is hypothesized that there are isoform-specific effects of the DOA kinase on the autophagy of the larval salivary glands in *Drosophila.*

RNAi constructs, encoding shRNAs, can be used to silence the expression of a specific *Doa* isoform, given that each isoform has different N-termini due to the use of alternative promoters. The RNAi constructs, on the second or third chromosome, are first crossed to *T(2;3) TSTL, CyO; TM6B, Tb*, and the segregation of markers (*Tb* on *TSTL* or *TM6*) can be used to follow the genotypes of overexpression/RNAi for *Doa*.

Since RNAi in *Drosophila* is cell-autonomous, the *GAL4-UAS* system can be used to target the expression of the RNAi constructs. By mating female flies with a *UAS-GFP* responder to male flies carrying a *SG-GAL4* driver, it is possible to drive expression of GFP in the salivary glands. Since the salivary glands of the progeny will be labelled with GFP, the salivary glands can be visualized through the use of fluorescence microscopy.

Images of the salivary glands were taken every 6 hours, from 12 to 24 hours APF, to demonstrate the effects of overexpression/RNAi constructs for *Doa* on autophagy. Discrepancies within the amount of autophagy of the salivary glands, when comparing the pupae carrying the modifier construct to the wild-type, could be indicative of an isoform's involvement in the promotion or inhibition of cell death.

MATERIALS AND METHODS

Drosophila Husbandry and Maintenance

Stock Genotypes

1. *UAS Doa-IR* Constructs (Table 1)
2. *T(2;3) TSTL, CyO; TM6B, Tb*
3. *UAS-GFP; SG-GAL4*

Maintenance

Drosophila melanogaster stocks were maintained at 25°C (in 50% humidity) unless otherwise noted. When collecting virgin female flies, stocks were kept at 18°C, and females were collected every 6-10 hours as needed.

Genesee Scientific supplied standard Drosophila food, consisting of agar, yeast, cornmeal, malt, and corn syrup. The food was prepared in 1000 mL of water, with the addition of 7.5 mL of Propionic Acid and 10 mL of Tegosept (in 95% EtOH), which both act as mold inhibitors. Vials were left overnight to harden, then stored in a refrigerator until needed.

Injection of the RNAi Constructs

The *Doa* RNAi constructs each encoded shRNAs, about 20 bp long, and were expressed under the *UAS-GAL*4 system. The constructs were injected by Christians Villalta in Norbert Perrimon's laboratory. The injection was done via a site-directed transposition at the attP site, which is specific to bacteriophage phiC31 (Figure 6). Embryos were injected in the posterior pole plasm with a pool of 7 DNA constructs (one corresponding to each *Doa* isoform). A pooled injection was possible given that this was a phiC31 transposition. While each transformant is an independent event, a site-specific insertion should result in the equal expression of all injected constructs at the same, unique site.

The injected host fly stock carried the attp40 site on chromosome 2L. The attp40 site and integrase carry the *yellow* (y^+) gene, and the VALIUM20 vector (Figure 7), used for cloning the shRNA constructs, carries the *vermillion* (v^+) gene. The v^+ gene, carried by the Vermillion-AttB-Loxp-Intron-UAS-MCS (VALIUM) transgene, rescues the *vermillion (v)* allele inherited from the injection stock. Therefore, the G1 transformants will display the dark red wild-type eye color phenotype among flies with *vermillion* colored eyes.

Each female G_0 fly from the injected larvae was crossed individually to a male fly with the genotype *yv; Gla*/CyO. The progeny was screened for y^+v^+ transformants, which were then individually backcrossed with *yv; Gla*/CyO. This rids the source of the integrase from the X chromosome, and balances the

chromosome with the transgene. From there, $y^{+}v^{+}$*; shRNA/CyO* stocks were established.

Once balanced stocks of the transgenic flies were generated, the DNA was sent for sequencing. Since this was a pooled injection, each G1 transformant could carry a different construct, so each line had to be sequenced in order to determine which line corresponded to which *Doa* isoform.

All crosses were maintained at 25°C unless otherwise noted. Balanced stocks were kept at 18°C, and virgin female flies were collected every 6-10 hours.

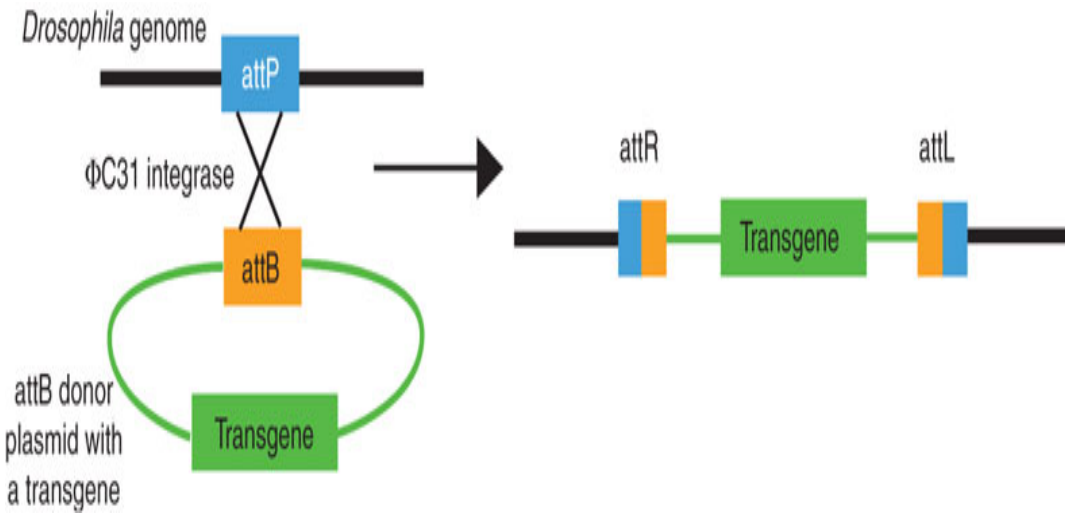


Figure 6. PhiC31 integrase vector system. A sequence-specific integrase, phiC31, is encoded within the phiC31 bacteriophage, and mediates recombination between attachment sites (att) in the bacteriophage and bacterial host. The preplaced attP sequence acts as a recipient site in the *Drosophila* genome. PhiC31 integrase mRNA is coinjected into a plasmid containing both the donor sequence (attB) and the transgene, which results in the site-specific insertion of the transgene at the attP site. Further integrase-catalyzed movement of the integrated transgene is prevented by the hybrid sites attL and attR, which were created during the process (Fish *et al*., 2007).

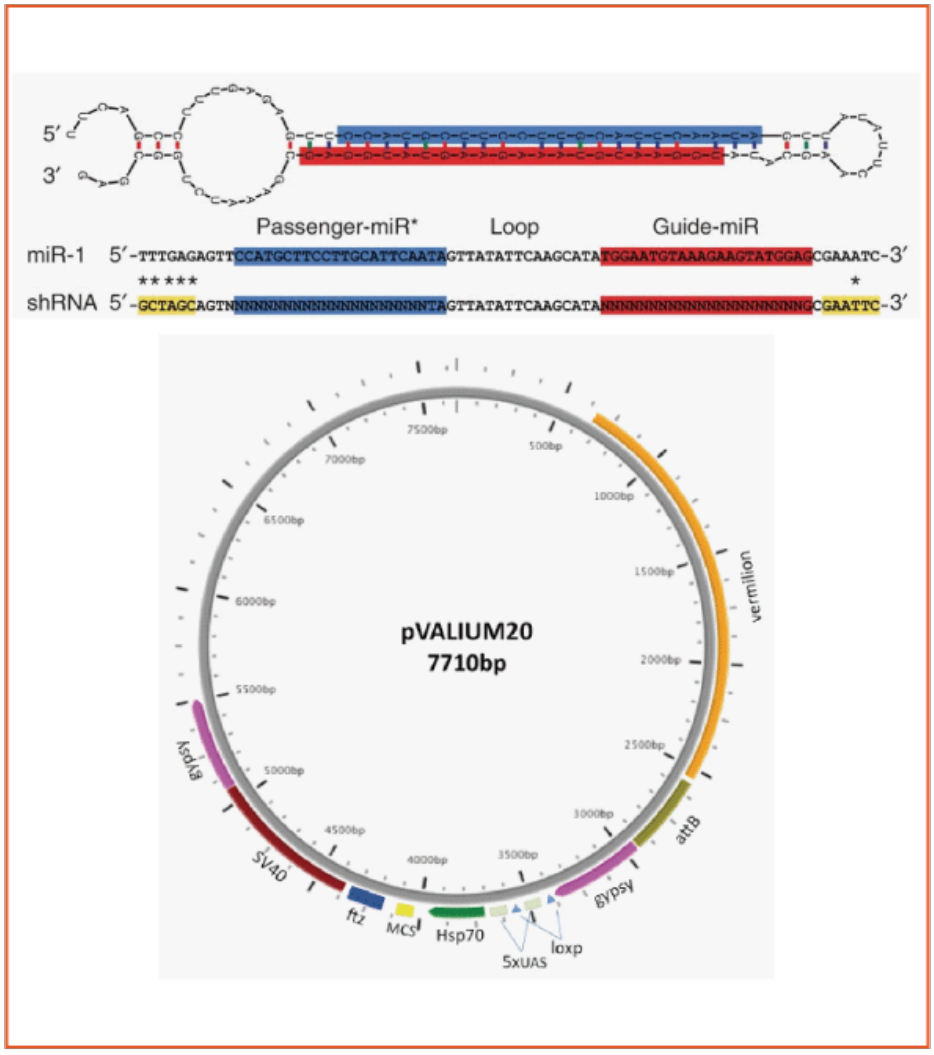


Figure 7. The VALIUM 20 vector. Due to a large number of random insertions generated by P-element transformations, which rendered many RNAi lines ineffective, a series of VALIUM site-specific insertion vectors were generated by TRiP (Dietzl *et al*., 2007). A second generation of vectors, including the VALIUM20 vector, were produced after discovering a more effective way to knock down genes in the stoma and germline (Ni *et al*., 2011). This new method uses shRNAs with a 21 bp targeting sequence embedded into a miRNA (miR-1) backbone (shown at the top of this image) (Haley *et al*., 2008; Ni *et al*., 2010; 2011). The bottom of this image shows the structure of the VALIUM20 vector, which carries the *vermillion* (v^{+}) gene (shown in orange), as well as the attB sequence (shown in olive green) necessary for the phiC31-target integration at the attP landing site (Groth *et al*., 2004). The vector also contains two gypsy insulators (shown in purple), which enhance hairpin DNA transcription (Ni *et al*., 2010). In the *GAL4-UAS* system, two *5xUAS*s (shown in white) can be removed using the Cre/loxP method to control expression levels (Ni *et al*., 2010). The vectors can be used to incorporate RNAi hairpins into attP landing sites (Ni *et al*., 2008, 2009, 2011). RNAi constructs are integrated into sites in the genome where induced GAL4-driven gene expression is high and optimal expression can occur (Markstein *et al*., 2008; Ni *et al*., 2008; 2009).

Preparation of DNA for Sequencing

Genomic DNA Purification

5 male transformant flies were collected and ground using a pestle in 50 µl of a 'Squishing Buffer and Proteinase K' solution. The Squishing Buffer consists of 10 mM Tris pH 8.0, 1mM EDTA, and 25 mM NaCl. To make the SB + ProK solution, 1 mL of SB buffer is mixed with 1 µl of Proteinase K (20 ng/µl).

The samples were incubated at 37°C for 50 minutes to digest the proteins, then spun in a centrifuge at 13,200 rpm for 2 minutes to pellet large debris and insoluble materials, before transferring the supernatant into strip tubes. The gDNA was heated at 95°C for 5 minutes to inactivate the proteinase K, before either continuing onto PCR, or freezing the samples at -20°C.

PCR

Typically, a PCR reaction mix includes the template DNA, free nucleotides, Taq Polymerase (the enzyme), and a primer. The primers are small oligonucleotide fragments of single-stranded DNA, which are able to hybridize with the template DNA at specific sites. Once heated, the strands of DNA will denature, allowing the primer to anneal to its target location. The DNA polymerase can then elongate the primer and form new double-stranded DNA. By the time the PCR reaction is finished, many identical copies of the original template DNA will have been created.

Each PCR sample consisted of 2.5 µl of the gDNA template and 27.5 µl of master mix. To make master mix for 50 samples, you use 600 µl of the enzyme-buffer mix GoTaqGreen from Promega, 466 µl nuclease free water, 17 µl (1:10 dilution) of the forward primer, and 17 µl (1:10 dilution) of the reverse primer. The primers are based on the vector sequences, not the inserted shRNAi coding sequences, so they are universal for the Valium vector used (Table 3).

The PCR program was set to the following temperature profile: 1. 95°C for 2 minutes (*Taq* activation), 2. 95°C for 30 seconds (denaturation), 3. 55°C for 30 seconds (annealing), 4. 72°C for 40 seconds (extension), 5. Repeat steps 2-4 for 35 cycles, 6. 72°C for 10 minutes (final extension).

Gel Electrophoresis

To make the agarose gel, 1.5 g of agarose was mixed with 150 mL of 1x TBE and microwaved until completely dissolved. 5 µl of ethidium bromide was added to 11 mL of the hot agarose solution, then poured and left to harden for 30 minutes.

Each lane was loaded with a mix of 5 µl of DNA loading buffer and 4 µl of the DNA sample. The DNA loading buffer consists of 1 mL of 2x TBE, 0.5 mL Glycerol, and 0.02 g Bromophenol Blue. 0.7 µl of the DNA ladder was injected into one lane. Gels were run at 75V for 30 minutes, and imaged afterwards to determine the presence of the desired product. Samples were either prepared for purification or frozen at -20°C.

PCR Product Purification

PCR samples were purified using the Qiagen DNA Purification Kit and individual QIAquick spin columns. The desired final DNA concentration was between 10-20 ng/µl. Purified DNA samples were run on a gel, and imaged to determine the presence of the desired product. Samples were either prepared for sequencing or frozen at -20°C.

Preparing Primers for Sequencing

A 1:50 dilution was performed to get the primer stock (100 µM) to the desired final concentration (2 µM). The primer mix consisted of 1.6 µl of nuclease free water and 0.4 µl (2 µM) of either the forward or reverse primer. Each strip tube being sent for sequencing contained 2 µl of primer mix and 12 µl of purified DNA. The samples were prepared for sequencing following guidelines set by the DF/HCC DNA resource core at the Dana-Farber Cancer Institute.

Analyzing DNA Sequencing Results

Upon getting the sequence analysis back, a BLAST search was run to see where the sequences aligned on the Drosophila genome. *Doa* is located on chromosome 3R around the 28 million nucleotide mark, so alignments around this area were of particular interest. Based on the results from the BLAST search, the

flies were labeled with the *Doa* isoform they corresponded to. 2 fly lines for each of the isoforms were maintained at 18°C. Any additional duplicate lines were thrown away.

Following the first injection, only 6 of the 7 isoforms had been isolated, as the 69 kD isoform did not appear in any of the sequenced fly lines, and had to be re-injected.

UAS-GFP; *SG-GAL4*

RNAi Constructs

Table 1. List of the RNAi constructs used in this experiment. The 'old' RNAi constructs were either constructed or obtained by Leonard Rabinow (sources listed), and the 'new' shRNAi constructs were isolated by myself. The 69kD isoform is listed in red, as it was not isolated in any of the transformants resulting from the pooled injection, and had to be re-injected at a later date.

OLD CONSTRUCTS	NEW CONSTRUCTS (shRNA)
55 kD-IR / CyO; TM6 / MKRS	55 kD
UAS Dicer 69 kD-IR #1 / TM6	69 kD
UAS 69 kD-IR #29 (II)	105 kD
UAS 69 kD-IR #45 / TM3	138 S + L kD
UAS 91 kD-IR #3 / TM6	138 L kD
UAS 91 kD-IR #1 / CyO (II)	91 + 227 kD
Fimi 105 kD-IR (III) (National Inst. Genetics, Japan)	227 kD

Mimi / 105 kD-IR (III) (National Inst. Genetics, Japan)	
EY08857 (III) (Bloomington Indiana *Drosophila* Stock Center. This construct permits GAL4-driven overexpression of the 105 kD isoform from the endogenous locus. This is a P-element insertion, carrying a UAS, in the *Doa* gene itself.)	
138 kD-IR / TM6 (Vienna *Drosophila* RNAi Stock Center)	
227 kD-IR #1 / TM3	
227 kD-IR #3 / TM3	
UAS Doa CAT wt (II) (This construct is an overexpression of only the catalytic domain of the DOA kinase. N-terminal elements, which are assumed to be regulatory in nature, are not included. When the wild-type construct is expressed in the eye, it results in a rough, shiny phenotype indicative of cell death.)	
UAS Doa CAT K199R (This construct is identical to the one above, except for a one amino-acid substitution from Lysine to Arginine at position 199 in DOA. This change results in the inactivation of the kinase function. Unlike the previous construct, when expressed in the eye, this does not show any phenotype. This suggests that kinase catalytic activity is required to produce the rough, shiny phenotype found when DOA is overexpressed.)	
UAS NLS Doa CAT/CyO (II) (This construct is identical to the UAS Doa CAT wt construct, but contains an additional Nuclear Localization Sequence. The NLS was added to the ORF, so the protein should be directed to the nucleus. The rough, shiny eye phenotype is present again, which suggests that DOA can function in the nucleus.)	
UAS NLS Doa CAT K199R (II) (This construct is identical to the UAS Doa CAT K199R construct, but also contains an additional Nuclear Localization signal. Just like the previous construct, the protein should be directed to the nucleus. No rough, shiny phenotype in the eye is present.)	

Experimental Crosses

The *Doa* RNAi constructs (Table 1) were crossed to *T(2:3) TSTL, CyO; TM6B, Tb*, which is a translocation between the second and third balancer chromosomes *CyO* and *TM6*. While all of the 'new' RNAi lines are on the second chromosome at the insertion site attP40, the 'older' constructs are on either the second or third chromosome. Since markers such as *Curly (Cy,* on the *CyO* balancer chromosome) or *Tubby (Tb,* on the *TM6* balancer chromosome) are dominant, one can recognize the marker's presence in the crosses. Segregation of *Tb* on either the *TM6* or *TSTL* (Figure 8, 9), from the *Doa* overexpression or RNAi constructs, permits the identification of the *Doa* modifier chromosome in both the larvae and pupae. The wild-type larvae and pupae and the 'tubby' pupae are easy to differentiate, as the *TM6* balancer carries the *Tb* dominant mutation, which makes the pupae appear shorter and more 'tubby' in comparison to the wild-type pupae (Lattao *et al*., 2011).

The balancers suppress recombination events, but any progeny with chromosomes that did happen to recombine will be lethal. Once the respective genotypes of interest were isolated, 1-2 male flies were crossed to 5 virgin female *UAS-GFP; SG-GAL4* flies. The salivary glands in the progeny, which are labeled with GFP, are examined using a fluorescence microscope.

All crosses were performed at 25^{o}C.

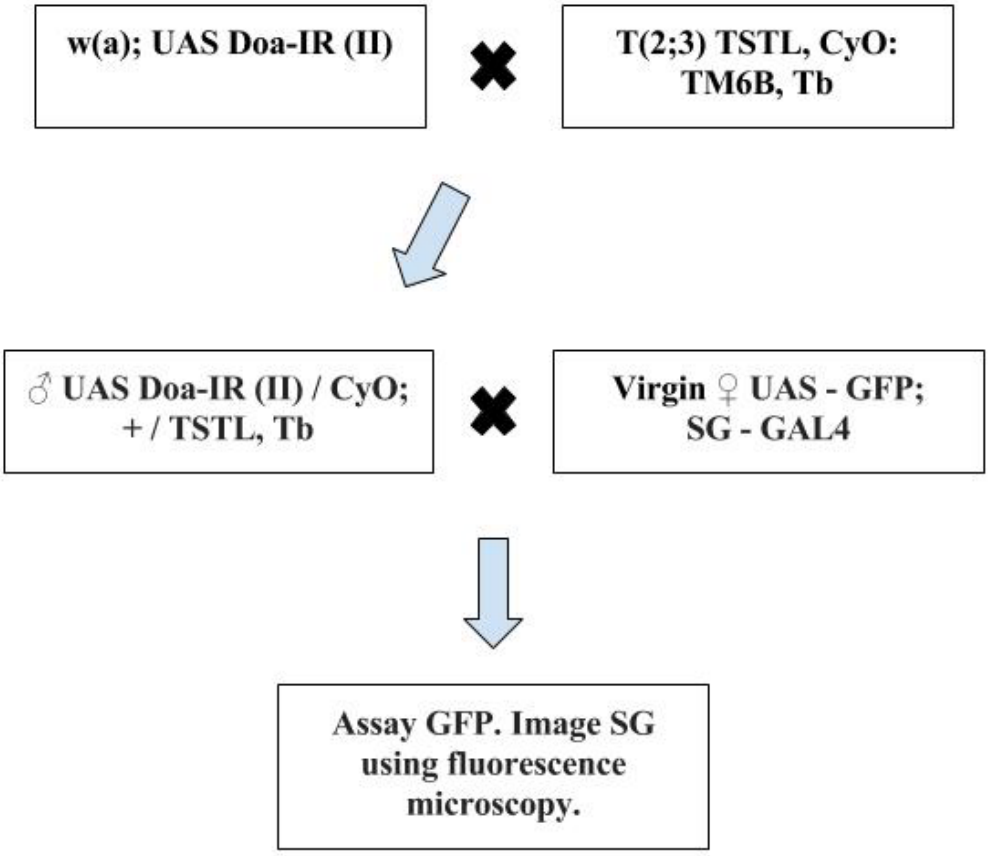


Figure 8. RNAi constructs on the second chromosome. Overview of the crosses involved in the *UAS-GFP; SG-GAL4* experiment. The RNAi constructs on second chromosome were crossed to *T(2;3) TSTL, CyO; TM6B, Tb*. 1-2 of the male progeny were then crossed to 5 virgin female *UAS-GFP; SG-GAL4* flies. The segregation of markers, in this case *Tb* on the *TSTL* balancer chromosome, was used to follow the genotypes of overexpression/RNAi for *Doa*, and the effects on autophagy are shown via the GFP-marked expression of the salivary glands.

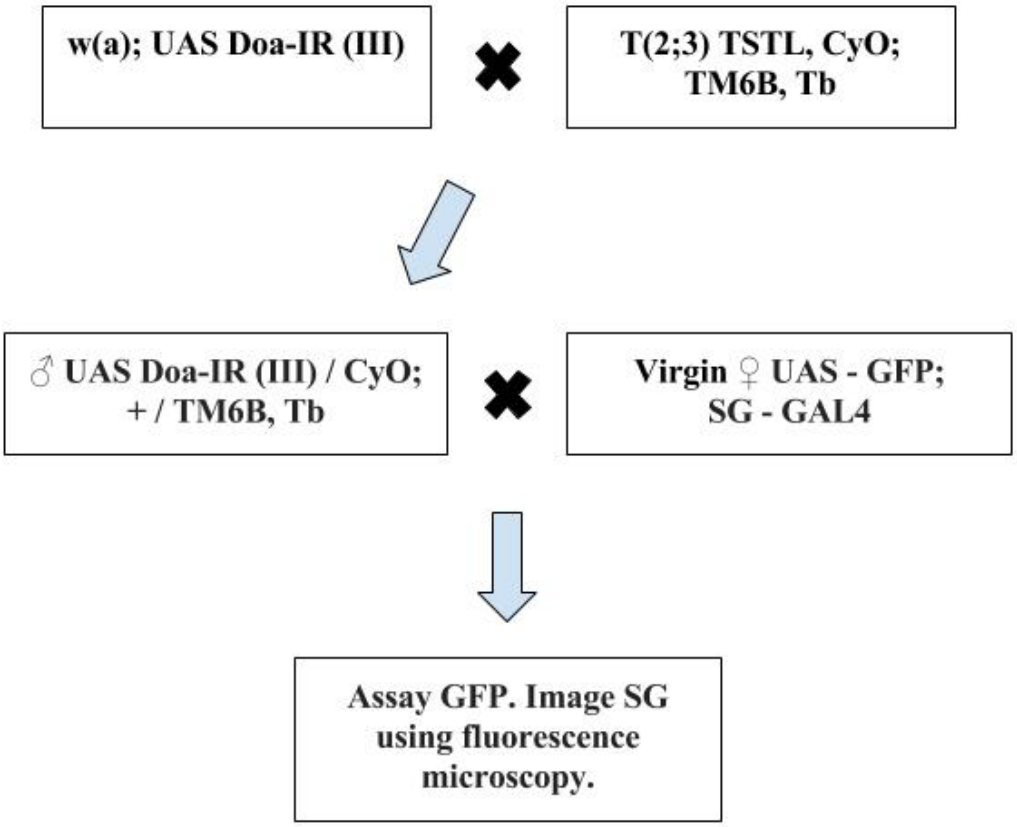


Figure 9. RNAi constructs on the third chromosome. Overview of the crosses involved in the *UAS-GFP; SG-GAL4* experiment. The RNAi constructs on third chromosome were crossed to *T(2:3) TSTL, CyO; TM6B, Tb.* 1-2 of the male progeny were then crossed to 5 virgin female *UAS-GFP; SG-GAL4* flies. The segregation of markers, in this case *Tb* on the *TM6* balancer chromosome, was used to follow the genotypes of overexpression/RNAi for *Doa*, and the effects on autophagy are shown via the GFP-marked expression of the salivary glands.

Fluorescence Microscopy

Images of the salivary glands were taken in 6 hour intervals, at 12, 18, and 24 hours APF, to illustrate the effects of each RNAi construct on autophagy of the salivary glands. The salivary glands were not dissected out, but rather, images were shot through the pupal case. Differences in the amount of cell death in the salivary glands when comparing the modifier construct and the wild-type (balancer) pupae could be indicative of a specific isoform's involvement in the inhibition or promotion of autophagy.

Fluorescent images were captured using a Nikon TE20000-U microscope, under the 4x objective lens. The FW-FITC filter was used to visualize the GFP-labelled salivary glands. The image software NIS was used to capture images at a set exposure time of 1.5 seconds, and to add scale bars 250 µl in length. Image resolution was set to 300 dpi.

RESULTS

The salivary glands were visualized through the pupal case, due to endogenous GFP, and driven from a *UAS-GFP* construct using a *Salivary-Gland-GAL4 (SG-GAL4)* driver. Fluorescent images were taken every 6 hours, from 12 to 24 hours APF, to show the effects of blocking expression of a specific *Doa* isoform with RNAi or shRNA on the promotion or inhibition of autophagy in the larval salivary glands in a qualitative manner.

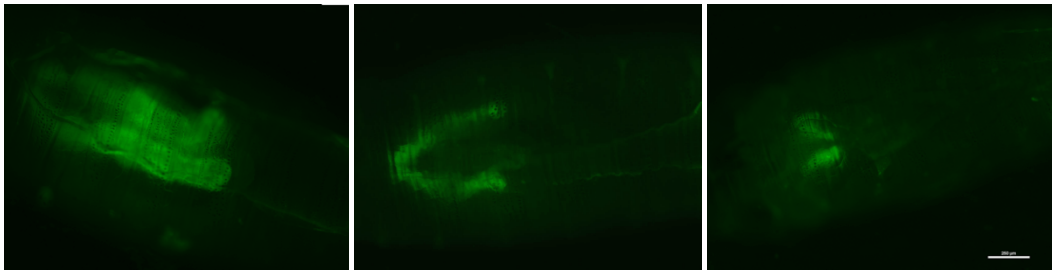
Overall, the majority of images taken for each RNAi or shRNA construct showed no apparent differences in level of GFP expression between the modifier construct and wild-type pupae at any given time point. There were, however, some noticeable differences in the levels of GFP expression between the modifier construct and wild-type pupae when RNAi and shRNA constructs were used to block the expression of the 105 kD *Doa* isoform (Fimi 105 kD-IR, Mimi/105 kD-IR III, and 105 kD shRNA constructs). These differences are illustrated in Figure 12a-c below, and are most striking at 18 hours APF.

By 24 hours APF, there is often little to no GFP expression left in the salivary glands.

10a: UAS 55 kD-IR/CyO; MKRS/TM6

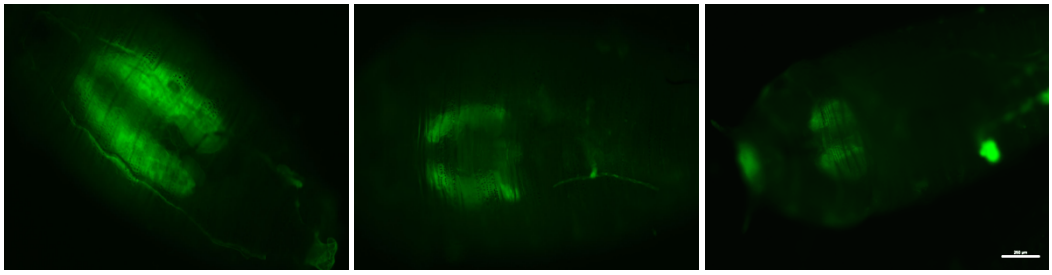
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

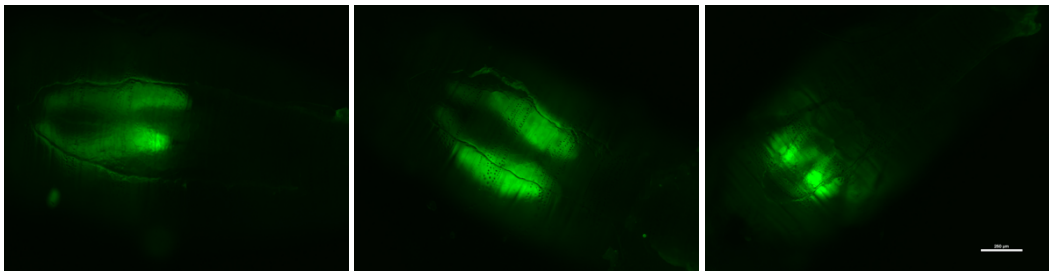
12hr APF | 18hr APF | 24hr APF



10b: 55 kD shRNA

Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

12hr APF | 18hr APF | 24hr APF

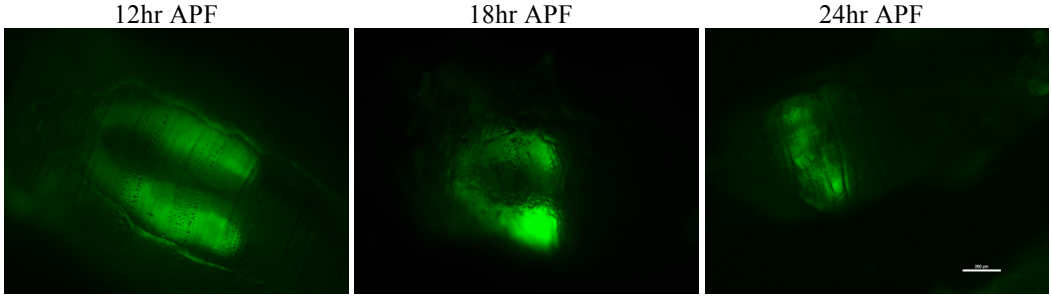
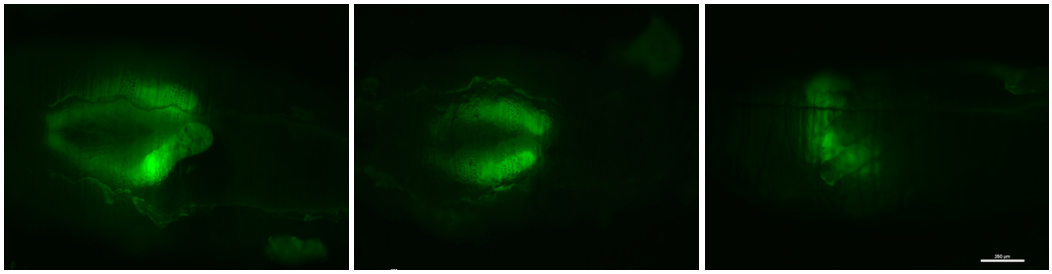


Figure 10a-b. Blocking expression of the 55 kD isoform with RNAi or shRNA. There seem to be no noticeable differences in the level of GFP expression in the salivary glands between the modifier construct and wild-type pupae. Scale bars are 250 µl in length.

11a. UAS Dicer 69 kD-IR #1/TM6

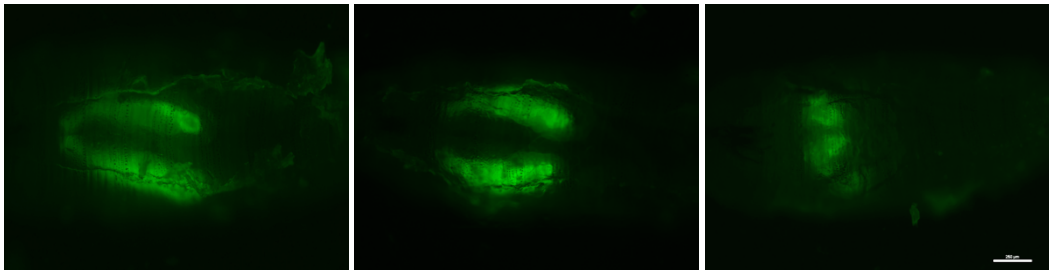
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

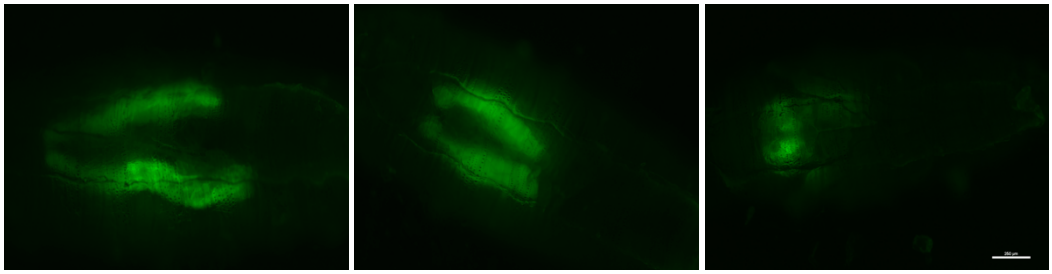
12hr APF | 18hr APF | 24hr APF



11b. UAS 69 kD-IR #29

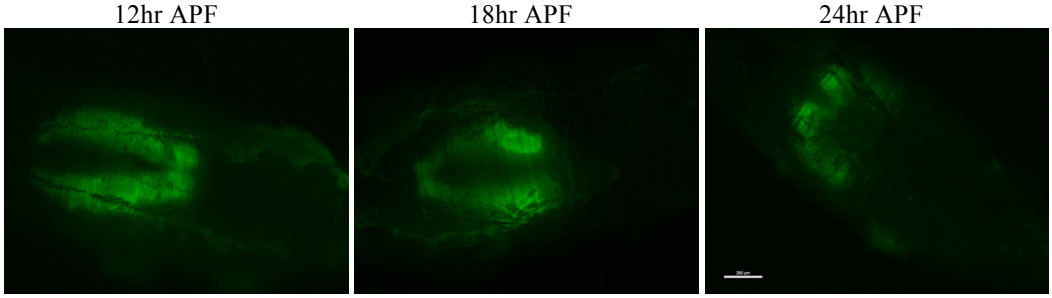
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild-Type:

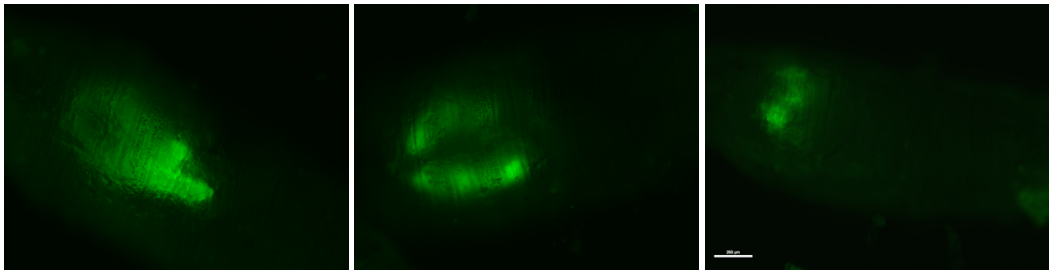
12hr APF | 18hr APF | 24hr APF



11c. UAS 69 kD-IR #45/TM3

Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

12hr APF | 18hr APF | 24hr APF

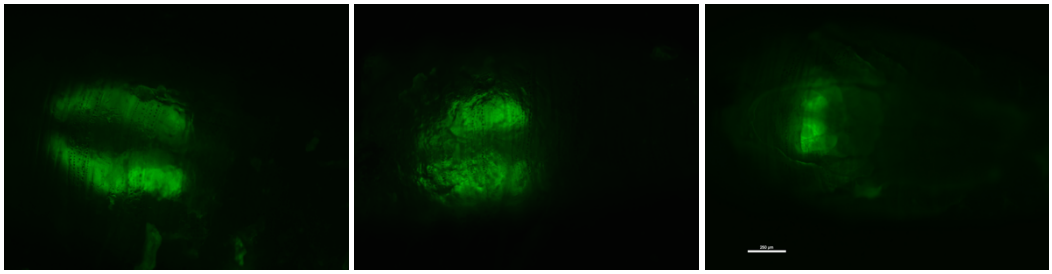
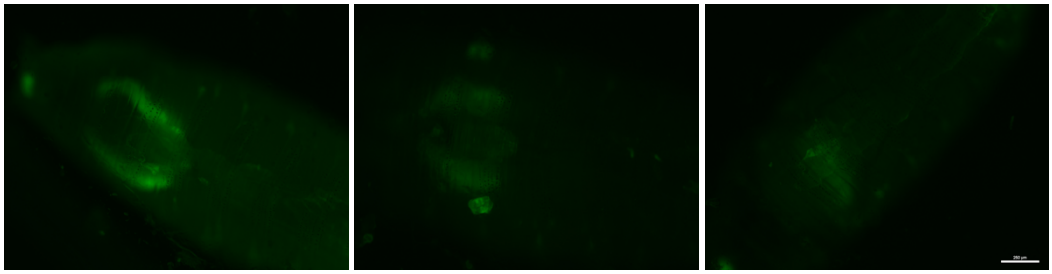


Figure 11a-c. Blocking expression of the 69 kD isoform with RNAi. There seem to be no noticeable differences in the level of GFP expression in the salivary glands between the modifier construct and wild-type pupae. There was no shRNA construct available for the 69 kD isoform, as the isoform was not isolated from the previous pooled injection of shRNA constructs, and had to be re-injected. Scale bars are 250 µl in length.

12a. Fimi 105 kD-IR

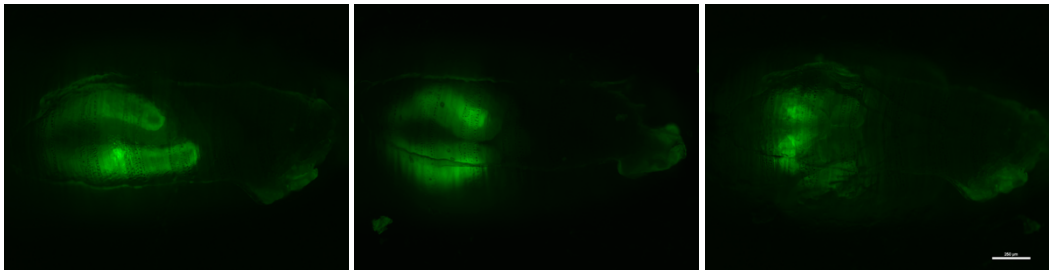
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

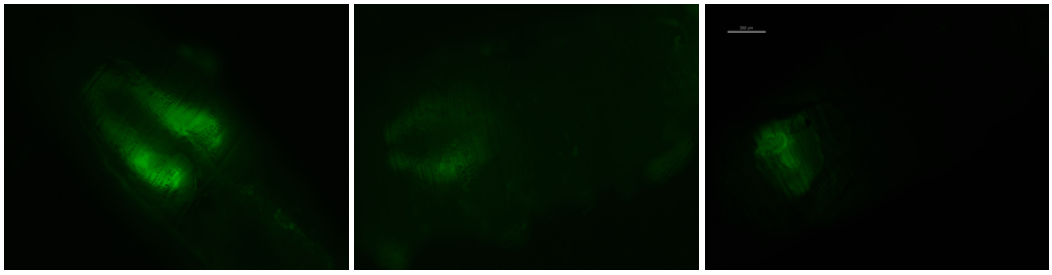
12hr APF | 18hr APF | 24hr APF



12b. Mimi/105 kD-IR III

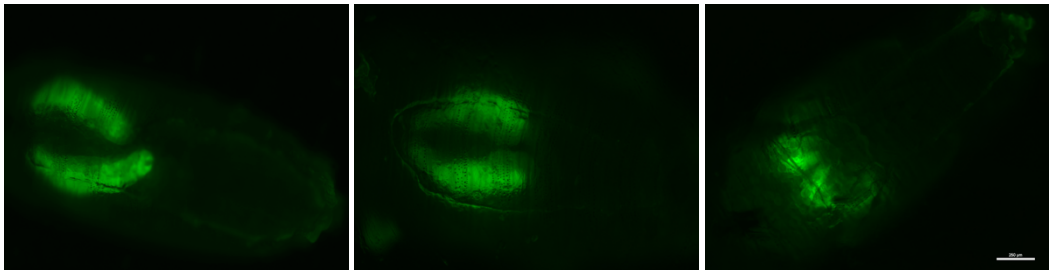
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

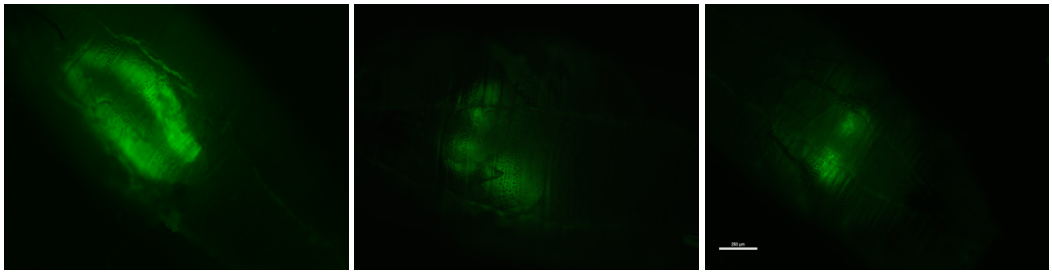
12hr APF | 18hr APF | 24hr APF



12c. 105 kD shRNA

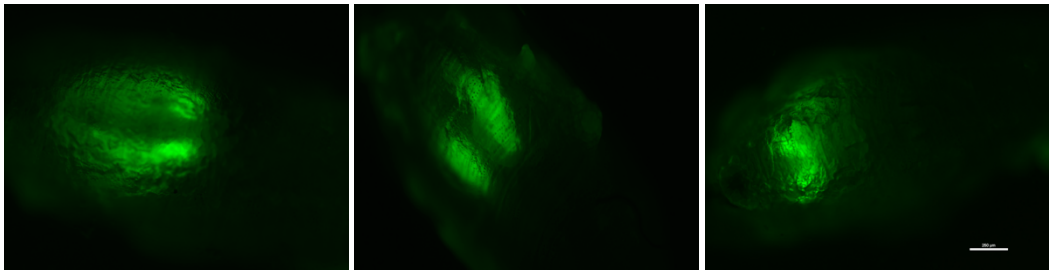
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

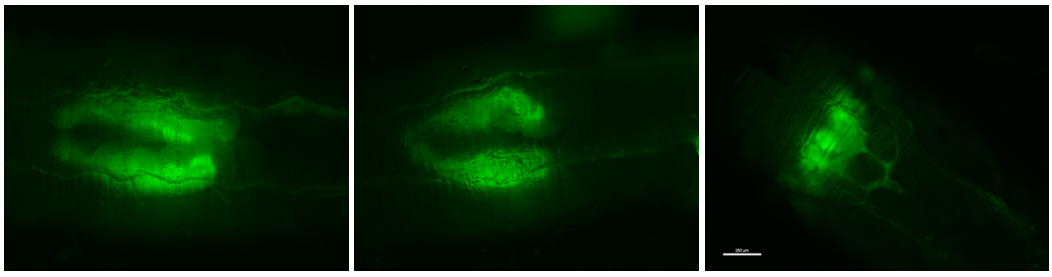
12hr APF | 18hr APF | 24hr APF



12d. EY08857 III (Overexpression of the 105 kD)

Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

12hr APF | 18hr APF | 24hr APF

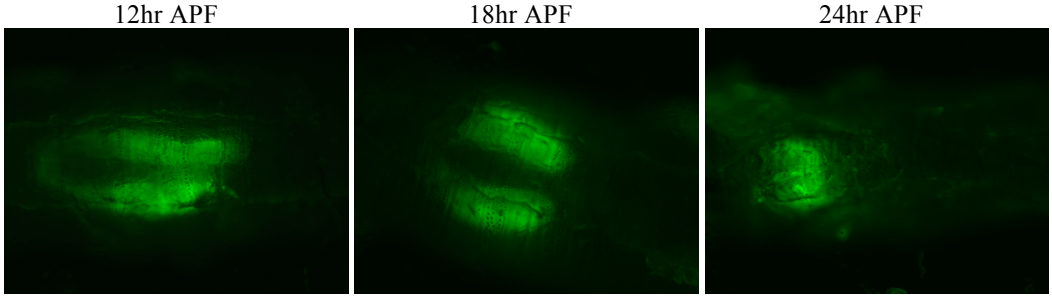
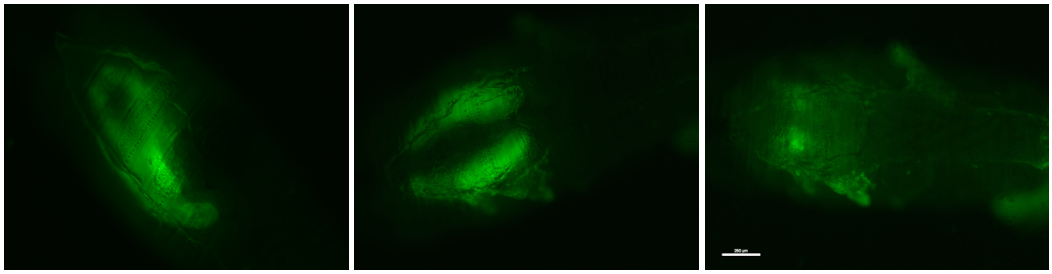


Figure 12a-d. Blocking expression of, or overexpressing, the 105 kD isoform with RNAi or shRNA. There are very noticeable differences in the level of GFP expression in the salivary glands between the modifier construct and wild-type pupae. The differences are quite stark at 18 hours APF, as the modifier construct pupae display a very weak GFP signal in comparison to the wild-type pupae. However, in the RNAi construct driving the overexpression of the 105 kD isoform, the level of GFP expression in the modifier construct and wild-type pupae are almost identical. Scale bars are 250 µl in length.

13a. 138 kD-IR/TM6

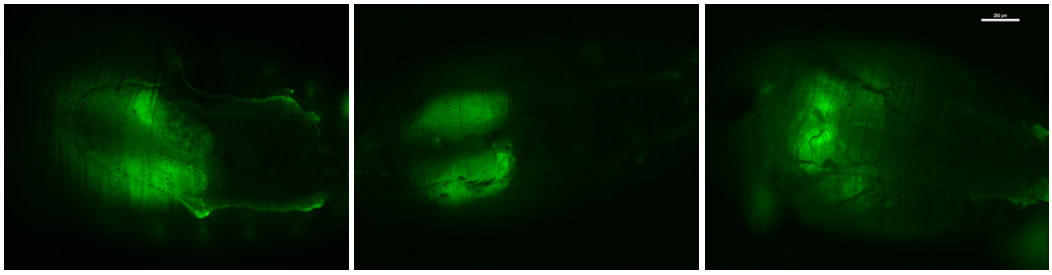
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

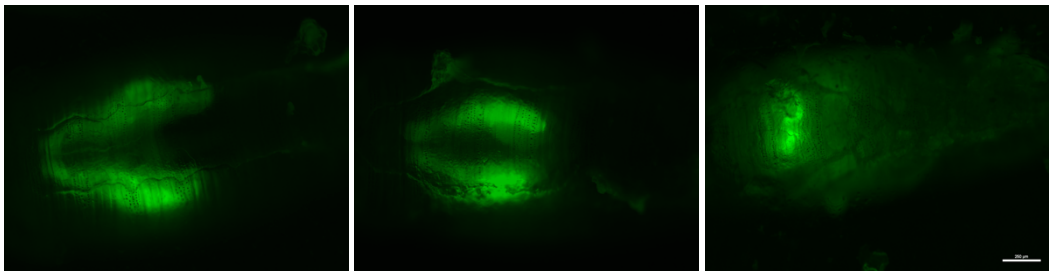
12hr APF | 18hr APF | 24hr APF



13b. 138 S+L kD shRNA

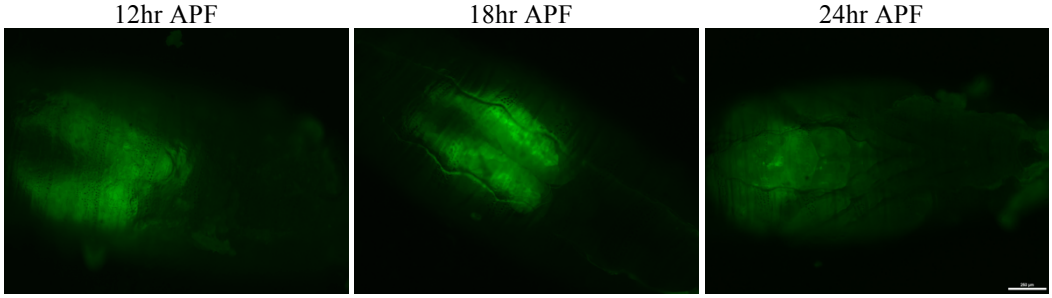
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

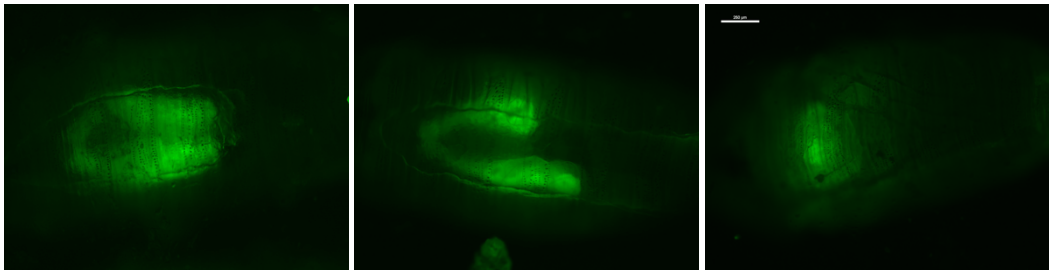
12hr APF | 18hr APF | 24hr APF



13c. 138L kD shRNA

Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

12hr APF | 18hr APF | 24hr APF

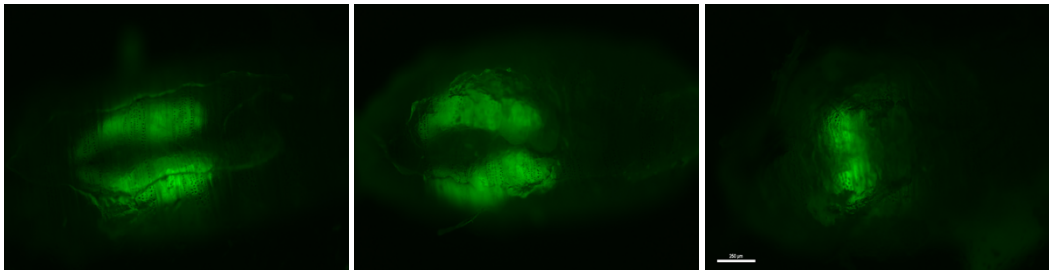
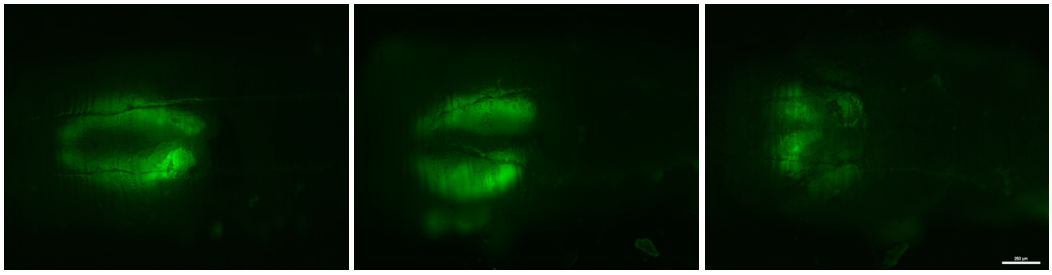


Figure 13a-c. Blocking expression of the 138 S+L kD and 138 L kD isoforms with RNAi or shRNA. There seem to be no noticeable differences in the level of GFP expression in the salivary glands between the modifier construct and wild-type pupae. Scale bars are 250 µl in length.

14a. UAS 91 kD-IR #1/Cyo II

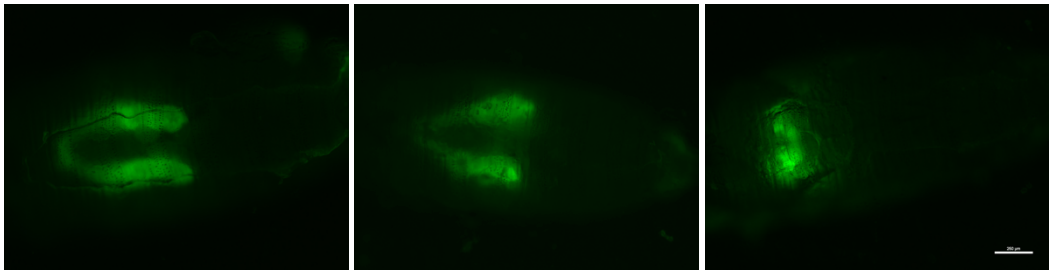
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

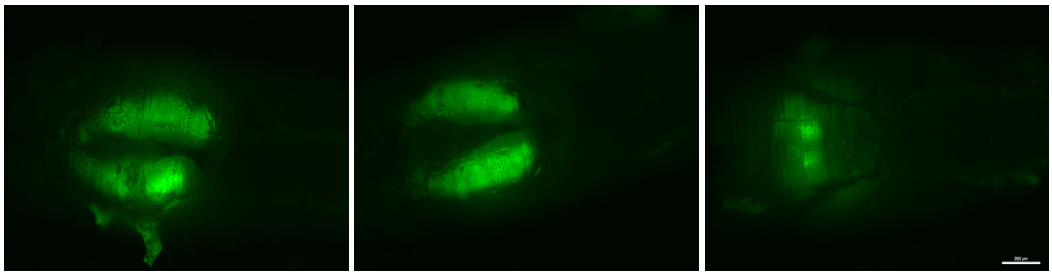
12hr APF | 18hr APF | 24hr APF



14b. UAS 91 kD-IR #3/TM6

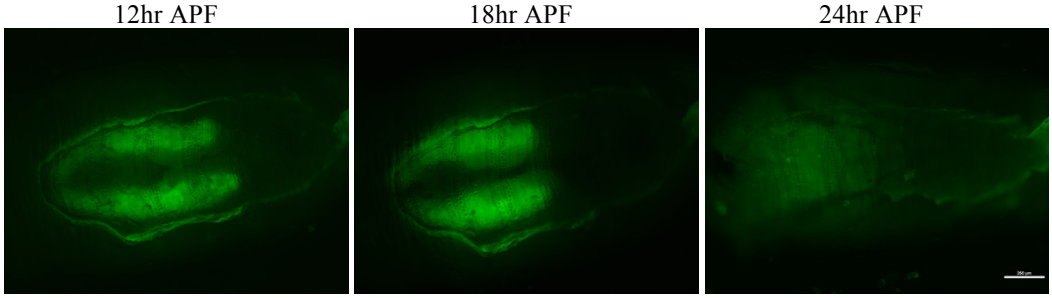
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

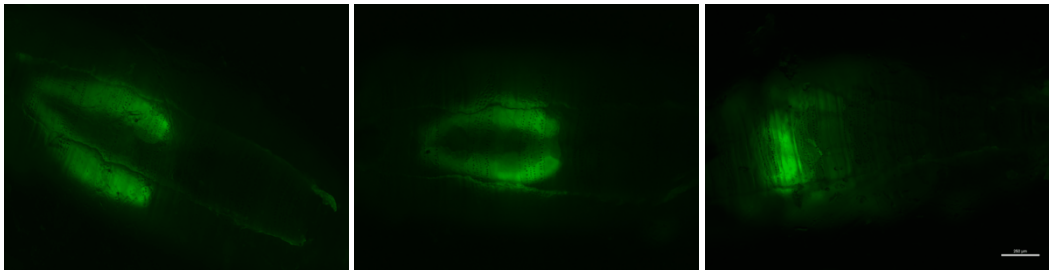
12hr APF | 18hr APF | 24hr APF



14c. 91+227 kD shRNA

Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

12hr APF | 18hr APF | 24hr APF

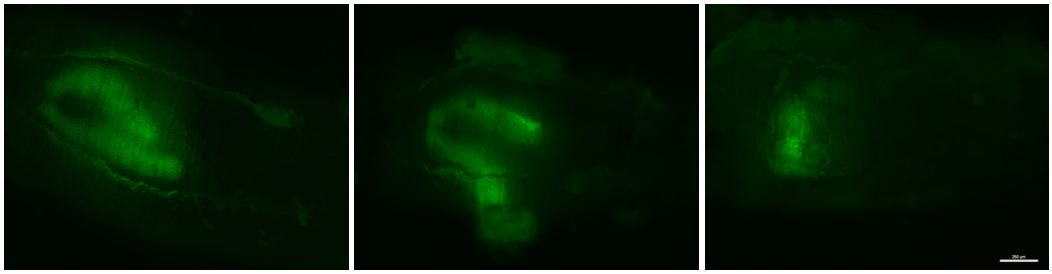
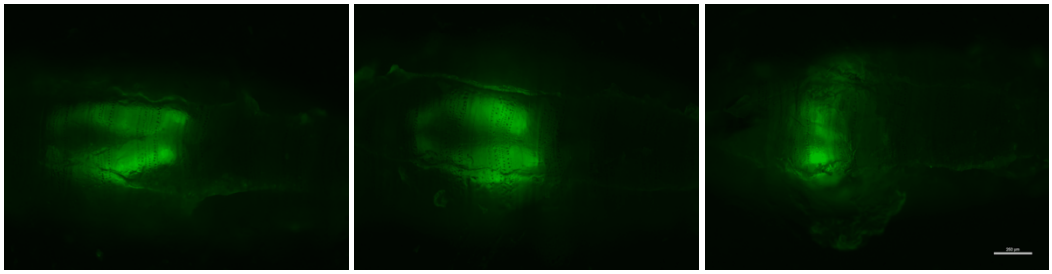


Figure 14a-c. Blocking expression of the 91+227 kD isoform with RNAi or shRNA. There seem to be no noticeable differences in the level of GFP expression in the salivary glands between the modifier construct and wild-type pupae. It is not possible to target just the 91 kD isoform of *Doa*, as it is completely overlapped by the 227 kD isoform. Scale bars are 250 µl in length.

15a. 227 kD-IR #1/TM3

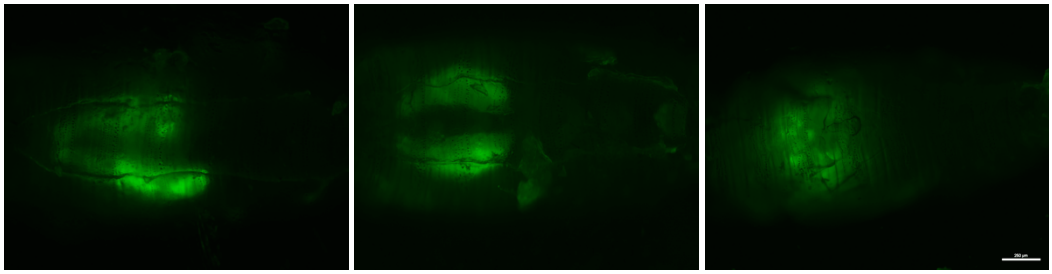
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

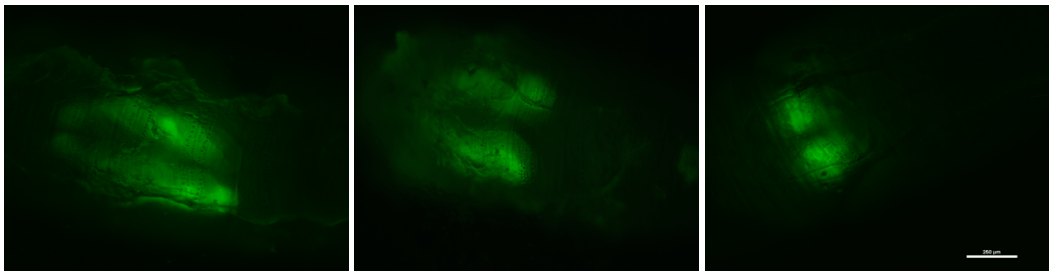
12hr APF | 18hr APF | 24hr APF



15b. 227 kD-IR #3/TM3

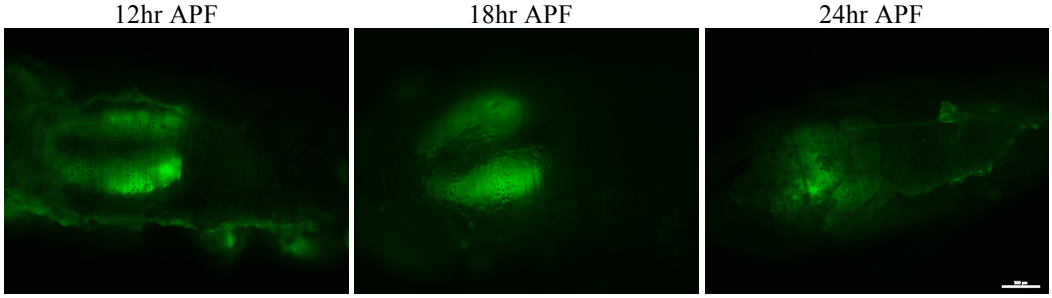
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

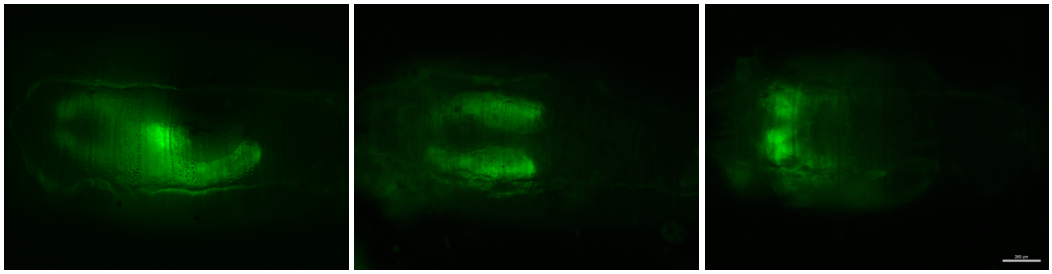
12hr APF | 18hr APF | 24hr APF



15c. 227 kD shRNA

Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

12hr APF | 18hr APF | 24hr APF

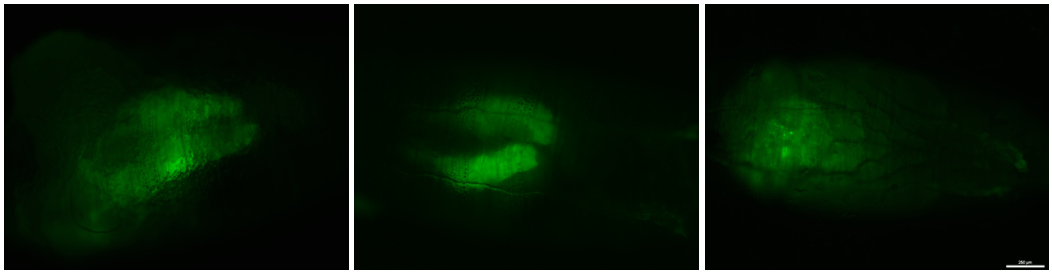
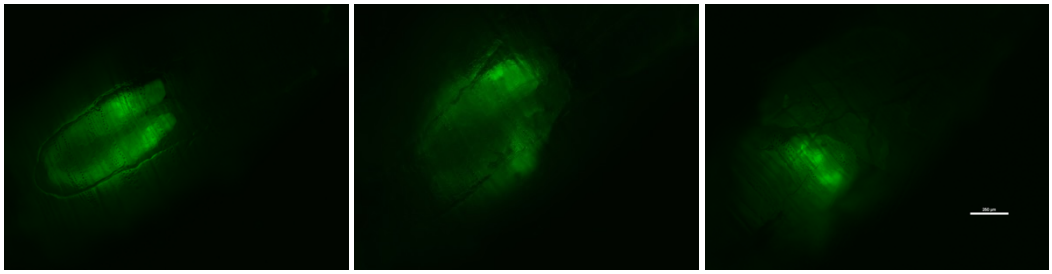


Figure 15a-c. Blocking expression of the 227 kD isoform with RNAi or shRNA. There seem to be no noticeable differences in the level of GFP expression in the salivary glands between the modifier construct and wild-type pupae. Scale bars are 250 µl in length.

16a. UAS Doa CAT/CyO II

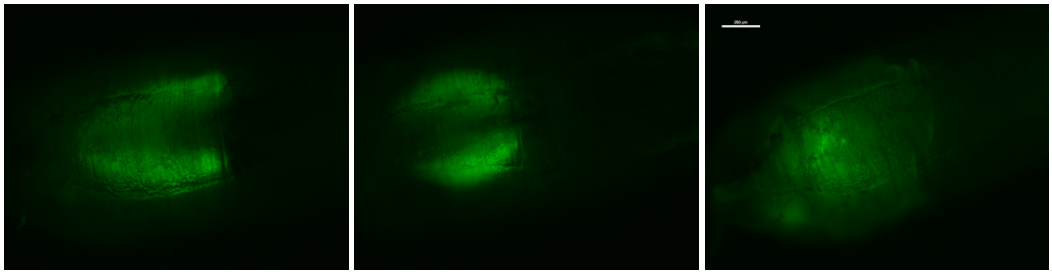
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

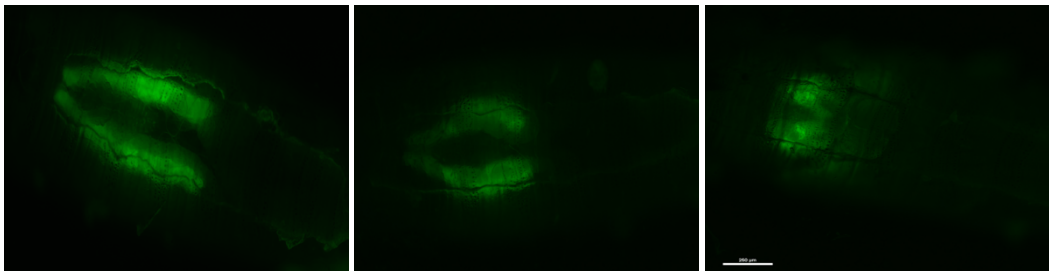
12hr APF | 18hr APF | 24hr APF



16b. UAS NLS Doa CAT/CyO II

Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

12hr APF | 18hr APF | 24hr APF

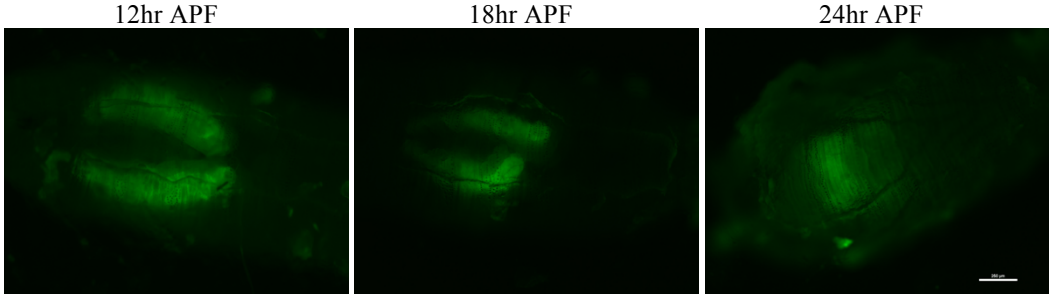
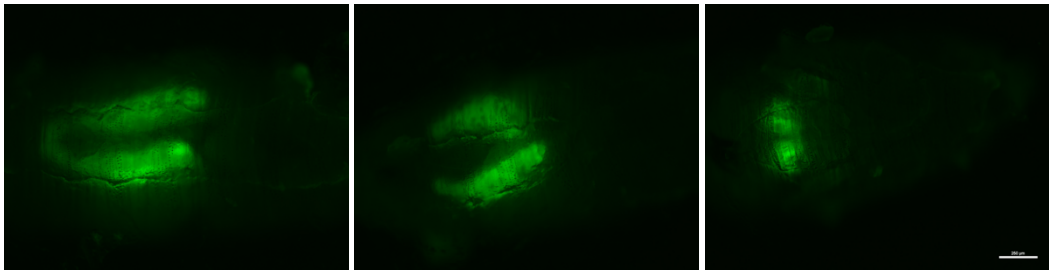


Figure 16a-b. Overexpression of the catalytic domain of DOA kinase. There seem to be no noticeable differences in the level of GFP expression in the salivary glands between the modifier construct and wild-type pupae. This result is shown in the constructs both with and without the additional Nuclear Localization Sequence (NLS) added to the ORF. Scale bars are 250 µl in length.

17a. UAS Doa CAT K199R II

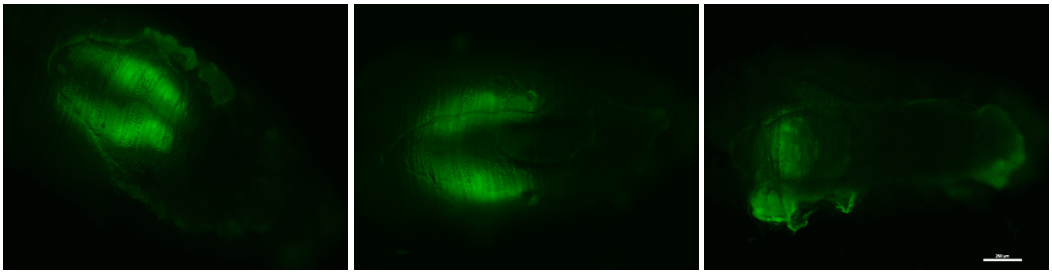
Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

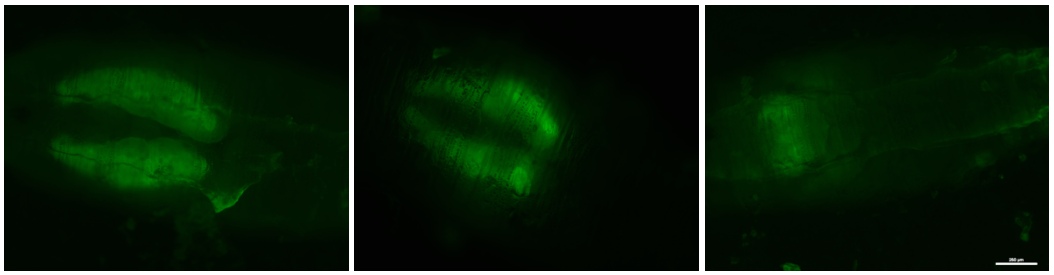
12hr APF | 18hr APF | 24hr APF



17b. UAS NLS Doa CAT K199R II

Modifier Construct:

12hr APF | 18hr APF | 24hr APF



Wild Type:

12hr APF | 18hr APF | 24hr APF

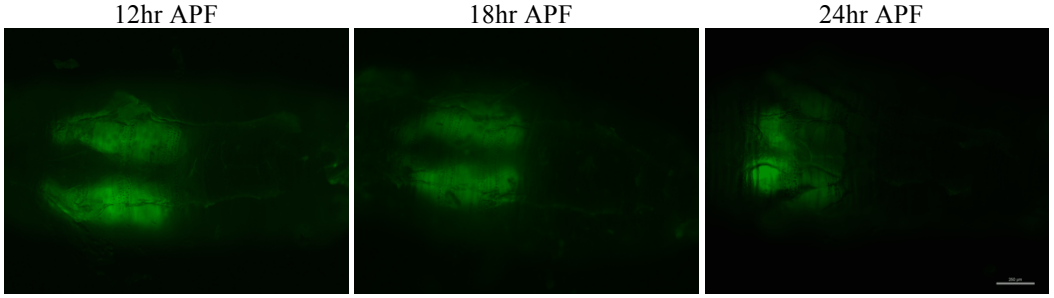


Figure 17a-b. Overexpression of the catalytic domain of DOA kinase with a one amino-acid substitution which renders the kinase inactive. There seem to be no noticeable differences in the level of GFP expression in the salivary glands between the modifier construct and wild-type pupae. This result is shown in the constructs both with and without the additional Nuclear Localization Sequence (NLS) added to the ORF. Scale bars are 250 µl in length.

DISCUSSION

The results from these experiments support the hypothesis that there could be isoform-specific effects of DOA kinase on autophagic cell death of the larval salivary glands. Blocking the expression of most of the *Doa* isoforms with RNAi, or overexpressing those with constructs available, seemed to have no effect on the amount of cell death present between the pupae carrying the modifier construct versus the wild-type. While these results are important to note, they suggest that the majority of the *Doa* isoforms do not appear to play a role in the promotion or inhibition of developmentally-induced autophagy within the larval salivary glands. By 24 hours APF, there is often little to no GFP expression left, as the larval salivary glands are almost completely degraded, and may then be replaced by the adult salivary glands (Robertson, 1936).

However, by blocking the expression of the 105 kD isoform with RNAi (Fimi 105 kD-IR, Mimi/105 kD-IR III, 105 kD shRNA), autophagic cell death of the salivary glands appears to be promoted. This is illustrated by the weak expression of GFP in the salivary glands in the pupae carrying the modifier construct in comparison to the wild-type (*TM6* balancer), especially at 18 hours APF (Figure 12a-c). A weaker GFP signal indicates that more degradation (hence more cell death) of the salivary glands has occurred. On the other hand, in the EY08857 construct (Figure 12d), which overexpresses the 105 kD isoform, the salivary glands do not exhibit the same level of cell death as shown when the

construct is blocked by RNAi. Instead, the level of GFP expression shown in pupae carrying the modifier construct or the wild-type are the same as the balancer sibling controls.

This could suggest that the normal role of the 105 kD isoform inhibits autophagic cell death in the salivary glands, and could be protective in nature. In a starvation model of autophagy, Hong Wen Tang found that *Doa* regulates autophagy (Tang *et al*., unpublished). While this particular experiment focuses on developmentally-induced autophagy instead of on a starvation-induced model of autophagy, the results suggest that DOA might in fact function in both. While there is not currently any indication as to how DOA is regulating cell death, these results in combination with Hong Wen Tang's findings suggest that there could be multiple ways of leading to the same autophagic cell-death pathway.

Interestingly, when observing the development of the male genitalia in *Drosophila*, the Fimi 105 kD-IR, Mimi/105 kD-IR III, 105 kD shRNA, and the 138 kD-IR/TM6 constructs all appear to inhibit apoptotic cell death in the male genital disc (Rabinow, unpublished). However, the relationship these observations have to one another, and the reason why the 138 kD RNAi construct has no noticeable effect on cell death in this experiment, has yet to be determined.

Although the noncatalytic sequences of LAMMER kinases are most likely regulatory and less important to overall kinase activity (Kpebe and Rabinow, 2008), perhaps there are differences within the N-termini of the 105 kD *Doa*

isoform that are important to, or necessary for, its normal role in the inhibition of autophagy.

Future Directions

Quantitative PCR

Given more time, the first thing I would like to do would be to perform qPCR, so that I could determine that the RNAi constructs are all working the way I have assumed them to be throughout this project. It would be reassuring to know that the RNA hairpins are actually affecting the *Doa* isoforms in the correct manner. If after doing qPCR the RNAi constructs did not appear to be working correctly, then perhaps the next step would be to generate a CRISPR-Cas9 construct for each of the *Doa* isoforms. A CRISPR for the 69 kD isoform has already been created, but the remaining 6 isoforms would have to follow.

Another use for qPCR could be to measure the expression of Anti-Microbial Peptides (AMPs) in the flies carrying the RNAi constructs. Although high levels of *Doa* are induced by *Relish*, an inducer of AMPs in the IMD pathway, elevated levels of the peptides are still found within *Doa* mutants (Zhao *et al*., 2015; Zhao and Rabinow, unpublished). This observation suggests that a negative autoregulatory loop could exist, however this has yet to be shown. AMP overexpression has also previously been linked to cell death (Cao *et al*., 2013).

Future Crosses

While the scope of the genetic crosses involved in this experiment was quite narrow, if this project were to continue, there are many other crosses involving *Doa* that would be of interest. The experiment described in this project could be performed again using *UAS-LC3-GFP*, which is another GFP-marker for salivary gland autophagy, and could potentially work better than the *SG>GFP* used in this experiment. It would also be interesting to repeat this experiment using *Atg8-mCherry* instead of *UAS-GFP; SG-GAL4*, and compare the effects on autophagy in the salivary glands to the findings presented in here. Since this is a *UAS*-directed construct, the effects could be tested in the fat body as well, or under a starvation-induced autophagy model.

Crossing the *UAS-Doa* RNAi and shRNA constructs against *UAS*-apoptotic inducers, such as *Atg1*, may generate interesting results. As the larval salivary glands are degraded during metamorphosis, several cell death genes, including *Atg* genes, are transcriptionally upregulated (Jiang *et al*., 1997; Lee *et al*., 2002). The overexpression of *Atg1* is sufficient to promote cell death in the salivary glands independent from caspase activation (Scott *et al*., 2007). Loss-of-function mutations in *Atg* genes can inhibit autophagy, and delay the degradation of the larval salivary glands (Berry and Baehrecke, 2007). The transcription factor FoxO could be responsible for the activation of *Atg1* and other *Atg* genes via JNK signaling (Chang and Neufeld, 2010). Overexpression of FoxO in the eye results in a small, rough eye phenotype, which is suppressed by a reduction in JNK

activity (Wang *et al*., 2005). FoxO is both necessary and sufficient for the induction of autophagy (Juhasz *et al*., 2007), and the effects of JNK on autophagy could be mediated via FoxO-dependent transcription of *Atg* genes (Chang and Neufeld, 2010). GMR-driven overexpression of the *Doa* RNAi constructs on other proapoptotic genes such as *rpr* and *hid*, or the overexpression of *egr*, an extracellular liquid inducing JNK-driven apoptosis, could yield interesting results.

There are numerous crosses that could be performed between the *Doa* RNAi lines and various different drivers that could be used, such as *nub-GAL4*, *da-GAL4*, *GMR-GAL4*, or *dsx-GAL.* Under the *GAL4-UAS system,* if female flies with the gene of interest linked to a *UAS* sequence are crossed to male flies with a *GAL4* driver, the progeny will express the gene of interest in a transcriptional manner specific to the driver chosen (Duffy, 2002). By using a *GMR-GAL4*, it is possible to see what effects, if any, the *UAS-Doa* RNAi constructs may have on the suppression or enhancement of ectopic cell death in the eye. The effects of the *UAS-Doa* RNAi constructs on wing phenotypes could be shown using a *nub-GAL4* driver, or ubiquitously throughout the organism using a *da-GAL4* driver. The effects of the RNAi constructs on the induction of mild female-to-male sex transformations could also be examined using the *dsx-GAL4* driver.

Crossing *Drosophila* paralogues of *NF-kB* such as *dl-GFP*, *dif-GFP*, or *Rel-GFP* to the RNAi and *UAS-Doa* constructs might reveal some interesting phenotypes. These proteins are localized in the cytoplasm until activated, at which point they translocate into the nucleus (Rushlow *et al*., 1989; Roth *et al*., 1989),

and will induce cell death when hyperactivated (Meyer *et al*., 2014). It is possible that *Doa* activity could affect the nuclear localization of *NF-kB* proteins *Rel*, *Dif*, and *dl* in the salivary glands or fat body, and that one or more *Doa* isoforms may be able to block cell death induced by *Rel*, *Dif*, or *dl*.

Activation of the Ras/mitogen-activated protein kinase (MAPK) pathway can induce cell cycle regulators, such as E2F, and cyclin-dependent kinases (CDKs) (Prober and Edgar, 2002). E2F transcription factors are key targets of the retinoblastoma (Rb) family of proteins, and they play an important role in cell cycle progression from the G1 to S phase (Gaubatz *et al*., 2000). While E2F normally activates transcription and promotes cell cycle progression, when in complex with Rb proteins, E2F actually represses target gene expression and negatively regulates cell cycle progression (Weintraub *et al*., 1992; Gaubatz *et al*., 2000; He *et al*., 2000). In *Drosophila*, E2F1 is a cell cycle regulator demonstrated to modulate the expression of genes required for the G1 to S phase progression (Neufeld *et al*., 1998). *Doa* has also been linked to cell cycle progression, and as it invokes the DNA damage checkpoint from both the G1 to S phase (Bettencourt-Dias *et al*., 2004) and the G2 to M phase *(Bjorklund et al*., 2006).

There are other crosses that could be done pertaining to various phenotypic, complementation, and sex transformation tests, which would help further characterize both the ‘old’ *Doa* RNAi constructs as well as the ‘new’ *Doa* shRNA constructs. However, the crosses described above would be of particular and more immediate interest moving forward.

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APPENDIX

Table 2. List of abbreviations. The full names of abbreviated words found in this project.

Abbreviation	**Full Name**
APF	After Puparium Formation
atg	Autophagy Gene
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
Caspase	Cysteine-Aspartic Protease
CLK	CDC Like Kinase
C-terminal	Catalytic Domain
CyO	Name of Balancer Chromosome
Doa, DOA	Darkener of Apricot
dsRNA	Double-Stranded RNA
dsx	Doublesex
Ecdysone	20-Hydroxyecdysone
gDNA	Genomic DNA
GFP	Green Fluorescent Protein
IR	Interfering RNA
kD	Kilodalton

N-terminal	Noncatalytic Domain
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
ProK	Proteinase K
qPCR	Quantitative Polymerase Chain Reaction
RISC	RNA-Induced Silencing Complex
RNAi	Interfering RNA
rpm	Rotations Per Minute
shRNA	Short Hairpin RNA
shmiRNA	Small Hairpin Micro RNA
siRNA	Small Interfering RNA
SB	Squishing Buffer
Ser	Serine
SG	Salivary Gland
SR	Serine, Arginine
Tb	Tubby
Thr	Threonine
TM3, TM6	Name of Balancer Chromosome (T = Third Chromosome; M = Metacentric Inversion; Number = 3, 6)
tra	Transformer Gene
Tyr	Tyrosine
UAS	Upstream Activation Sequence

v	Vermillion (Eye Color)
VALIUM vector	Vermillion-AttB-Loxp-Intron-UAS-MCS Vector
w^a	White Apricot (Allele of the *white* locus attributing a reduction in red pigment to an "apricot" colored orange)
wt	Wild-Type
y	Yellow (Body Color)
+	Denotes Wild-Type Gene

Table 3. Primer sequences. These primers are based on the vector sequences, not the inserted shRNAi coding sequences, so they are universal for the VALIUM20 vector.

Primer ID	**Sequence**
Forward Primer	5'-CGCAGCTGAACAAGCTAAAC-3'
Reverse Primer	5'-TAATCGTGTGTGATGCCTACC-3'