Molecular mechanisms underlying the crosstalk between autophagy and apoptosis

by

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ABSTRACT

Macroautophagy (hereafter referred to as autophagy) is an evolutionary conserved mechanism for the degradation of long-lived proteins and organelles inside lysosomes. Autophagy functions as an adaptive survival response to starvation and other cellular stresses, but recent studies also demonstrated a role for autophagy in cell death. Functional studies indicate that a complex relationship exists between autophagy and apoptosis, the most common form of cell death, but the regulatory mechanisms underlying their interactions are largely unknown.

To advance our understanding of the links between these two processes, I conducted a RNAi screen of Drosophila melanogaster cell death-related genes for their requirement in the regulation of starvation-induced autophagy. I discovered that six cell death genes, Dcp-1, hid, Bruce, Buffy, debcl, and p53 as well as Ras/Raf/MAPK signaling pathway components function in autophagy regulation in Drosophila l(2)mbn cells.

I used Drosophila genetics to investigate a role for effector caspase Dcp-1 and inhibitor of apoptosis protein Bruce during autophagy in vivo. During Drosophila oogenesis, I found that autophagy is induced at two nutrient status checkpoints, the gerarium and mid-oogenesis. Degenerating mid-stage egg chambers in DmAtg1 and DmAtg7 mutants exhibited reduced DNA fragmentation, suggesting autophagy may contribute to cell death during oogenesis. At the two nutrient status checkpoints in the developing ovary, Dcp-1 and Bruce function to regulate both autophagy and starvation-induced cell death, and epistasis analysis showed that Dcp-1 is downstream of Bruce, indicating that Bruce may negatively regulate Dcp-1 activity. In addition, I found that the catalytic activity of
Dcp-1 is essential for autophagy regulation, suggesting that Dcp-1 dependent proteolysis may serve as a regulatory mechanism by which Dcp-1 mediates a cellular switch between autophagy and apoptosis. To identify potential substrates of Dcp-1, I employed an immuno-precipitation and mass spectrometry assay, the results of which can be used to generate a working model for how Dcp-1 controls autophagy.

In summary, I developed an efficient screening method that resulted in the identification of several cell death-related regulators of autophagy. Further genetic and biochemical analyses of the effector caspase Dcp-1 in autophagy regulation provided new insights into the relationships between autophagy and apoptosis.
TABLE OF CONTENTS

ABSTRACT........................................................................................................................ ii
TABLE OF CONTENTS......................................................................................................... iv
LIST OF TABLES................................................................................................................ vi
LIST OF FIGURES ............................................................................................................. vii
ACKNOWLEDGEMENTS................................................................................................. viii
DEDICATION.................................................................................................................... ix
CO-AUTHORSHIP STATEMENT..................................................................................... xii

Chapter 1 Introduction

1.1 The autophagy process.............................................................................................. 1
1.2 The molecular machinery of autophagy ................................................................. 2
1.3 Roles of autophagy in Drosophila melanogaster development................................. 4
1.4 Role of autophagy in cancer ..................................................................................... 5
1.5 Apoptosis .................................................................................................................. 6
1.6 Caspase activation and substrate recognition ........................................................... 9
1.7 The Drosophila melanogaster ovary model system and starvation-induced cell death.............................................................................................................................. 11
1.8 Relationship between autophagy and apoptosis ..................................................... 12
1.9 Objectives and hypotheses...................................................................................... 14
   1.9.1 Rationale .......................................................................................................... 14
   1.9.2 Hypotheses and specific aims .......................................................................... 15
1.10 References............................................................................................................. 27

Chapter 2 Effector caspase Dcp-1 and IAP protein Bruce regulate starvation-induced autophagy during Drosophila oogenesis......................................................... 43

2.1 Introduction............................................................................................................. 43
2.2 Materials and methods ............................................................................................ 46
   2.2.1 Cell culture conditions ..................................................................................... 46
   2.2.2 dsRNA synthesis.............................................................................................. 47
   2.2.3 RNA interference (RNAi)................................................................................ 47
   2.2.4 Flow cytometry based LysoTracker Green (LTG) assay................................. 48
   2.2.5 GFP-LC3 detection .......................................................................................... 48
   2.2.6 Statistical analysis............................................................................................ 49
   2.2.7 Generation of transgenic flies .......................................................................... 49
   2.2.8 Fly strains......................................................................................................... 50
   2.2.9 Generation of Atg 1 germline clones (GLCs).................................................. 50
   2.2.10 LysoTracker Red staining.............................................................................. 50
   2.2.11 TUNEL assay................................................................................................. 51
   2.2.12 Quantitative RT-PCR..................................................................................... 52
2.3 Results..................................................................................................................... 53
   2.3.1 RNAi screening assay identifies known positive and negative regulators of starvation-induced autophagy in Drosophila l(2)mbn cells........................................... 53
   2.3.2 Identification of cell death-related genes that regulate starvation-induced autophagy in l(2)mbn cells....................................................................................... 56
   2.3.3 Autophagy occurs in response to nutrient deprivation in germaria and mid-stage egg chambers in the Drosophila melanogaster ovary..................................................... 58
2.3.4 Dcp-1 and Bruce regulate autophagy in germaria and degenerating mid-stage egg chambers ................................................................. 59
2.3.5 Dcp-1 and Bruce mutants have altered TUNEL staining in germaria and degenerating mid-stage egg chambers ................................................. 61
2.3.6 Autophagy contributes to cell death in nutrient-deprived ovaries .............. 62
2.4 Discussion ........................................................................................................ 63
2.5 References ......................................................................................................... 91

Chapter 3 The effector caspase Dcp-1 catalytically regulates starvation-induced autophagy ................................................................. 98
3.1 Introduction ....................................................................................................... 98
3.2 Materials and methods ..................................................................................... 101
  3.2.1 Cell culture and transfection ..................................................................... 101
  3.2.2 Immunofluorescence ............................................................................... 102
  3.2.3 LysoTracker Red (LTR) and DAPI staining ............................................. 103
  3.2.4 Immunoprecipitation (IP) and MS/MS analysis ...................................... 103
  3.2.5 Western Blot ............................................................................................. 105
  3.2.6 Computational analyses of protein sequences ......................................... 105
3.3 Results ............................................................................................................... 106
  3.3.1 Effector caspase Dcp-1 genetically interacts with IAP protein Bruce and functions downstream of Bruce ......................................................... 106
  3.3.2 IAP protein Bruce delays starvation induced autophagic responses in the larval fat body and midgut .............................................................. 107
  3.3.3 The effector caspase Dcp-1 accelerates the starvation-induced autophagic response but is not required for autophagy in the larval fat body ............... 108
  3.3.4 Starvation has a rapid effect on activation of Dcp-1 but not drICE ............ 109
  3.3.5 The catalytic activity of Dcp-1 but not drICE is required for the induction of starvation-induced autophagy ......................................................... 110
  3.3.6 Potential regulators or substrates of Dcp-1 in cells undergoing autophagy 111
3.4 Discussion ........................................................................................................ 112
3.5 References ....................................................................................................... 134

Chapter 4 Conclusions and future research .......................................................... 140
4.1 Overall summary and significance of the thesis research ............................... 140
4.2 Current limitations and summary of future research directions .................... 150
4.3 Potential applications of the research findings .......................................... 151
4.4 References ...................................................................................................... 159

Appendices .......................................................................................................... 166
Appendix A: abbreviations list ............................................................................ 166
LIST OF TABLES

Table 2.1 Primer sequences for the preparation of dsRNAs .................................................. 85
Table 2.2 Comparison of essential autophagy genes in the Drosophila larval fat body and
l(2)mbn cells .................................................................................................................... 86
Table 2.3 Quantification of autophagy in region 2 germaria ................................................ 87
Table 2.4 Quantification of autophagy in stage 8 degenerating egg chambers .................. 88
Table 2.5 Quantification of cell death in region 2 germaria .................................................. 89
Table 2.6 Quantification of cell death in stage 8 degenerating egg chambers .................... 90

Table 3.1 Potential Dcp-1 substrates .................................................................................. 133
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Model of the autophagy process</td>
<td>17</td>
</tr>
<tr>
<td>1.2</td>
<td>Molecular machinery of autophagy</td>
<td>19</td>
</tr>
<tr>
<td>1.3</td>
<td>Core apoptosis signaling pathways in nematodes, fruitflies and mammals.</td>
<td>21</td>
</tr>
<tr>
<td>1.4</td>
<td>Conserved features of effector caspases in <em>Drosophila melanogaster</em> and mechanism of caspase 7 activation</td>
<td>23</td>
</tr>
<tr>
<td>1.5</td>
<td><em>Drosophila melanogaster</em> ovariole structure</td>
<td>25</td>
</tr>
<tr>
<td>2.1</td>
<td>Quantification of starvation induced autophagy in <em>Drosophila l(2)mbn</em> cells</td>
<td>68</td>
</tr>
<tr>
<td>2.2</td>
<td>Identification of known cell death related genes in autophagy regulation in <em>l(2)mbn</em> cells using RNAi</td>
<td>70</td>
</tr>
<tr>
<td>2.3</td>
<td>Validation using 2\textsuperscript{nd} set of dsRNAs</td>
<td>73</td>
</tr>
<tr>
<td>2.4</td>
<td>Nutrient deprivation induces autophagy at region 2 within the gerarium and in dying mid-stage egg chambers</td>
<td>75</td>
</tr>
<tr>
<td>2.5</td>
<td>The effector caspase <em>Dcp-1</em> is not only required for nutrient starvation induced autophagy but also is sufficient for the induction of autophagy during <em>Drosophila</em> oogenesis</td>
<td>77</td>
</tr>
<tr>
<td>2.6</td>
<td>Bruce suppresses autophagy at region 2 within gerarium and in dying stage 8 egg chambers.</td>
<td>79</td>
</tr>
<tr>
<td>2.7</td>
<td><em>Dcp-1</em> is required for nutrient starvation induced gerarium cell death and IAP protein Bruce inhibits gerarium and mid-oogenesis cell death.</td>
<td>81</td>
</tr>
<tr>
<td>2.8</td>
<td>Lack of <em>Atg7</em> or <em>Atg1</em> function reduces DNA fragmentation during mid-oogenesis cell death.</td>
<td>83</td>
</tr>
<tr>
<td>3.1</td>
<td>Ovarian atrophy phenotype resulting from <em>Bruce</em> mutations is rescued by <em>Dcp-1</em></td>
<td>119</td>
</tr>
<tr>
<td>3.2</td>
<td>Bruce postpones starvation-induced autophagy in the larval fat body and midgut.</td>
<td>121</td>
</tr>
<tr>
<td>3.3</td>
<td>Expression of <em>Dcp-1</em> accelerates autophagy in response to nutrient withdrawal but is not essential for autophagy in the fat body and midgut.</td>
<td>124</td>
</tr>
<tr>
<td>3.4</td>
<td>Time course analyses of <em>Dcp-1</em> and drICE cleavage at the inter-domain site during nutrient deprivation.</td>
<td>126</td>
</tr>
<tr>
<td>3.5</td>
<td>The catalytic activity of <em>Dcp-1</em> but not drICE is required for starvation-induced autophagy.</td>
<td>128</td>
</tr>
<tr>
<td>3.6</td>
<td>Putative model of <em>Dcp-1</em> mediated autophagy</td>
<td>130</td>
</tr>
<tr>
<td>3.7</td>
<td>Bruce expression increase in fat body in response to nutrient withdrawal</td>
<td>131</td>
</tr>
<tr>
<td>4.1</td>
<td>A hypothetical pathway for the regulation of sensitivity thresholds leading to autophagy or apoptosis.</td>
<td>153</td>
</tr>
<tr>
<td>4.2</td>
<td>The effector caspase <em>Dcp-1</em> is sufficient for the induction of autophagy during <em>Drosophila</em> oogenesis</td>
<td>155</td>
</tr>
<tr>
<td>4.3</td>
<td>Possible relationships between autophagy and DNA degradation in <em>Drosophila</em> oogenesis</td>
<td>157</td>
</tr>
</tbody>
</table>
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Now it is your turn.

I will support you to pursue your dream of getting a PhD degree.
CO-AUTHORSHIP STATEMENT


I performed 90% of the experiments presented in this paper. Dr. S. Chittaranjan assisted in the construction of the Drosophila GFP-LC3 transfected l(2)mbn cell line. Analyses of ectopic expression of Dcp-1 during mid-oogenesis using the LysoTracker Red assay, and generation of Atg1 germline clones were conducted in collaboration with Dr. S. González Barbosa and Dr. K. McCall. I prepared the manuscript in collaboration with Dr. S. Gorski.


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A. Hannigan assisted in preparation of the last paragraph of the manuscript. I prepared the manuscript in collaboration with Dr. S. Gorski.
Chapter 1 Introduction

1.1 The autophagy process

Autophagy (self eating) is a process for the degradation of cytosolic components inside lysosomes. Based on the mechanisms by which substrates are delivered to lysosomes, three major forms of autophagy, macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) have been described. During the macroautophagy process, cytosolic components and intracellular organelles are first sequestered within double-membrane vesicles called autophagosomes. The origin of the pre-autophagosomal structure, also known as the phagophore assembly site, has been a topic of debate; however, two models for its origin, termed the maturation or assembly models, have been proposed. Based on the maturation model, the origin of the autophagosomal membrane is the endoplasmic reticulum (ER), whereas the assembly model suggests that the autophagosomal membrane assembles de novo from localized lipid synthesis. The recent identification of a novel double FYVE domain containing protein 1 (DFCP1) that co-localizes with ER membrane and autophagosomal proteins provides support for the maturation model. The autophagosome subsequently fuses with a lysosome to become an autolysosome where the cellular components are degraded (Figure 1.1). Microautophagy is involved in a direct uptake of inclusions (e.g., glycogen) and organelles (e.g., ribosomes and peroxisomes) at the lysosomes by invagination of the lysosomal membrane without the formation of intermediate transport vesicles. CMA involves the recognition and transport of protein substrates to the lysosome, and is responsible for degradation of approximately 30% of cytosolic proteins in response to
nutrient deprivation. Substrate proteins of CMA contain a particular pentapeptide motif (KFERQ) that are recognized by a cytosolic chaperone hsc70 and subsequently bind to a receptor, lysosome-associated membrane protein type 2a (lamp2a), at the lysosomal membrane which mediates lamp2a protein complex translocation into the lysosomes for degradation.

Macroautophagy is the most studied form of autophagy and hereafter is referred to as autophagy. Autophagy occurs at basal levels in normal conditions and is induced by various stimuli including starvation, hypoxia, intracellular stress, hormones and growth factor deprivation. The most fundamental role of autophagy is to provide an internal source of nutrients under starvation conditions. Nucleotides, amino acids, and free fatty acids are generated from the degradation of the sequestered materials and further recycled for the synthesis of macromolecules and ATP production. Basal autophagy also plays a key role in eliminating defective organelles (e.g. mitochondria) or aggregated proteins that may be resistant to the ubiquitin-proteosome degradation pathway. In addition, autophagy is involved in other processes including cellular differentiation, tissue remodeling, growth control, elimination of pathogens, and clearance of apoptotic cells.

1.2 The molecular machinery of autophagy

The discovery and characterization of autophagy-related (Atg) genes in yeast, Saccharomyces cerevisiae, have contributed to the understanding of the molecular mechanisms involved in autophagy. There are 31 autophagy-related genes in yeast, and 18 Atg proteins are essential for autophagosome formation. The Atg genes encode proteins which are required for the induction of autophagy, autophagosome nucleation,
vesicle expansion and completion, and final retrieval of Atg proteins from mature autophagosomes.\textsuperscript{16} Most of the \textit{Atg} genes are conserved in higher eukaryotes, including mammals. The molecular mechanisms of autophagy are illustrated in Figure 1.2.

TOR kinase and class III phosphatidylinositol 3 kinase (PI3K) are two important regulators of autophagy.\textsuperscript{17, 18} TOR kinase is a sensor of amino acid deprivation, and is believed to be a “gatekeeper” for the induction of autophagy.\textsuperscript{17} Under nutrient-rich conditions, TOR kinase phosphorylates Atg13 (autophagy-related gene-13).\textsuperscript{19} The hyperphosphorylated form of Atg13 has a low affinity for its interacting proteins Atg1 and Atg17.\textsuperscript{19} This results in a reduction in Atg1 kinase activity which ultimately results in inhibition of autophagy.\textsuperscript{19, 20} Conversely, during amino acid deprivation, TOR activity is inhibited, resulting in Atg 13 interaction with Atg1 and Atg17, which activates autophagy (Figure 1.2 A).\textsuperscript{19, 20}

The initial step of vesicle nucleation is the activation of the class III PI3K, also known as Vps34, to generate phosphatidylinositol 3-phosphate (PI3P) in both yeast and mammals.\textsuperscript{18} In yeast, Vps34 activation depends on the formation of a multi-protein complex that includes Atg6, the myristoylated Vps15 kinase, and Atg14 (Figure 1.2 B).\textsuperscript{1} In mammals, activation of Vps34 is dependent on the formation of a multi-protein complex that consists of Atg6/Beclin-1, Ultraviolet irradiation resistance-associated tumour suppressor gene (UVRAG), Bif-1, Ambra1, and a myristylated serine kinase Vps15 (Figure 1.2 B).\textsuperscript{21-23}

Two ubiquitin-like conjugation systems are involved in autophagosome membrane elongation and completion. Atg12 is activated by an E1-like enzyme called Atg7, followed by the transfer of Atg12 by Atg7 to an E2-like enzyme, Atg10.\textsuperscript{24-26} Later,
Atg12 is conjugated to Atg5 and forms a larger protein complex with Atg16 through oligomerization of Atg16 monomers.\textsuperscript{24-26} The Atg5/Atg12/Atg16 complex asymmetrically localizes to the outer side of the autophagosomal membrane throughout the elongation process and dissociates from the autophagosome upon completion (Figure 1.2 C).\textsuperscript{26} Atg8 is cleaved by Atg4 to produce the cytosolic form Atg8-glycine and is activated by Atg7. Another E2-like protein Atg3 is responsible for Atg8 conjugation.\textsuperscript{26} Finally Atg8 is lipidated with phosphatidylethanolamine (PE) and inserted in the autophagosome membrane.\textsuperscript{27} In contrast to the Atg5/Atg12/Atg16 system, Atg8 remains on the autophagosomal membrane after the completion of autophagosome formation.\textsuperscript{27} Microtubule associated protein 1 light chain 3B (MAP1LC3B or LC3) is the functional human homologue of Atg8.\textsuperscript{28} Based on the observations that LC3 displays different intracellular locations and an increased electrophoretic mobility on SDS-polyacrylamide gels during autophagic membrane recruitment, it has been employed as a marker for autophagosomes and autophagic activity.\textsuperscript{28, 29} A recycling pathway that is composed of Atg9 and Atg18 mediates the disassembly of Atg proteins from mature autophagosomes (Figure 1.2 D).\textsuperscript{30, 31} However, the retrieval mechanism which the Atg9-Atg18 complex mediates is still poorly understood.

1.3 Roles of autophagy in \textit{Drosophila melanogaster} development

Autophagy is observed in several \textit{Drosophila melanogaster} larval tissues, including salivary glands, fat body and midgut, and genetic studies in flies provided evidence that autophagy plays a role in \textit{Drosophila melanogaster} development. Mutations in some of the autophagy genes result in lethality at various stages of \textit{Drosophila melanogaster} development.\textsuperscript{7} For example, null mutations in \textit{Atg1} exhibit a
highly penetrant lethal phenotype in the pupal stage, prior to eclosion.\textsuperscript{32} Lethality at the larval stage is observed in flies carrying mutations in \textit{Atg18} or \textit{Atg6} genes.\textsuperscript{32} However, flies carrying mutations in \textit{Atg7} or \textit{Atg8a} develop to adulthood, despite an essential role for both genes in autophagy.\textsuperscript{32-34} Autophagy also plays an important role in adaptation to starvation in the larval fat body. The larval fat body is a nutrient storage organ that stores lipids and glycogen.\textsuperscript{35} Nutrients in the larval fat body are degraded by autophagy to support development of imaginal tissues which turn into adult fly tissues\textsuperscript{36} and to sustain larval survival for two or more weeks in the absence of amino acids.\textsuperscript{37} \textit{Atg1}, \textit{Atg5}, and \textit{Atg7} mutants were shown to die earlier than wild-type flies in response to starvation conditions.\textsuperscript{32, 33} Autophagy also promotes survival in response to a number of other stresses, including oxidative stress, chill-induced coma and \textit{CO\textsubscript{2}} anesthesia.\textsuperscript{33} Further, autophagy appears to contribute to cell death in several tissues of \textit{Drosophila melanogaster}. Deficiencies in \textit{Atg} genes prevent complete destruction of larval salivary glands\textsuperscript{38} and suppress cell death of the aminoserosa which is eliminated during embryogenesis.\textsuperscript{39} In \textit{Atg7} mutants, the larval midgut shows reduced DNA fragmentation, an indicator of cell death, and its complete destruction is delayed for four hours during metamorphosis.\textsuperscript{33} Overexpression of \textit{Atg1} results in cell death in both the larval fat body and salivary glands.\textsuperscript{34, 38} Together, these examples illustrate multiple roles for autophagy during \textit{Drosophila melanogaster} development.

\subsection*{1.4 Role of autophagy in cancer}

Autophagy has been linked to various human diseases, including cancer. Autophagy functions as a survival mechanism when cells encounter nutrient deprivation. When cancer cells encounter hypoxia, metabolic stress, or nutrient starvation, autophagy may
aid in cell survival by providing nutrients and/or energy, and thus promote tumorigenesis.\textsuperscript{40} Autophagy has been shown to maintain tumour survival in response to metabolic stress \textit{in vitro} and in hypoxic regions of tumours \textit{in vivo}.\textsuperscript{41-44} Paradoxically, genetic studies show that autophagy can act as a tumour suppression mechanism. Beclin-1 is the human orthologue of \textit{Atg6} in yeast and is involved in the early steps of autophagic vesicle formation. Allelic loss of \textit{Beclin1} is observed in human breast, ovarian and prostate cancers, and \textit{Beclin1} heterozygous mice have a high incidence of spontaneous tumours, indicating that \textit{beclin-1} is a haplo-insufficient tumour suppressor gene.\textsuperscript{45, 46} Autophagy related-4C (Atg4C) deficient mice are prone to tumour formation following exposure to chemical carcinogens.\textsuperscript{47} Autophagy is known to help degradation of damaged organelles such as depolarized mitochondria.\textsuperscript{48} Defective mitochondria lead to accumulation of reactive oxygen species leading to genotoxic damage, which may explain the potential tumor-suppressive effects of autophagy.\textsuperscript{49} A recently described role of autophagy in maintaining genome stability could also explain the tumour suppression mechanism of autophagy.\textsuperscript{42} Alternatively autophagy may promote cell death as reported in some systems.\textsuperscript{38, 39, 50, 51} The survival and death promoting roles of autophagy in tumour progression are still far from being understood. A better understanding of the roles of autophagy in both tumour survival and tumour suppression may ultimately provide novel approaches for cancer prevention and treatment strategies.

1.5 Apoptosis

Programmed cell death maintains the homeostasis of metazoans by removing unwanted or damaged cells during development and adulthood.\textsuperscript{52, 53} Apoptosis, also known as type I programmed cell death, has morphological characteristics of membrane
blebbing, chromatin condensation, cell shrinkage and DNA fragmentation.\textsuperscript{54, 55} Cells undergoing apoptosis become fragmented and form apoptotic bodies which are engulfed by neighboring phagocytotic cells.\textsuperscript{56} Apoptosis is important for developmental processes, including embryogenesis, nervous system development, and immune system differentiation.\textsuperscript{57-59} In addition, apoptosis is involved in disease processes such as autoimmune diseases, neurodegenerative disorders, immunologic deficiencies, and cancers.\textsuperscript{52}

A family of proteases known as caspases is at the core of the apoptosis machinery.\textsuperscript{59, 60} Caspases are expressed ubiquitously and are synthesized as inactive zymogens.\textsuperscript{60} In response to various death signals, caspases become activated, cleave multiple cellular substrates and lead to cell death.\textsuperscript{61, 62} Proteins which induce caspase-dependent cell death and control levels of active caspases in cells are evolutionarily conserved (Figure 1.3).\textsuperscript{60, 63} In \textit{Caenorhabditis elegans}, the sole caspase CED-3 and its activator, the adaptor protein CED-4 are expressed ubiquitously.\textsuperscript{64} Activation of CED-3 is controlled by CED-4, but no inhibitors of activated CED-3 have been identified.\textsuperscript{64} Expression of the anti-apoptotic multi-domain Bcl-2 family protein, CED-9, prevents cell death by sequestering CED-4 at mitochondria and preventing CED-4 oligomerization, which is required for CED-3 activation.\textsuperscript{65} In dying cells, a small pro-apoptotic protein EGL-1 (a member of the BH3 domain only Bcl-2 family) binds to CED-9 and changes its conformation resulting in the release of CED-4 from the inhibitory CED-4-CED-9 complex.\textsuperscript{65}

In \textit{Drosophila melanogaster}, molecular mechanisms governing apoptosis have been studied extensively. There are seven caspases, and three of them, Dronc, Strica, and
Dredd, are initiator caspases, which are molecularly characterized by the presence of a long N-terminal prodomain. The remaining four caspases, Dcp-1, drICE, Decay and Daydream contain a short prodomain suggesting that they function as effector caspases. Cell death is triggered by activation of the apical caspase Drone (encoded by \( Nc \)) and is mediated by the adaptor protein Dark, the fly homologue of CED-4 in worms and Apaf-1 in mammals. Expression of the anti-apoptotic protein DIAP1 (encoded by \( th \)) suppresses Drone activity and also downstream effector caspases (Dcp-1 and drICE). The pro-apoptotic proteins, Reaper (Rpr), Grim, Hid, and Sickle, disrupt the interaction between DIAP1 and caspases. Further, *Drosophila melanogaster* has two Bcl-2 family proteins, encoded by *debcl* and *buffy*, and these proteins have pro- and anti-apoptotic activity respectively, suggesting that they might be the upstream regulators of caspase activation, but direct evidence for this interaction is still lacking. In addition, several upstream signaling pathways have been shown to regulate the cell death machinery. For example, the EGF receptor/Ras pathway specifically inhibits Hid activity through MAPK-mediated phosphorylation. DNA damage up-regulates expression of *Drosophila melanogaster* p53 which then binds to the cis-regulatory region of *rpr* and induces apoptosis.

In mammals, activation of caspase 9, the mammalian homolog of Drone in *Drosophila melanogaster*, is regulated by Bcl-2 family proteins and dependent on Apaf-1. Cell death stimuli activate BH3-only family members which facilitate pro-apoptotic Bcl-2 family member BAX and BAK-dependent release of pro-apoptotic mitochondria proteins, including cytochrome c and Smac (also known as DIABLO). Cytochrome c binds to and activates Apaf-1 in the cytoplasm which results in a
conformational change in Apaf-1. Apaf-1 further binds to ATP/dATP and forms the apoptosome which mediates the activation of caspase-9 and triggers a cascade of caspase activation.90-92 The tetrapeptide, Ala-Val-Pro-Ile, at the N terminus of mature Smac is an IAP-binding motif which binds to XIAP and relieves XIAP-mediated inhibition of caspase 9.93-95 The tetrapeptide is conserved between flies and humans, since the *Drosophila melanogaster* RHG proteins, Reaper, Hid, Grim and Sickle, all contain a conserved IAP-binding tetrapeptide motif.60

Defective apoptosis is a hallmark of most, if not all, types of cancers and activation of apoptosis is one of the current strategies to eliminate cancer cells.96 The tumour suppressive role of apoptosis is well established, and pathways and genes associated with apoptosis have been studied extensively. Overexpression of the apoptosis inhibitors (e.g. Bcl-2 or IAP family members) or deletion of apoptosis effectors (e.g. caspases) suppresses apoptosis and allows tumour cells to survive the stresses of oncogene activation, uncontrolled proliferation and chemotherapy.97, 98 Antagonists of Bcl-2 and IAP proteins have either entered the clinic or are under clinical trials as therapeutics to restore the apoptotic pathway of resistant tumours.99, 100 The finding that the IAP binding motif of Smac acts as a potent mediator of caspase activation makes this tetrapeptide a promising candidate for a therapeutic agent and efforts have been put into the development of small molecule Smac mimetics for cancer treatment.100

1.6 Caspase activation and substrate recognition

Caspases are the executioners of the apoptotic response. Caspases are a family of cysteine proteases that cleave after an aspartate residue in their substrates.101 Each caspase monomer contains an N-terminal pro-domain followed by a 20kDa (p20) and a
10kDa (p10) subunit. Caspases exist constitutively as homodimers. Caspases can be divided into two classes: the initiator caspases have extended N terminal pro-domains which contain caspase recruitment domains (CARD) or death effector domains (DED) to interact with other molecules, while the effector caspases have a pro-domain that is relatively short. All caspases are synthesized as inactive zymogens and their catalytic activities are increased by several orders of magnitude after inter-domain cleavages.\textsuperscript{60, 102}

In \textit{Drosophila melanogaster}, Dcp-1 was shown to undergo inter-domain cleavage at Asp33 and Asp215, and the proteolytic cleavage of drICE occurs at Asp28 and Asp230 during Rpr-mediated apoptosis (Figure 1.4 A).\textsuperscript{103} The backbone configuration of all caspases is highly conserved and their catalytic groove is composed of four surface loops (L1-L4) as revealed by crystal structures of several active caspases (for review see Shiozaki 2004).\textsuperscript{102} Analyses of the human caspase-7 zymogen (Figure 1.4 B) and active caspase-7 (Figure 1.4 C) helped to reveal the mechanisms of caspase activation and substrate binding.\textsuperscript{104} The L1 and L4 loops constitute two parallel sides of the substrate binding groove and L3 serves as the base. The inter-domain cleavage allows the L2 loop and the L2’ loop of the neighboring caspase monomer to switch to an open conformation and expose the catalytic residue cysteine.\textsuperscript{104} A similar conformation change was also observed in \textit{Drosophila melanogaster} effector caspase drICE.\textsuperscript{80} Caspases recognize at least four amino acids, P4-P3-P2-P1, in their substrates and cleave after the C-terminal residue (P1) which is usually an Asp residue. However, the initiator caspase Dronc has an equal specificity for either an Asp or Glu residue in P1.\textsuperscript{105} The most frequent residues at the P4-P1 position are Asp, Glu, Val, and Asp (DEVD), the canonical caspase cleavage motif, as determined with \textit{in vitro} positional scanning peptide libraries. However, there
are significant differences between the actual cellular caspase substrate cleavage sites and the in vitro substrate specificity profiles;\textsuperscript{106, 107} thus, the prediction of a caspase substrate based on caspase cleavage motifs in their amino acid sequences needs to be carefully validated.

1.7 The Drosophila melanogaster ovary model system and starvation-induced cell death

Cell death in the Drosophila melanogaster ovary has been studied for more than a century.\textsuperscript{108} The appearance of vacuoles in the dying cells\textsuperscript{109} and the induction of cell death in nurse and follicle cells in response to nutritional changes at distinct stages during oogenesis make the ovary a potential model system to study the relationship between autophagy and cell death.\textsuperscript{110} Each adult Drosophila melanogaster ovary contains 15 to 20 ovarioles (for review see Spradling 1993)\textsuperscript{108}. Each ovariole contains a series of developing egg chambers that consist of 16 germline cells (15 nurse cells and 1 oocyte) surrounded by a layer of somatic follicle cells (Figure 1.5). Germline stem cells are located at the anterior end of each ovariole in a specialized region called the germarium. The germline stem cells develop into cystoblasts which undergo four rounds of mitosis to form 16-cell cysts. These cysts then undergo incomplete cytokinesis and remain connected with each other through ring canals. Somatic follicle cells surround the germline cysts when they migrate into region 2b (Figure 1.5). Each germline cyst then becomes a complete egg chamber, moves out of the germarium, and progresses through 14-defined developmental stages. During pre-vitellogenic stages, the relative sizes of the oocyte and nurse cells remain similar. At stage 8, the oocyte increases its volume and undergoes vitellogenesis, a process of yolk protein synthesis and uptake. Toward the
late stage of oogenesis, the nurse cells support the development of the oocyte by transferring to it their cytoplasmic contents via the ring canals. The nurse cells eventually undergo cell death and dying cells are engulfed by the surrounding follicle cells during late oogenesis.  

When animals encounter nutrient deprivation, chemical insults or hormonal changes, egg chambers undergo cell death at two earlier stages, germarium and mid-oogenesis. Thus, cell death at these two stages has been proposed to act as checkpoints, where the nutrient and/or environmental status of egg chambers are monitored prior to investing energy into egg production. The core apoptotic activators of \textit{Drosophila melanogaster}, \textit{rpr}, \textit{hid}, \textit{grim} and \textit{sickle}, are not required for cell death in mid-oogenesis, and cell death genes such as \textit{debc1}, \textit{ark} and \textit{p53} also are not required for mid-oogenesis cell death. However, one essential player in mid-oogenesis cell death is the effector caspase Dcp-1. Thus, mid-oogenesis cell death has been described as “non-canonical” and the genes required for cell death at this stage are still largely unknown.

### 1.8 Relationship between autophagy and apoptosis

A complex inter-connection exists between components of apoptosis and autophagy. Two apoptotic inducers, sphingolipid and ceramide, have been shown to activate autophagy in mammalian cells. Components of the apoptotic pathway, including \textit{TRAIL}, \textit{FADD}, \textit{Bad} and \textit{DAPK}, were shown to be positive regulators of autophagy. In contrast, the class I PI3K/Akt/Tor signaling pathway, an anti-apoptotic pathway, acts to suppress autophagy (for reviews see Levine 2005 and Lum 2005). Anti-apoptotic proteins, Bcl-2 and Bcl-X\textsubscript{L}, were shown to suppress autophagy through a direct interaction with Beclin-1 which contains a BH3 domain.
Pro-apoptotic BH3-only proteins such as BNIP3, Bad and Puma, as well as pharmacological BH3 mimetics, also function to induce autophagy through the competitive disruption of the interaction between Beclin1 and Bcl-2 or Bcl-XL. An Atg5 cleavage product, which is generated by calpain cleavage, moves to mitochondria and associates with Bcl-xL leading to caspase activation. In addition, autophagy was shown to contribute to cell death during developmental programs in *Drosophila melanogaster* as discussed previously, and in apoptosis-deficient mammalian cells in response to cytotoxic stress. Mouse embryonic fibroblasts (MEFs) from double-knockout Bax<sup>−/−</sup> Bak<sup>−/−</sup> mice are resistant to various apoptosis inducers, and when Bax<sup>−/−</sup> Bak<sup>−/−</sup> MEFs are treated with DNA damaging agents such as etoposide (a topoisomerase-2 inhibitor), autophagy is induced in Bax<sup>−/−</sup> Bak<sup>−/−</sup> MEFs followed by non-apoptotic cell death. Knockdown of *Atg5* or *Atg6/Beclin1* by RNAi reduces the etoposide-induced cell death of Bax<sup>−/−</sup> Bak<sup>−/−</sup> MEFs. Inhibition of caspase 8 by treatment with the pan-caspase inhibitor z-VAD or RNAi triggers autophagy and cell death concurrently in L929 mouse fibrosarcoma cells. z-VAD-induced cell death is rescued by knockdown of key autophagy genes such as *Atg6/Beclin1* or *Atg7* by RNAi. Cell death induced by zVAD was shown to be associated with the accumulation of reactive oxygen species (ROS) that is caused by the autophagy mediated-depletion of the major enzymatic ROS scavenger, catalase, and RNAi of *Atg7* or *Atg8* blocks both ROS accumulation and cell death. Autophagy also plays a role in eliminating apoptotic corpses by providing an energy source (ATP) to facilitate the generation of engulfment signals, including lysophosphatidylcholine secretion (come-get-me signal) and phosphatidylserine exposure (eat-me signal) in a mouse embryoid body cavitation model and in a mouse
neuroepithelium model. These findings clearly show a complex inter-connection between apoptosis and autophagy; however, the molecular mechanisms underlying the relationships between apoptosis and autophagy pathways are still poorly understood.

1.9 Objectives and hypotheses

1.9.1 Rationale

Several apoptotic components have been shown to play a role in the regulation of autophagy. Autophagy was shown to contribute to cell death during development or in apoptosis-deficient mammalian cells in response to cytotoxic stress (for reviews see Baehrecke 2002, Levine 2005, and Thorburn 2008). Autophagy has also been linked to cancer; however, the survival and death promoting roles of autophagy in tumour progression are still far from being resolved. A better understanding of the interplay between autophagy and apoptosis will provide insight into their relationship during both development and disease, and aid in the design of cancer treatments. Numerous studies have focused on characterizing the function of Atg genes in cell death, but a systematic approach to determine the involvement of cell death genes in autophagy has not been employed. To address this question, I used Drosophila melanogaster as a model system and performed an RNAi gene silencing screen on cell death genes. I chose Drosophila melanogaster as the model system on the basis of its less redundant but evolutionary conserved cell death pathway and the availability of numerous molecular/genetic tools. For example, during evolution, an expansion of caspase family members took place. There are 11 caspases that play a critical role in apoptosis in mammals, but only 4 caspases are required for apoptosis in Drosophila melanogaster. Carrying out the
RNAi screen in a less complex model organism may thus provide a higher probability of identifying evolutionary conserved genes/pathways that regulate autophagy, since there is a less chance of functional redundancy in any given tissue of cell line. Findings in *Drosophila melanogaster* will also benefit our understanding of the crosstalk between autophagy and apoptosis in mammals.

1.9.2 Hypotheses and specific aims

**Hypothesis 1:** Given that some apoptotic genes were shown to play a role in autophagy regulation, I hypothesized that a systematic screening approach would identify additional cell death genes involved in autophagy regulation in *Drosophila l(2)mbn* cells

**Specific Aim 1:** Design a systematic approach to identify cell death genes involved in the regulation of autophagy

A systematic RNAi screen coupled with flow cytometry-based LysoTracker Green and cell-based GFP-LC3 assays was employed to investigate the involvement of cell death genes in starvation induced autophagy in *Drosophila l(2)mbn* cells, which are haemocytes derived from *lethal (2) malignant blood neoplasm* mutants.

**Hypotheses 2:** Given that amino acid starvation was shown to trigger cell death in the *Drosophila melanogaster* ovary, I hypothesized that starvation would induce the autophagic response and autophagy related genes would contribute to cell death in the ovary. Genes that were required for regulation of autophagy in *l(2)mbn* cells were predicted to have a similar function in autophagy regulation during *Drosophila melanogaster* oogenesis.
Specific Aim 2: Determine the roles of \textit{Atg} genes (\textit{Atg1} and \textit{Atg7}) in starvation-induced cell death and the roles of \textit{Dcp-1} and \textit{Bruce} in autophagy regulation during \textit{Drosophila melanogaster} oogenesis.

LysoTracker Red (LTR) and GFP-LC3 assays were employed to determine whether autophagy was induced in response to nutrient deprivation in the germaria and mid-oogenesis egg chambers in the ovary. The TUNEL assay, an indication of DNA fragmentation, was employed to assess the function of two autophagy related genes, \textit{Atg1} and \textit{Atg7}, in germarium and mid-oogenesis cell death. The effects of \textit{Dcp-1} and \textit{Bruce} mutations on autophagy and cell death were examined in germaria and mid-oogenesis egg chambers.

\textbf{Hypotheses 3:} First, I hypothesized that \textit{Bruce} and \textit{Dcp-1} interact genetically and that \textit{Bruce} functions as a negative regulator of \textit{Dcp-1} activity. Second, I hypothesized that the catalytic activity of \textit{Dcp-1} plays a regulatory function in the autophagy process, and substrates of \textit{Dcp-1} have a functional significance in mediating autophagy.

Specific Aim 3: Determine the genetic interaction between \textit{Dcp-1} and \textit{Bruce} and characterize the molecular mechanisms of \textit{Dcp-1} in the autophagy process

To examine the genetic relationship between \textit{Dcp-1} and \textit{Bruce}, I created \textit{Dcp-1; Bruce} double mutants. Analyses of double mutants during starvation-induced autophagy and cell death in the ovary were performed. I employed the catalytically inactive construct of \textit{Dcp-1} to determine whether \textit{Dcp-1} dependent proteolytic events were required for starvation-induced autophagy. To identify potential substrates of \textit{Dcp-1} in \textit{l(2)mbn} cells undergoing starvation-induced autophagy, I employed an immunoprecipitation and mass spectrometry (IP-MS) strategy.
Figure 1.1 Model of the autophagy process.
Cytoplasmic components and organelles are sequestered into a double-membrane structure called an autophagosome. The autophagosome fuses with a lysosome to create an autolysosome where the inner membrane and cellular contents are degraded by lysosomal enzymes. During autophagy, Atg8 (LC3 in mammals) is cleaved, lipidated and conjugated to autophagosomes. The membrane translocation event of Atg8 makes it a useful marker to monitor autophagy. [Figure adapted from Klionsky 2008].
Figure 1.1

- Cytosolic proteins and organelles
- Preautophagosomal structure
- Isolation membrane
- Autophagosome
- Autolysosome

- Lysosome
- Atg8 (LC3)
Figure 1.2 Molecular machinery of autophagy.
The formation of an autophagosome can be divided into four distinct steps: induction, vesicle nucleation, vesicle elongation, and membrane retrieval. (A) The phosphorylation (P) state of Atg13 is controlled by Tor kinase. Inactivation of Tor results in the dephosphorylation of Atg13, and the subsequent formation of a protein complex that includes Atg1, Atg13 and Atg17 for the induction of autophagy. (B) The activation of class III phosphatidylinositol 3 kinase (PI3K), also known as Vps34, generates phosphatidylinositol 3-phosphate (PI3P) and is important for the vesicle nucleation step. In yeast, Atg6, Atg14, and Vps15, are part of the class III PI3K complex. In mammals, the PI3K complex consists of Beclin1/Atg6, Vps15, UVRAG, Ambra1, and Bif-1. Bcl-2 and Bcl-X<sub>L</sub> interact constitutively with Beclin1, and dissociation of Beclin1 from its inhibitors Bcl-2 or Bcl-X<sub>L</sub> is required for autophagy induction. (C) Two ubiquitin-like conjugation systems, the Atg12 conjugation system (Atg12, Atg5, and Atg16) and the Atg8 lipidation system (Atg8, Atg3 and Atg7) are involved in vesicle expansion. (D) The Atg9-Atg18 dependent membrane retrieval complex is required for the disassembly of Atg protein complexes from mature autophagosomes. [Figure adapted from Maiuri 2007 and Melendez 2008].<sup>7,131</sup>
Figure 1.2

A. Regulation of induction

Inactive

Atg13

P

P

Nutrients → Tor

Atg13

P

Atg17

Active

Atg1

B. Vesicle nucleation

Yeast

Atg6

Atg14

Vps34

Vps15

P(3)P

Mammals

Bif-1

UVRAG

Ambra1

Beclin1

Vps34

Vps15

P(3)P

Bcl-2, Bcl-XL

C. Vesicle expansion and completion

Atg12

Atg10

E2

Atg12

Atg12

Atg5

Atg5

Atg12

Atg12

Atg5

Atg16

Atg16

Atg8

Gly

E1

Atg7

E2

Atg3

PE

D. Retrieval

Atg9

Atg18
Figure 1.3 Core apoptosis signaling pathways in nematodes, fruitflies and mammals.

In all three species, the core apoptotic components are conserved. However, mammals and the fruitfly Drosophila melanogaster have both initiator (purple) and effector (orange) caspases, and there is only a single caspase CED-3 that carries out both functions (initiator and effector) in the nematode worm Caenorhabditis elegans. The activity of caspases is restrained by inhibitor of apoptosis proteins (IAP) (grey). IAP binding proteins (blue) remove the IAP-mediated negative regulation of caspases. The adaptor protein CED-4/Dark/Apaf-1 (green) promotes the activation of caspases. CED-4/Dark/Apaf-1 activity is inhibited by anti-apoptotic Bcl-2 family members (light yellow). The activation of Dark might be regulated by the Drosophila melanogaster Bcl-2 family members, Debcl and Buffy, but direct evidence for this interaction is still lacking. [Figure adapted from Hay 2004].67
Figure 1.4 Conserved features of effector caspases in *Drosophila melanogaster* and mechanism of caspase 7 activation

(A) A schematic representation of *Drosophila melanogaster* Dcp-1 and drICE. The red ovals indicate the catalytic cysteine residue. The four surface loops (L1-L4) and the p20 and p10 subunits are indicated for each caspase. Arrows indicate the sites of inter-domain cleavage that results in generation of the p20 and p10 subunits. Caspases exist as homo-dimers and their catalytic activity is increased after cleavage at the inter-domain sites that result in an allosteric conformational change of surface loop 2 (L2). L2 contains the catalytic residue cysteine and L2’ stabilizes the catalytic site of the adjacent caspase monomer. The conformation of the L2’ loop is dramatically different between mammalian inhibitor (I) bound procaspase-7 zymogen (B) and active caspase-7 (C). The L2’ loop of the procaspase-7 zymogen is locked in a closed conformation by covalent linkage. After inter-domain cleavage, the L2’ and L2 loops switch to their open conformation allowing substrate binding. L1 and L4 constitute two parallel sides of the substrate binding groove and L3 serves as the base. [Figure 1.4 B and C from (Shiozaki and Shi, 2004)]
Figure 1.4
Figure 1.5 *Drosophila melanogaster* ovariole structure

A schematic representation of a *Drosophila melanogaster* ovariole. Each ovariole is composed of the gerarium in the anterior, followed by a row of progressively developing stages (2-14) of egg chambers. A cystoblast (cb) is derived from a germline stem cell (gsc) and undergoes four rounds of mitosis to form a 16-cell cyst (cc). While the germline cyst migrates from region 2a to 2b, it loses contact with the inner gerarium sheath cells (isc) and is surrounded by a layer of follicle cells (fc) which are derived from the somatic stem cells (ssc). In region 3, a germline cyst surrounded by follicle cells moves out of the gerarium and becomes an egg chamber. In each egg chamber, the most posterior germline cell becomes the oocyte (oo) and the remaining 15 cells are nurse cells (nc). Vitellogenesis (yolk synthesis and uptake) takes place at stage 8 of oogenesis. [Figure and text from Drummond-Barbosa and Spradling, 2001].\(^\text{110}\)
Figure 1.5
1.10 References


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Chapter 2 Effector caspase Dcp-1 and IAP protein Bruce regulate starvation-induced autophagy during *Drosophila* oogenesis

2.1 Introduction

Autophagy is an evolutionarily conserved mechanism for the degradation of long-lived proteins and organelles. During autophagy, cytoplasmic components are sequestered into double membrane structures called autophagosomes which then fuse with lysosomes to form autolysosomes, where degradation occurs. Currently, there are 31 *Atg* (autophagy-related) genes in yeast, and 18 Atg proteins are essential for autophagosome formation. Most yeast *Atg* genes have orthologues in higher eukaryotes and encode proteins required for autophagy induction, autophagosome nucleation, expansion and completion, and final retrieval of Atg protein complexes from mature autophagosomes (for review see Levine and Yuan, 2005).

Depending on the physiological and pathological conditions, autophagy has been shown to act as a pro-survival or pro-death mechanism in vertebrates. In the case of growth factor withdrawal, starvation and neurodegeneration, autophagy has been shown

to function in cell survival. In contrast, autophagy has been reported to act as a cell death mechanism in derived cell lines where caspases or apoptotic regulators are impaired. The nature and perhaps level of the stress stimulus may also be important in determining whether autophagy promotes cell survival or cell death.

Overlaps between components in apoptosis and autophagic pathways have been described. Upstream signal transducers in apoptotic pathways, including TRAIL, TNF, FADD and DAPK, have been shown to play a role in autophagy regulation, and two apoptotic inducers, including sphingolipid and ceramide, can activate autophagy in mammalian cells. In addition, two recent studies demonstrate physical and functional interactions between components of apoptosis and autophagy. First, the anti-apoptosis protein, Bcl-2, suppresses autophagy through a direct interaction with Beclin 1, a protein required for autophagy. Second, the autophagy related protein 5 (Atg5), which is cleaved by calpain, associates with Bcl-XL leading to cytochrome c release and caspase activation. Further examples and discussion of the connections between apoptosis and autophagy can be found in several recent reviews on this topic. The current findings indicate that there is a complex relationship between apoptosis and autophagy, but the regulatory mechanisms underlying the cross-talk between the two processes are still largely unknown.

Autophagy is observed in several Drosophila tissues during development, and thus Drosophila is useful as a model to study autophagy in the context of a living organism. Fourteen Drosophila annotated genes share significant sequence identity with the yeast Atg genes and, overall, 8 Drosophila Atg homologues have already been shown to be required for autophagy function. In addition, recent studies demonstrated the
role of autophagy in *Drosophila* physiological cell death. Loss of *Atg* genes, including *Atg1, Atg2, Atg3, Atg6, Atg7, Atg8, Atg12* and *Atg18*, inhibited proper degradation of salivary glands during development. Overexpression of *Atg1* induced premature salivary gland cell death in a caspase-independent manner.\(^{23}\) In contrast, caspase activity was required for *Atg1*-mediated apoptotic death in the fat body.\(^{24}\) Mutation of *Atg7* resulted in an inhibition of DNA fragmentation in the midgut but led to an increase of DNA fragmentation in the adult *Drosophila* brain.\(^{25}\) Together these results suggested that the mechanistic role of autophagy in cell death and the interrelations between autophagy and apoptosis may be tissue and/or context dependent.

The adult *Drosophila* ovary contains 15 to 20 ovarioles comprised of developing egg chambers which consist of 16 germ line cells (15 nurse cells and 1 oocyte) surrounded by a layer of somatic follicle cells. The germline cells originate from stem cells which undergo mitosis to form 16-cell cysts in a specialized region called the germarium. In the late stage of oogenesis, the nurse cells support the development of the oocyte by transferring to it their cytoplasmic contents. After this “dumping” event, the nurse cells undergo cell death and their remnants are engulfed by the surrounding follicle cells.\(^{26, 27}\) In addition to this late stage developmental cell death, egg chambers can be induced to die at two earlier stages, during germarium formation (in region 2) and mid-oogenesis, by factors such as nutrient deprivation, chemical insults, and altered hormonal signaling.\(^{28-30}\) In some respects, cell death during *Drosophila* oogenesis is similar to the death of *Drosophila* larval salivary glands. Both nurse cells and salivary gland cells are large and polyploid, and the entire tissues undergo cell death simultaneously.\(^{28}\) Notably, morphological features of autophagy have been described during mid-oogenesis cell
death in a related species, *Drosophila virilis*, suggesting that the cell death process in ovaries and salivary glands share additional similarities.

Previous studies have focused on characterizing the role of autophagy genes in cell death and determining the paradoxical functions of autophagy (pro-survival and pro-death) in various cell lines and organisms. However, a systematic approach that investigates the involvement of cell death genes in starvation-induced autophagy has not been conducted. Here we present RNAi analyses to determine whether known cell death related genes in *Drosophila* play a role in autophagy regulation in the *l(2)mbn* cell line.

We chose the *l(2)mbn* cell line based on the following reasons. First, unlike other *Drosophila* cell lines (S2 and Kc), the origin tissue of the *l(2)mbn* cell line is known and was a sample of haemocytes from the *Drosophila* mutant lethal malignant blood neoplasm. Second, *l(2)mbn* cells showed lysosome bodies and autophagosome like structures after addition of the steroid hormone 20-hydroxyecdysone. Finally our QRT-PCR results showed the expression of core apoptotic and autophagic genes in *l(2)mbn* cells indicating both apoptosis and autophagy pathways are competent in the *l(2)mbn* cell line. We also utilize *Drosophila* genetics to investigate a role for the effector caspase *Dcp-1* and the IAP family member *Bruce* in autophagy regulation in vivo during *Drosophila* oogenesis. Further, we examine the function of autophagy genes *Atg7* and *Atg1* in starvation-induced germline cell death in the *Drosophila* ovary.

### 2.2 Materials and methods

#### 2.2.1 Cell culture conditions

*Drosophila l(2)mbn* cells were maintained in Schneider’s *Drosophila* medium (Gibco-Invitrogen) supplemented with 10% fetal bovine serum (FBS) in 25-cm²
suspension flasks (Sarstedt) at 25°C. All the experiments were carried out 3 days after passage and the cells were discarded after 25 passages.

2.2.2 dsRNA synthesis

Individual PCR products containing coding sequences for the transcripts to be targeted were generated by RT-PCR using the Superscript one-step RT-PCR kit with platinum taq (Invitrogen). Each primer used in RT-PCR contained a 5' T7 RNA polymerase binding site (TAATACGACTCAGAGG) followed by sequences specific for the targeted genes (Table 2.1). For *in vitro* transcription reactions, 50µl of each RT-PCR product was ethanol-precipitated and resuspended in 8µl of nuclease free water and then used as a template. *In vitro* transcription reactions were carried out using T7 RiboMax Express RNAi systems (Promega) according to the manufacturer’s instructions. dsRNAs were ethanol precipitated and resuspended in 50µl of nuclease free water. A 5µl aliquot of 1/100 dilution was analysed by 1% agarose gel electrophoresis to determine the quality of dsRNA. The dsRNA was quantitated using the PicoGreen assay (Invitrogen) and adjusted to 200ng/µl with nuclease free water.

2.2.3 RNA interference (RNAi)

66µl of cells (1 x 10^6 cells/mL) in SFM922 medium were seeded into each well of a 96 well plate. 2µg of dsRNA (20µM) was added into each well. After one hour incubation at room temperature, the cells received Schneider’s medium supplemented with 10% FBS to achieve a final 200µl volume. Cells were incubated for 72 hours at 25°C. Since RNAi of th triggered a massive amount of apoptosis at the standard incubation time of 72 hours, we instead used a 24 hour incubation period. After 24 hours
of \( th \)-dsRNA treatment, there was already a significant number of apoptotic cells present indicating an efficient knock-down by RNA interference, but a sufficient number of healthy cells (more than 10,000) remained for LTG analysis.

### 2.2.4 Flow cytometry based LysoTracker Green (LTG) assay

For drug treatments, 3-methyladenine (3MA; 10mM) or Bafilomycin A1 (Baf; 0.1\( \mu \)M) was added when nutrient full medium was replaced with 2mg/ml glucose in PBS. After 4 hours incubation at 25°C, cells were incubated for 20 minutes at room temperature with LysoTracker Green (LTG; 50nM) for quantification of autophagy levels and propidium iodide (PI; 2\( \mu \)g/mL) to eliminate dead cells. The cells were then analyzed using flow cytometry (FACSCalibur; Becton Dickinson). A minimum of 10,000 cells per sample were acquired for triplicate samples per experiment. LTG fluorescence levels of cells (excluding PI positive cells) were analyzed using Flowjo software.

For RNAi experiments, the RNAi-treated cells after 72hrs incubation were transferred into a U bottom 96 well plate and centrifuged at 800 rpm for 5 minutes. Nutrient full medium was replaced with 2mg/mL glucose/PBS with dsRNA (20\( \mu \)M) for 4 hour starvation treatment and cells were labeled with LTG and PI for 20 minutes at room temperature, and analyzed as described above.

### 2.2.5 GFP-LC3 detection

The p2ZOp2F-eGFP-LC3 plasmid was generated by restriction digestion of eGFP-LC3 from pUASP-eGFP-LC3\(^{33}\) and cloning into the p2ZOp2F vector\(^{34}\). To create a stable cell line, \textit{Drosophila l(2)mbn} cells were transfected with p2ZOp2F-eGFP-LC3 and selected for the presence of the construct using zeocin. The resulting p2ZOp2F-
eGFP-LC3 stable (GFP-LC3) l(2)mbn cells were maintained in Schneider’s *Drosophila* medium (Gibco-Invitrogen) supplemented with 5% fetal bovine serum (FBS) and zeocin (10μg/mL). 66μl of these GFP-LC3 l(2)mbn cells (2.5 x 10^6 cells/mL) in SFM922 medium were seeded into each well of an 8 well CC2 coated chamber slide (LabTek system). Cells were incubated with dsRNAs as described above, followed by 4 hour starvation treatment. Cells were fixed with 2% paraformaldehyde for 20 minutes and incubated with anti-GFP antibody (1:200; JL8; Clontech), followed by anti-mouse immunoglobulin Alexa 488 conjugates (Molecular Probes). Cells were mounted with SlowFade Gold antifade reagent with DAPI at room temperature (Invitrogen). Images were obtained using a 63x objective on a Zeiss Axioplan2 microscope and captured with a cooled mono 12 bit camera (Qimaging) and Northern Eclipse image analysis software (Empix Imaging Inc.) Cells with more than three GFP-LC3 punctate dots were considered as GFP-LC3 positive cells. A minimum of 200 cells per sample were counted manually for triplicate samples per experiment.

### 2.2.6 Statistical analysis

Two tailed student’s t-test (equal variances) was used to compare mean levels. n=3. A value of P < 0.05 was considered statistically significant.

### 2.2.7 Generation of transgenic flies

The *UASp-full-length-Dcp-1* construct was generated by PCR amplification of the coding region from a *Dcp-1* cDNA clone and subsequent cloning of the amplicon into the *UASp* vector. Transgenic flies were generated using standard procedures. To express full-length *Dcp-1* in the germline, flies were crossed to NGT; nanosGAL4 flies.
and resulting progeny were analyzed. To express truncated Dcp-1 and GFP-LC3 in the germline, yw; nanosGAL4 UASp-tDcp-1 were crossed to UASp-GFP-LC3; nanos GAL4 and resulting progeny were analyzed.38

2.2.8 Fly strains

w1118 was used as the wild-type stock. Other fly stocks used were as follows: Dcp-1Prev1 and UASp-GFP-LC3; nanos-GAL4, Bruce E81, Bruce E16, Atg777, Atg714, CG5335d30, and Atg1Δ3D.

2.2.9 Generation of Atg 1 germline clones (GLCs)

To generate germline clones, FRT2A was recombined onto the Atg1Δ3D chromosome. Correct recombinants were confirmed by failure to complement Df(3L)Bsc10 and Atg100305. Germline clones were generated with the FLP/FRT/ovoD technique as described.39 Larvae of the genotype HSflp; ovoD FRT2A/Atg1Δ3DFRT2A were heatshocked on day 4 and 5 for 1 hour at 37°C.

2.2.10 LysoTracker Red staining

For nutrient deprivation experiments, flies were conditioned on yeast paste for 2 days and then placed in a dry vial with access to a 10% sucrose solution for 4-5 days.38 Ovaries were dissected in PBS and immediately transferred into PBS containing 0.8 μM LysoTracker Red (LTR) (Invitrogen) for 5 min at room temperature in the dark. The ovaries were then stained with 0.1mg/mL DAPI for 30 sec. The ovaries were washed three times with PBS, and mounted with SlowFade antifade reagent (Invitrogen) at room temperature. Images were obtained using a 20 or 40x objective on a Zeiss Axioplan2 microscope and captured with a cooled mono 12 bit camera (Qimaging) and Northern
Eclipse image analysis software (Empix Imaging Inc.). Egg chambers with more than ten LTR positive spots were considered as LTR positive. Stage 8 degenerating egg chambers in \emph{w^{118}} flies were scored by the presence of condensed nurse cell nuclei. The degenerating egg chambers in \emph{Dcp-1} \textsuperscript{Prey} files were characterized by a disappearance of follicle cells and a persistence of nurse cell nuclei, as reported previously.\textsuperscript{40}

For expression of \emph{Dcp-1} in ovaries, \emph{NGT/+; nanos-GAL4/UASp-fl-Dcp-1} flies were conditioned on wet yeast paste for 2-4 days and dissected in Ringers\textsuperscript{41}. Ovaries were incubated with 50µM Lysotracker Red DND-99 in PBS for three minutes, washed three times for five minutes each time in PBS, fixed ten minutes in 1:1 heptane:3.7% formaldehyde in Pipes buffer (0.1M Pipes, 2mM MgSO\textsubscript{4}, 1mM EGTA, pH 6.9), washed three times for five minutes each time in PBT (PBS +0.1% Triton-X), and mounted in anti-fade + 1.5µg/ml Hoechst 33258. Egg chambers were viewed at room temperature using an Olympus UPlanFl 20X, 0.50 objective on an Olympus BX50 confocal microscope. Images were captured using Olympus Magnafire SP model#S99810 (Hoechst) and Olympus Fluoview (LTR) cameras. DABCO was used as the imaging medium. Egg chambers with more than ten LTR positive spots were considered as LTR positive. All figures were processed with Photoshop 7.0 (Adobe). Color was added on LysoTracker image using ImageJ.

\subsection*{2.2.11 TUNEL assay}

Ovaries were dissected in Schneider’s \emph{Drosophila} medium (Gibco-Invitrogen) supplemented with 10% fetal bovine serum (FBS). The ovaries were fixed in PBS containing 4% paraformaldehyde. The ovaries were then washed two times (5 min each) in PBS, permeabilized with 0.2% triton X-100 for 5 minutes, followed by two washes in
PBS. TUNEL reaction was carried out with the DeadEnd fluorometric TUNEL system (Promega). The ovaries were then stained with 0.1mg/mL DAPI for 30 sec at room temperature, mounted in SlowFade antifade reagent (Invitrogen) and observed under a Zeiss Axioplan 2 microscope. Images were obtained using a 10, 20 or 40x objective on a Zeiss Axioplan2 microscope and captured with a cooled mono 12 bit camera (Qimaging) and Northern Eclipse image analysis software (Empix Imaging Inc.)

2.2.12 Quantitative RT-PCR

Cells (approximately 2x10^5 cells in 600µL) were incubated with dsRNAs as described above at 25°C for 72 hours, followed by 4 hour starvation treatment. Cell cultures were transferred to RNAse free eppendorf tubes (Ambion) and cells were pelleted at 1000 rpm for 10 min. Cells were lysed in 1ml Trizol (Invitrogen) and total RNA was extracted according to manufacturer’s instructions. Isolated RNA was treated with RNAse free DNAse and 50ng of total RNA was used in a 15µL QRT-PCR reaction. QRT-PCR was performed using the one-step SYBR green RT-PCR Reagent Kit (Applied Biosystems) on an Applied Biosystems 7900 Sequence Detection System. Expression levels were calculated using the Comparative C_T method with Drosophila rp49 as the reference. All samples were analyzed in triplicate. The knock-down efficiency was determined by comparing the fold change in expression between target dsRNA treated and untreated cells.
2.3 Results

2.3.1 RNAi screening assay identifies known positive and negative regulators of starvation-induced autophagy in Drosophila l(2)mbn cells.

To quantify starvation induced autophagy we used a Drosophila tumorous larval hemocyte cell line, lethal (2) malignant blood neoplasm (l(2)mbn)\(^{32}\), and employed LysoTracker Green dye (LTG) shown previously to label lysosomes and autolysosomes in Drosophila.\(^{22, 42}\) Flow cytometry was employed to acquire LTG fluorescence of individual cells. Under nutrient full medium conditions, we detected a basal level of LTG labeling in l(2)mbn cells (Figure 2.1A). When cells were transferred into amino acid deprived medium for 2 hours, we observed a detectable increase in LTG labeling. After 4 hours of amino acid deprivation, a further increase in the percentage of cells with high LTG fluorescence levels (LTG\(^{\text{high}}\) population) was observed (Figure 2.1A). To confirm that autophagy is indeed upregulated under nutrient deprived conditions in l(2)mbn cells, we constructed a stable l(2)mbn cell line expressing mammalian microtubule associated protein 1 light chain 3 (LC3)/ATG8 fused to GFP protein, a widely used marker for autophagy. During autophagy, LC3 conjugates to phosphatidylethanolamine (PE) which then inserts into the autophagosomal membrane. Thus, localization of GFP-LC3 changes from a diffuse cytoplasmic pattern to a punctate autophagosomal membrane-bound pattern that can be monitored by microscopy.\(^{42, 43}\) As expected, the percentage of cells with more than three GFP-LC3 puncta (GFP-LC3 positive) was increased from 9% (n=216) in the nutrient-full condition to 32% (n=200) in the nutrient-starved condition for 2 hours (Figure 2.1B). Further, to confirm that LTG labeling correlates with autophagy levels in l(2)mbn cells, we employed the
pharmacological autophagy inhibitors, 3-methyladenine (3MA) and Bafilomycin A1 (Baf). 3MA blocks autophagy by inhibiting PI3-kinase activity.\textsuperscript{44} Baf is a specific inhibitor of lysosomal proton pumps and prevents the fusion of autophagosomes with lysosomes.\textsuperscript{45} In \textit{l(2)mbn} cells, both autophagy inhibitors significantly reduced LTG fluorescence levels following starvation treatment (Figure 2.1C and D). Consistently, addition of 3MA also decreased the numbers of GFP-LC3 positive cells following starvation treatment. As expected, the addition of Baf, which is known to increase the numbers of autophagosomes by preventing their lysosomal degradation, resulted in increased GFP-LC3 puncta in starved cells (Figure 2.1B). These results indicate that our flow cytometry based LTG assay is able to detect changes in the autophagy levels of \textit{l(2)mbn} cells in response to starvation and autophagy-inhibiting drug treatments.

To further validate our flow cytometry based LTG assay and determine its sensitivity, we used RNAi to specifically inhibit \textit{Drosophila} autophagy genes. Currently, there are 14 \textit{Drosophila} genes that share significant sequence identity with yeast \textit{Atg} genes, and 7 \textit{Drosophila Atg} homologues have already been shown to be essential for starvation-induced autophagy in the larval fat body.\textsuperscript{22} In our assay, RNAi of 11 \textit{Drosophila Atg} homologues individually resulted in a statistically significant reduction in the LTG\textsuperscript{high} population following starvation treatment (Figure 2.1E and Table 2.2). The effects on LTG staining by RNAi of \textit{Atg} genes or 3-MA were modest in size (e.g. 30-48% change relative to the Hs-RNAi negative control in Figure 2.1E), but reproducible and statistically significant. The limited magnitude of the detectable effects may be due, at least in part, to the nature of this dye as an acidophilic probe which detects autolysosomes but also background lysosomal staining. As with any RNAi-based screen,
incomplete target knockdown may also be a contributing factor. Comparison of our RNAi-mediated results with previous *in vivo* results from the *Drosophila* larval fat body (Table 2.2) indicates that *l(2)mbn* cells require the same autophagy genes as the fat body.\(^{22}\)

In higher eukaryotes, starvation induced autophagy is suppressed by components of the insulin/class I phosphoinositide 3-kinase (PI3K) and TOR pathways (for reviews see Klionsky, 2007; Levine and Yuan, 2005; Maiuri et al., 2007).\(^1, 3, 4\) To determine whether PI3K and TOR pathways are required for starvation-induced autophagy in *l(2)mbn* cells, we designed dsRNAs against several genes in these pathways. RNAi of *Tor* or *RheB*, negative regulators of autophagy, showed an increase in LTG \(^{\text{high}}\) cells compared to *Hs*-dsRNA (negative control) treated cells following starvation treatment (Figure 2.1F). In contrast, RNAi of *Pten*, *Tsc1 Tsc2* and *S6k*, positive regulators of autophagy, showed a reduction in the LTG \(^{\text{high}}\) population (Figure 2.1F). These results indicate that components of TOR and PI3K pathway are essential to regulate starvation-induced autophagy in *Drosophila l(2)mbn* cells. These results also demonstrate that our primary screening method, a flow cytometry-based LTG assay, is capable of detecting alterations induced by RNAi-mediated knockdown of positive and negative regulators of autophagy. Thus, this method can be employed to identify novel components in starvation-induced autophagy. To ensure the changes in LTG fluorescence levels were due to alterations in autophagy, we used GFP-LC3 to track changes in autophagosome formation in cells. RNAi of *Tor* showed an increase in the numbers of GFP-LC3 positive cells following starvation treatment (Figure 2.2G). In contrast, reduction of *Pten* expression by RNAi resulted in a decrease in the number of the GFP-LC3 positive cells.
(Figure 2.2G). Together these two methods allow us to monitor the dynamic steps, formation of autophagosomes (GFP-LC3) and autophagosome-lysosome fusion (LTG), during autophagy.

2.3.2 Identification of cell death-related genes that regulate starvation-induced autophagy in \( l(2)mbn \) cells.

To better understand the relationship between autophagy and apoptosis, we investigated whether known cell death genes were required for starvation-induced autophagy in \( l(2)mbn \) cells. dsRNAs were designed against the \( Drosophila \) core cell death effectors, \textit{rpr}, \textit{hid}, \textit{grim} and \textit{skl}, and autophagy was evaluated by the flow cytometry based LTG assay. Only dsRNA corresponding to \textit{hid}, but not \textit{rpr}, \textit{grim} or \textit{skl} showed an effect on autophagy by this assay. RNAi of \textit{hid} decreased the percentage of LTG\textsuperscript{high} cells following starvation treatment (Figure 2.2A). Previous studies showed that the Ras/Raf/MAPK pathway specifically inhibits the pro-apoptotic activity of \textit{hid}.\textsuperscript{46} To determine whether the Ras/Raf/MAPK pathway also plays a regulatory role in autophagy in \( l(2)mbn \) cells, we designed dsRNAs to target these three components. RNAi of \textit{Ras}, \textit{phl} (also known as \textit{raf}) or \textit{rl} (also known as \textit{MAPK}) all further enhanced the LTG fluorescence levels suggesting that, like in apoptosis, they have an inhibitory role in autophagy regulation (Figure 2.2B). A second set of dsRNAs, non-overlapping with the first set of dsRNAs, was designed to validate these new findings and consistent results were observed (Figure 2.3A). In addition, GFP-LC3 was employed to track changes in autophagosome formation in cells. RNAi of \textit{hid} showed a decrease in the numbers of cells with GFP-LC3 puncta (Figure 2.2F and G), while RNAi of \textit{Ras}, \textit{phl} or \textit{rl} all resulted
in a significant increase in the numbers of GFP-LC3 positive cells following starvation treatment (Figure 2.2G).

All RHG family members, Rpr, Hid, Grim and Skl, bind to Drosophila Inhibitor of Apoptosis Protein-1 (DIAP1) and inhibit its anti-apoptotic activities. To test whether DIAP1 (encoded by th) is a putative downstream mediator of Hid-dependent autophagy in l(2)mbn cells, dsRNA was designed specifically to target th. We found that th-dsRNA treated cells showed no difference in LTG fluorescence levels compared to Hs-dsRNA (negative control) treated cells (Figure 2.2C). Interestingly, our data showed that reduced expression of Bruce, another IAP family member protein, further increased the LTG fluorescence levels following starvation treatment (Figure 2.2C) (confirmed using non-overlapping dsRNAs; see Figure 2.3B). RNAi of Bruce expression also resulted in an increase in GFP-LC3 puncta following starvation treatment (Figure 2.2G). These results suggest that Bruce, instead of DIAP1, could be the downstream target of Hid during starvation induced autophagy in l(2)mbn cells.

Next we investigated whether the transducers of apoptotic signals, Ark, Buffy, and debcl are required for starvation- induced autophagy. Reduced expression of Ark, the Drosophila homologue of mammalian Apaf-1, did not affect the LTG fluorescence levels (Figure 2.2D). RNAi of two Bcl-2 family members Buffy or debcl, resulted in a decrease in the percentage of LTG^high^ cells following starvation treatment (Figure 2.2D). Consistently, reduction of Buffy and debcl expression by RNAi decreased the percentage of GFP-LC3 positive cells following starvation treatment (Figure 2F and G). Reduced expression of Ark, Buffy, and debcl was determined using quantitative RT-PCR (Figure 2.3C). In addition, we reduced expression of the tumor suppressor p53 by RNAi and
found that starvation-induced autophagy was inhibited (Figure 2.2D). Results were further confirmed using non-overlapping dsRNAs (Figure 2.3B).

To investigate the requirement of caspases, the final effectors of apoptosis, in starvation-induced autophagy we designed gene specific dsRNAs corresponding to seven different Drosophila caspases. RNAi of just one caspase, Dcp-1 but not others resulted in a decrease in the percentage of LTG\textsuperscript{high} cells following starvation treatment (Figure 2.2E). A second dsRNA against Dcp-1, non-overlapping with the first dsRNA, yielded a similar result (Figure 2.3B). Reduction of Dcp-1 expression by RNAi was determined using quantitative RT-PCR (Figure 2.3C). Consistent with the LTG derived data, RNAi-mediated knock-down of Dcp-1 resulted in a decrease in GFP-LC3 positive cells following starvation treatment (Figure 2.2F and G). These results indicate that Dcp-1 functions as a positive regulator of autophagy in Drosophila l(2)mbn cells.

2.3.3 Autophagy occurs in response to nutrient deprivation in germaria and mid-stage egg chambers in the Drosophila melanogaster ovary.

To further characterize the requirement of Dcp-1 and Bruce in autophagy regulation, we studied Drosophila melanogaster oogenesis \textit{in vivo}. We used a transgenic fly line which expresses a GFP-LC3 fusion protein under the control of the UASp promoter.\textsuperscript{33} Co-expression of UASp-GFP-LC3 with the germline-specific nanos-GAL4 driver resulted in detectable GFP-LC3 expression in the germline (nurse cells and oocyte) cells but not in somatic (follicle) cells (Figure 2.4A).\textsuperscript{33,36} When UASp-GFP-LC3; nanos-GAL4 flies were subjected to nutrient deprivation, we observed numerous GFP-LC3 puncta in region 2 within the germarium (Figure 2.4B). In contrast, flies raised in the presence of yeast paste (well-fed) had a diffuse GFP-LC3 pattern (Figure 2.4B).
addition, we found an increase in punctate Lysotracker Red (LTR) staining in germaria of nutrient deprived wild-type (w^{118}) flies compared to well-fed wild-type flies (Figure 2.4B and Table 2.3). We also observed numerous GFP-LC3 puncta in nutrient-deprived degenerating stage 8 chambers, but a diffuse GFP-LC3 pattern was detected in healthy egg chambers (Figure 2.4 C). Similarly, degenerating stage 8 egg chambers had numerous LTR positive dots in the nurse cells, while healthy egg chambers had a low level of LTR staining (Figure 2.4 C and Table 2.4). In starved Atg7 mutants (Atg7^{d77}/Atg7^{d14}), there was a significant decrease in punctate LTR staining in region 2 of the germarium and in stage 8 degenerating egg chambers compared to flies with the genotype CG5335^{d30}/Atg7^{d14}, employed previously as controls in Juhasz et al. 2007 (Figure 2.4D, and Table 2.3 and 2.4). These results indicated that nurse cells lacking the core autophagy regulator Atg7 failed to induce autophagy in response to nutrient deprivation. Overall, our observations showed that nutrient deprivation induces autophagy in region 2 germaria and in degenerating stage 8 egg chambers in Drosophila melanogaster.

2.3.4 Dcp-1 and Bruce regulate autophagy in germaria and degenerating mid-stage egg chambers.

To determine whether Dcp-1 is required for autophagy in germaria and degenerating mid-stage egg chambers during oogenesis, we employed LTR staining in nutrient-deprived Dcp-1^{Prev} mutant flies. We observed a decrease in punctate LTR staining in region 2 of the germarium and in stage 8 degenerating egg chambers (Figure 2.5A and B, and Table 2.3 and 2.4) compared to nutrient-deprived wild-type flies. Consistent results were observed using GFP-LC3. Degenerating stage 8 egg chambers in
nutrient – deprived $Dcp-1^{Prev}$ mutants containing the GFP-LC3 transgene had a diffuse GFP-LC3 pattern instead of punctate GFP-LC3 structures (Figure 2.5C). Together, these results indicate that nurse cells lacking $Dcp-1$ function are severely impaired in the ability to induce autophagy in response to starvation.

To determine whether $Dcp-1$ was also sufficient to induce autophagy in vivo, we generated transgenic flies that express the full length $Dcp-1$ (fl-$Dcp-1$) under the control of the $UASp$ promoter. In the presence of a nutrient rich food source, degenerating stage 8 egg chambers are observed only rarely in wild-type flies. However, under nutrient rich conditions, we observed an abundance of degenerating stage 8 egg chambers in $nanos$-GAL4/$UASp$-fl-$Dcp-1$ flies with increased levels of punctate LTR staining (Figure 2.5D and Table 2.4). Further, we expressed an activated form of $Dcp-1$ (missing the prodomain) and GFP-LC3 in the germline using the $UASp/nanos$-GAL4 system and observed numerous degenerating stage 8 egg chambers with GFP-LC3 puncta (Figure 2.5E), indicating that activity of effector caspase $Dcp-1$ is sufficient to induce autophagy during mid-oogenesis even under nutrient rich conditions.

We identified the IAP protein Bruce as a negative regulator of autophagy in $l(2)mbn$ cells. We next asked whether Bruce is able to inhibit autophagy during Drosophila oogenesis. We monitored the LTR staining in ovaries of $Bruc_e^{E81}$ flies, which have a deletion in the Baculoviral IAP Repeat (BIR) domain that binds to caspases. In the presence of a nutrient rich food source, we observed an increase in punctate LTR staining in region 2 of the germarium in $Bruc_e^{E81}$ flies compared to controls ($Bruc_e^{E81}/TM3$) (Figure 2.6A and Table 2.3). Similarly, we observed numerous degenerating stage 8 egg chambers with increased levels of punctate LTR staining in
Bruce*E81 flies, resembling overexpression of Dcp-1 (Figure 2.6B and Table 2.4). In well-fed conditions, we observed no degenerating stage 8 egg chamber in control Bruce*E81/TM3 flies (n=187 ovarioles; Table 2.4). Punctate LTR staining was similarly observed in region 2 germaria (Table 2.3) and degenerating stage 8 egg chambers (data not shown) in well-fed Bruce*E16 flies which have a 10kb deletion in the 3’ end of the Bruce gene sequence.49 Our results demonstrate that Bruce is normally required to inhibit autophagy under nutrient rich conditions.

2.3.5 Dcp-1 and Bruce mutants have altered TUNEL staining in germaria and degenerating mid-stage egg chambers.

Our previous work showed that nutrient deprived Dcp-1 mutants (Dcp-1 Prev) have defects in mid-oogenesis germline cell death.40 To determine whether Dcp-1 is also required for germline cell death in region 2 within the gerarium, we employed the TUNEL assay to detect levels of DNA fragmentation as an indication of cell death. We found that nutrient deprived Dcp-1 mutants had decreased levels of TUNEL positive cells in region 2 within the gerarium compared to nutrient deprived wild-type flies (Figure 2.7A and Table 2.5), indicating that Dcp-1 is also required for gerarium stage cell death.

We also investigated the role of Bruce in cell death during oogenesis. In well-fed Bruce*E81 flies, we observed a degenerating ovary phenotype which has been shown previously in ovaries with partial loss of another IAP protein, DIAP1.50 This ovary phenotype may be a consequence of excess cell death. Consistent with this possibility, we observed an increased number of cells with TUNEL positive staining in region 2 within the gerarium compared to controls (Figure 2.7A and Table 2.5). Similar results were
obtained with Bruce\textsuperscript{E16} flies (Table 2.5). Numerous TUNEL positive dots were also observed in degenerating stage 8 egg chambers of Bruce\textsuperscript{E81} well-fed flies (Figure 2.7B). These findings demonstrate that Bruce acts as an inhibitor of cell death in germaria and mid-stage egg chambers.

2.3.6 Autophagy contributes to cell death in nutrient-deprived ovaries.

To assess the role of autophagy that we observed during the germarium and mid-oogenesis stages, we employed the TUNEL assay to detect DNA fragmentation in two \textit{Atg} gene mutants. Most \textit{Atg} gene knockouts in \textit{Drosophila} result in pupal or larval lethality, thus we first analyzed the fully viable \textit{Atg7} mutant flies\textsuperscript{22, 25}. We found that nutrient deprived \textit{Atg7} mutants had reduced levels of TUNEL positive cells in region 2 within the germarium compared to control flies (Table 2.5). Further, degenerating stage 8 egg chambers in starved \textit{Atg7} mutants showed low or no TUNEL positive staining compared to controls (Figure 2.8A and B and Table 2.6). However, nuclear DNA condensation was still observed in the degenerating stage 8 egg chambers of starved \textit{Atg7} mutants (Figure 2.8B). To further investigate the role of autophagy in starvation-induced germline cell death, we generated \textit{Atg1} germline clones (GLCs), since mutations in \textit{Atg1} result in lethality at the pupal stage of development\textsuperscript{22, 24}. Consistent with our \textit{Atg7} mutant observations, nutrient deprived \textit{Atg1} GLC ovaries had decreased levels of TUNEL staining in both germaria and degenerating stage 8 egg chambers, indicating a suppression of DNA fragmentation (Figure 2.8C and D and Table 2.5 and 2.6). Also consistent with \textit{Atg7}, we observed nuclear DNA condensation in the \textit{Atg1} GLC degenerating stage 8 egg chambers (Figure 2.8D). Our results show that lack of autophagy results in a reduction of DNA fragmentation following starvation-induced cell death.
death in the germaria and mid-stage egg chambers, suggesting that autophagy contributes to the cell death process at these stages.

2.4 Discussion

Key outstanding questions that need to be addressed are how autophagy and apoptosis pathways interact with each other, and whether common regulatory mechanisms exist between these two processes. I have shown here that six known cell death genes and the Ras/Raf/MAPK signaling pathway not only function in apoptosis but also act to regulate autophagy in *Drosophila* l(2)mbn cells. I cannot rule out the possibility that additional cell death genes that we screened may also function in autophagy but were not detected in our assay due to insufficient knockdown by RNAi, long half-life of the corresponding proteins, and/or functional redundancy.

Consistent with our *in vitro* data, the involvement of Hid in autophagy regulation has been demonstrated in *Drosophila*. Overexpression of Hid induced autophagy in the fat body, larval epidermis, midgut, salivary gland, Malpighian tubules, and trachea epithelium. Further, expression of the constitutively active Ras form (RasV12), which has been shown to inhibit Hid activity in apoptosis, can also block Hid-induced autophagy. In *Drosophila* salivary glands, the Ras signaling pathway has also been shown to inhibit the autophagy process. Based on our loss-of-function findings and these previous gain-of-function studies, we speculate that the Ras/Raf/MAPK pathway acts upstream to inhibit Hid activity in autophagy.

Poor nutrition has a dramatic effect on egg production in *Drosophila*. Flies fed on a protein-deprived diet showed an increase in cell death in germaria and midstage egg chambers. These two stages have been proposed to serve as nutrient status checkpoints.
where defective egg chambers are removed prior to the investment of energy into them. The molecular mechanisms of germarium cell death are still largely unknown, and Daughterless, a helix-loop-helix transcription factor, was the only known regulator involved in cell death of germaria. Daughterless, a helix-loop-helix transcription factor, was the only known regulator involved in cell death of germaria.\textsuperscript{53} Nurse cell death during mid-oogenesis is also different from most developmental cell death in other \textit{Drosophila} tissues, since apoptotic regulators such as \textit{rpr}, \textit{hid} or \textit{grim} are not required for cell death in these cells.\textsuperscript{54} However, the activity of caspases, particularly Dcp-1, was shown to be required for mid-oogenesis cell death.\textsuperscript{40, 55} My findings implicate several additional genes, \textit{Dcp-1}, \textit{Bruce}, \textit{Atg7}, and \textit{Atg1}, in nutrient deprivation induced cell death in the germarium, and also during mid-oogenesis.

Other forms of cell death, such as autophagic cell death, have been proposed previously to be involved in the elimination of defective egg chambers during mid-oogenesis. Known signaling pathways including insulin and ecdysone pathways have been shown to be required not only for the survival of nurse cells in mid-oogenesis, but are known to also regulate the autophagy process, supporting the notion that autophagy plays a role in mid-oogenesis cell death.\textsuperscript{28, 30} Features of autophagy were observed during \textit{Drosophila virilis} mid-oogenesis cell death as shown by monodansylcadaverine staining and transmission electron microscopy.\textsuperscript{31} Our results using GFP-LC3 and LTG demonstrate that autophagy occurs in degenerating mid-stage egg chambers and also in germaria of nutrient deprived \textit{Drosophila melanogaster}. We found that mutation of \textit{Atg7} results in a significant decrease of autophagy in dying mid-stage egg chambers and in germaria of starved flies, further supporting the presence of autophagy during these stages.
The role of autophagy in cell survival or cell death is still not well resolved and likely to be context dependent. Our results show that autophagy contributes to the cell death process in the ovary. Loss of Atg7 or Atg1 activity in both dying mid-stage egg chambers and germaria leads to decreased TUNEL staining, indicating a reduction in DNA fragmentation. Consistent results were observed previously in the larval midguts of Atg7 mutants, which also showed an inhibition of DNA fragmentation. Interestingly, lack of autophagy function does not appear to affect nuclear DNA condensation in nurse cells. Nurse cells in degenerating stage 8 egg chambers of starved Atg7 mutants or Atg1 GLCs appeared to still have condensed nuclei as shown by DAPI staining (Figure 2.8 B and D). Thus, based on Atg7 and Atg1 mutant analyses, autophagy contributes to DNA fragmentation but not all aspects of nurse cell death. Future studies are required to determine how autophagy is connected to known pathways leading to DNA fragmentation and chromatin condensation during cell death.

The IAP family member Bruce was shown previously to repress cell death in the Drosophila eye. Bruce was also shown to protect against excessive nuclear condensation and degeneration, perhaps by limiting excessive caspase activity, during sperm differentiation. Other IAP family members have been shown to bind caspases via a BIR domain and inhibit apoptosis. The presence of a BIR domain in Bruce suggests that it may also have caspase-binding activity. We found that lack of Bruce function resulted in an increase in both LTR and TUNEL staining in germaria and degenerating mid-stage egg chambers. Thus, the Bruce mutant degenerating phenotype in ovaries suggests that Bruce might function normally to restrain or limit caspase activity in this tissue. Since we found that Dcp-1 and Bruce are both required for the regulation of
autophagy and DNA fragmentation in germaria and dying mid-stage egg chambers, it is possible that Bruce acts to bind and degrade Dcp-1 in nurse cells under nutrient rich conditions. Future studies employing epistasis and protein interaction analyses will be required to test this prediction. We cannot rule out the possibility that other IAP proteins, such as DIAP1, and other caspases also play a role during these stages. However, at least in response to starvation signals, Bruce and Dcp-1 play a non-redundant dual role in the regulation of autophagy and cell death in the ovary.

Numerous studies have linked caspase function to apoptosis, but recent findings indicate that caspases are also required for non-apoptotic processes including immunity and cell-fate determination.\textsuperscript{57, 58} We have shown here that Dcp-1 is also required for starvation-induced autophagy. In the ovary, it appears that both apoptotic and autophagic events occur in the germaria and mid-stage egg chambers following nutrient deprivation. It is possible that Dcp-1 coordinates autophagy and apoptosis at these two nutrient status checkpoints to ensure elimination of defective egg chambers in the most efficient manner possible. \textit{Dcp-1} mutants exhibit intact nuclei in stage 8 defective egg chambers, indicating a block in both DNA fragmentation and nuclear condensation, and further supporting a dual regulatory role for Dcp-1 in mid-oogenesis cell death. Dcp-1 might function to induce autophagosome formation while coordinately acting upon alternate proteolytic targets to complete execution of apoptosis. Future studies to elucidate upstream regulators and downstream substrates of Dcp-1 in cells undergoing autophagy or apoptosis will help to establish the regulatory mechanisms governing the crosstalk between these two cellular processes. Given the multiple cellular effects associated with
autophagy, our results also have important therapeutic implications for the use of modulators of caspase or IAP activity in the treatment of cancer and other diseases.
Figure 2.1 Quantification of starvation induced autophagy in *Drosophila l(2)mbn* cells.

A. Flow cytometry analysis of *l(2)mbn* cells starved for 2 hours (2hr S; blue) or 4 hours (4hr S; red) showed an increase in LysoTracker Green (LTG) fluorescence levels (x axis) compared to control cells in full nutrient medium (C; brown). The gate shown on the histogram represents the LTG\textsuperscript{high} population.

B. Representative images of GFP-LC3 puncta in fed control (C) *l(2)mbn* cells, 2hr starved (S) cells, and starved cells treated either with 3 methyladenine (3MA) or Bafilomycin A1 (Baf). Note the increased accumulation of GFP-LC3 puncta in Baf treated cells. Scale bar, 10\(\mu\)m.

C. Flow cytometry analysis of 4 hour starved cells were incubated with 3 methyladenine (4hr S+3MA; blue) or Bafilomycin A1 (4hr S+Baf; green). Both autophagy inhibitors reduced the LTG fluorescence levels compared to starved cells (4hr S; red) Control cells in nutrient full medium (C; brown) are represented by the brown line.

D. Both autophagy inhibitors, 3MA and Baf, reduced the LTG\textsuperscript{high} population significantly. (3MA; \(P=0.00001\) and Baf; \(P=0.00006\)).

E. RNAi of representative *Atg* genes decreased the LTR fluorescence levels compared to control. (*Atg1*; \(P=0.01\), *Atg5*; \(P=0.01\), *Atg7*; \(P=0.002\), *Atg8a*; \(P=0.008\), *Atg8b*; \(P=0.006\) and *Atg12*; \(P=0.02\)).

F. RNAi of all tested genes in the TOR/PI3K pathways had a statistically significant effect on LTG fluorescence levels. Known negative regulators of autophagy are shown with grey bars; positive regulators are shown with white bars. (*Pten*; \(P=0.007\), *Tsc1*; \(P=0.027\), *Tsc2*; \(P=0.025\), *RheB*; \(P=0.005\), *Tor*; \(P=0.016\), and *S6k*; \(P=0.012\)).

Results represent the mean value ± standard deviation (S.D.) from at least three independent experiments. dsRNA corresponding to a human gene (Hs) was employed as a negative control in (E) and (F).
Figure 2.2 Identification of known cell death related genes in autophagy regulation in l(2)mbn cells using RNAi.

A. The percentage of LTG\textsuperscript{high} cells was reduced by hid-RNAi (P=0.006) but not by rpr, grim and skl-RNAi.

B. Knockdown of Ras, phl and rl expression by RNAi resulted in an increase in the percentage of LTG\textsuperscript{high} cells. (Ras; P= 0.003, phl; P=0.001, and rl; P=0.028).

C. th-RNAi treatment (24 hours) had no significant effect on LTG levels; in contrast, RNAi of Bruce resulted in an increase in LTG fluorescence levels (P=0.01).

D. Reduction of debcl, Buffy or p53 expression by RNAi resulted in a decrease in LTG fluorescence levels. (debcl; P=0.018, Buffy; P=0.006, and p53; P=0.004).

E. RNAi of effector caspase Dcp-1 resulted in a significant decrease in the LTG\textsuperscript{high} population (P =0.001).

F. Representative images of GFP-LC3 puncta in cells treated with the indicated RNAi following 2 hours starvation treatment. Scale bar, 10\textmu m.

G. Quantification of cells with GFP-LC3 puncta following RNAi treatment. Cells with more than three GFP-LC3 punctate dots were considered GFP-LC3 positive cells. Cells treated with the RNAi indicated here all showed a significant difference (P < 0.05) in percentage of GFP-LC3 positive cells compared to the human (Hs) RNAi control. (Pten; P=0.006, Tor; P=0.034, Buffy; P=0.005, debcl; P=0.003, Bruce; P=0.003, Dcp-1; P=0.007, hid; P=0.002, Ras; P=0.006, and phl; P=0.050).

Results represent the mean value ± S.D. from three independent experiments.
Figure 2.3 Validation using 2nd set of dsRNAs

A. Independent dsRNAs corresponding to Hid and Ras pathway components produced consistent results compared to those shown in Figure 2.2A and B.

hid_2; P=0.005, Ras_2; P=0.007, phl_2; P=0.032, and rl_2; P=0.019. Results represent the mean value ± S.D. from three independent experiments.

B. Independent dsRNAs, non-overlapping with the first set of dsRNAs, yielded reproducible results compared to those shown in Figure 2.2 C-E. dcp-1_2; P=0.024, debcl_2; P=0.0001, Buffy_2; P=0.030, p53_2; P=0.002, and Bruce_2; P=0.038. Asterix indicate P value <0.05 compared to Hs- RNAi control. Results represent the mean value ± S.D. from three independent experiments.

C. mRNA levels of the representative genes were analyzed by QRT-PCR. RNAi knockdown ranged from 61% to 96%. The comparative CT method was used to calculate fold differences relative to non-starved cells. rp49 was employed as an endogenous control. S = Starvation.
Figure 2.3

A

% of cells with LTG high

Hs  hid_2  Ras_2  phl_2  rl_2

RNAi

* P < 0.05

B

% of cells with LTG high

Hs  Dcp-1_2  debcl_2  Buffy  p53_2  Bruce_2

RNAi

* P < 0.05

C

Fold expression

Dcp-1  Ice  debcl  Bruce  Ark  Buffy

Gene

Legend:
- 4hr S
- RNAi + 4hr S
- RNAi_2 + 4hr S
Figure 2.4 Nutrient deprivation induces autophagy at region 2 within the germarium and in dying mid-stage egg chambers.

A. GFP-LC3 proteins were expressed in nurse cells (NC) but not in follicle cells (FC) by using the UASp/nanos Gal4 system. DAPI staining of nuclei is shown in blue. Scale bar, 20 μm.

B. UASp-GFP-LC3; nanos GAL4 flies were conditioned on yeast paste and had a diffuse GFP-LC3 pattern. Numerous GFP-LC3 puncta (green) at region 2 within germarium were observed in nutrient deprived flies. Ovaries were stained with LysoTracker Red (LTR) in w^{1118} flies. Germarium of nutrient deprived w^{1118} flies had an increase in punctate LTR staining (red) compared to well-fed germarium. Scale bar, 20 μm.

C. Degenerating stage 8 egg chambers (arrows) had numerous GFP-LC3 puncta (green) and an increase in LTR positive dots (red) compared to healthy egg chambers (arrowheads). DAPI (white) staining of nuclei is shown in the two panels on the right. Scale bar, 50 μm.

D. Degenerating stage 8 egg chambers (arrows) of nutrient deprived Atg7 mutants (Atg7^{d177}/Atg7^{d114}) showed a dramatic decrease in LTR staining. Scale bar, 50 μm. DAPI staining of nuclei is shown in white.

At least seven different animals from each strain were examined for each condition.
Figure 2.5 The effector caspase Dcp-1 is not only required for nutrient starvation induced autophagy but also is sufficient for the induction of autophagy during *Drosophila* oogenesis.

A. Germaria of the nutrient deprived *Dcp-1* \(^{Prev}\) files showed a dramatic decrease in LTR staining compared to nutrient deprived wild type flies shown in Fig. 2.4 B. Scale bar, 20 μm.

B. Degenerating stage 8 egg chambers (arrows) of nutrient deprived *Dcp-1* \(^{Prev}\) files showed a dramatic decrease in LTR staining compared to nutrient deprived wild type flies shown in Fig. 2.4 C. Scale bar, 50 μm.

C. Lack of Dcp-1 function (*UASp*-GFP-LC3 *Dcp-1* \(^{Prev}\) /*Dcp-1* \(^{Prev}\); nanos-*GAL4*/+) resulted in uniform diffuse staining of GFP-LC3, rather than the punctate pattern observed in wild type degenerating stage 8 egg chambers shown in Fig 2.4 C. Scale bar, 50 μm.

D. Dying egg chambers (arrows) of *NGT*/+; **nanos-GAL4/UASp-fl-Dcp-1* flies that were conditioned on yeast paste showed a significant increase in punctate LTR staining (red) compared to healthy egg chambers (arrowheads). Scale bar, 50 μm.

E. Expression of activated Dcp-1 (a truncated form) and GFP-LC3 in the germline (*UASp*-GFP-LC3/+; **nanos-GAL4/nanos-GAL4** *UASp-tDcp-1*) resulted in abundant degenerating stage 8 egg chambers (arrows) with numerous GFP-LC3 puncta (green). Scale bar, 50 μm.

DAPI staining of nuclei is shown in white. At least seven different animals from each strain were examined for each condition.
Figure 2.5

A. Germarium

B. Stage 8

C. Dcp-1

D. nanos-GAL4/UAS-fl-Dcp-1

E. UASp-GFP-LC3; nanos-GAL4/UASp-tDcp-1
Figure 2.6 Bruce suppresses autophagy at region 2 within germarium and in dying stage 8 egg chambers.

A. The germarium in well-fed Bruce$^{E81}$ flies showed an increase in LTR staining (red) compared to wild type well-fed flies shown in Fig. 2.4 B (upper right). DAPI staining (white) of nuclei is shown on the right. Scale bar, 20 μm.

B. In well-fed wild type flies, mid-oogenesis nurse cell death is a rare event. Lack of Bruce function resulted in an increase in dying stage 8 egg chambers (arrows) in ovaries under well fed condition and these degenerating stage 8 egg chambers had numerous LTR (red) punctate dots. DAPI staining (white) is shown on the right. Scale bar, 50 μm.

At least seven different animals were examined.
Figure 2.7 Dcp-1 is required for nutrient starvation induced germarium cell death and IAP protein Bruce inhibits germarium and mid-oogenesis cell death.

A. Ovaries were stained with TUNEL (green) to detect DNA fragmentation. Clusters of cysts with TUNEL staining were observed in region 2 in nutrient deprived w¹¹¹⁸ files. In Dcp-1 Prev flies, fewer TUNEL positive cysts in region 2 were observed. Under well-fed condition, numerous TUNEL positive cysts were observed in Bruce⁸⁻⁸ flies. DAPI staining of nuclei is shown in white. Scale bar, 20 μm.

B. Numerous degenerating stage 8 egg chambers (arrows) with TUNEL positive staining (green) were observed in well fed Bruce⁸⁻⁸ flies. DAPI staining of nuclei (white) is shown on the right. Scale bar, 50 μm.

At least seven different animals from each strain were examined for each condition.
Figure 2.7

A

\[ \begin{array}{ccc}
\text{W}^{\text{prev}} & \text{Dcp-1} & \text{E81} \\
\text{Starved} & \text{Starved} & \text{Fed} \\
\end{array} \]

B

\[ \begin{array}{cc}
\text{Fed} \\
\end{array} \]
Figure 2.8 Lack of *Atg7* or *Atg1* function reduces DNA fragmentation during mid-oogenesis cell death.

A. TUNEL positive staining was observed in dying stage 8 egg chambers (arrows) of starved control flies (*CG5335\textsuperscript{d30}/Atg\textsuperscript{7d14}*). DAPI staining of nuclei (white) is shown on the right.

B. In nutrient deprived *Atg7* mutants (*Atg\textsuperscript{7d77}/Atg\textsuperscript{7d14}*) degenerating stage 8 egg chambers (arrows) showed no or low levels of TUNEL staining. Nuclear DNA condensation, detected by DAPI, was still observed.

C. Dying stage 8 egg chambers from nutrient deprived control siblings (*Atg1 Δ\textsuperscript{3D}/TM3*) generated from the same cross in D had abundant TUNEL positive staining.

D. In nutrient deprived *Atg1* GLCs, degenerating stage 8 egg chambers (arrows) showed no or low levels of TUNEL staining. Nuclear DNA condensation (DAPI, right) in degenerating egg chambers appeared to occur as in the controls.

Scale bar, 50μm. At least seven different animals from each strain were examined.
Figure 2.8

A

\( d_{20} \)

\( d_{14} \)

CG5335 / Atg7

B

\( d_{77}/d_{14} \)

Atg7

TUNEL

DAPI

C

Atg1/TM3

TUNEL

DAPI

D

Atg1

TUNEL

DAPI
Table 2.1 Primer sequences for the preparation of dsRNAs

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RNAi of 11 *DmAtg* genes showed a significant reduction in LTG high cells (P<0.05), indicating that these *DmAtg* genes are required for autophagy in l(2)mbn cells.

Table 2.2 Comparison of essential autophagy genes in the *Drosophila* larval fat body and l(2)mbn cells

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<td>Yes</td>
</tr>
<tr>
<td></td>
<td>CG12334</td>
<td><em>DmAtg8b</em></td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td><em>Atg9</em></td>
<td>CG3615</td>
<td><em>DmAtg9</em></td>
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<td></td>
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<tr>
<td><em>Atg12</em></td>
<td>CG10861</td>
<td><em>DmAtg12</em></td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td><em>Atg18</em></td>
<td>CG7986</td>
<td><em>DmAtg18</em></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>CG8678</td>
<td></td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

*a* The fat body data was generated from a study by Scott et al.\(^{22}\)
Table 2.3 Quantification of autophagy in region 2 germaria

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Nutritional Status</th>
<th>LTR positive</th>
<th>Number</th>
<th>% of Autophagy</th>
</tr>
</thead>
<tbody>
<tr>
<td>w^{1118}</td>
<td>Fed</td>
<td>8</td>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td>w^{1118}</td>
<td>Nutrient deprivation</td>
<td>25</td>
<td>36</td>
<td>69</td>
</tr>
<tr>
<td>Dcp-1 _^{Prev}</td>
<td>Nutrient deprivation</td>
<td>17</td>
<td>53</td>
<td>32</td>
</tr>
<tr>
<td>Bruce _^{E81}</td>
<td>Fed</td>
<td>67</td>
<td>116</td>
<td>58</td>
</tr>
<tr>
<td>Bruce _^{E81/TM3}</td>
<td>Fed</td>
<td>18</td>
<td>55</td>
<td>33</td>
</tr>
<tr>
<td>Bruce _^{E16}</td>
<td>Fed</td>
<td>35</td>
<td>63</td>
<td>56</td>
</tr>
<tr>
<td>Bruce _^{E16/TM3}</td>
<td>Fed</td>
<td>13</td>
<td>71</td>
<td>18</td>
</tr>
<tr>
<td>Atg7^{d17}/Atg7^{d14}</td>
<td>Nutrient deprivation</td>
<td>14</td>
<td>65</td>
<td>22</td>
</tr>
<tr>
<td>CG533S^{d20}/Atg7^{d14}</td>
<td>Nutrient deprivation</td>
<td>37</td>
<td>68</td>
<td>54</td>
</tr>
</tbody>
</table>

Numbers in the fourth column refer to the numbers of individual germarium scored in at least seven different animals.
Table 2.4 Quantification of autophagy in stage 8 degenerating egg chambers

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Nutritional Status</th>
<th>LTR positive</th>
<th>Number</th>
<th>% of Autophagy</th>
</tr>
</thead>
<tbody>
<tr>
<td>( w^{1118} )</td>
<td>Nutrient deprivation</td>
<td>29</td>
<td>40</td>
<td>73</td>
</tr>
<tr>
<td>( Dcp-1^{prev} )</td>
<td>Nutrient deprivation</td>
<td>8</td>
<td>54</td>
<td>15</td>
</tr>
<tr>
<td>( Atg7^{d77}/Atg7^{d14} )</td>
<td>Nutrient deprivation</td>
<td>13</td>
<td>51</td>
<td>25</td>
</tr>
<tr>
<td>( CG5335^{d30}/Atg7^{d14} )</td>
<td>Nutrient deprivation</td>
<td>7</td>
<td>*14</td>
<td>50</td>
</tr>
<tr>
<td>( nanos-GAL4/UASp-fl-Dcp-1 )</td>
<td>Fed</td>
<td>62</td>
<td>74</td>
<td>84</td>
</tr>
<tr>
<td>( Bruce^{E81} )</td>
<td>Fed</td>
<td>43</td>
<td>52</td>
<td>83</td>
</tr>
<tr>
<td>( Bruce^{E81}/TM3 )</td>
<td>Fed</td>
<td>0</td>
<td>**0</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Number in column 4 refers to the number of individual individual degenerating stage 8 egg chambers scored in at least 7 different animals.

* n=4 animals scored for this genotype; ** No degenerating stage 8 egg chambers detected
Table 2.5 Quantification of cell death in region 2 germaria

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Nutritional Status</th>
<th>TUNEL positive number</th>
<th>% of TUNEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>w1118</td>
<td>Fed</td>
<td>9</td>
<td>50</td>
</tr>
<tr>
<td>w1118</td>
<td>Nutrient deprivation</td>
<td>34</td>
<td>51</td>
</tr>
<tr>
<td>Dcp-1 &lt;sup&gt;Prev&lt;/sup&gt;</td>
<td>Nutrient deprivation</td>
<td>22</td>
<td>69</td>
</tr>
<tr>
<td>Bruce &lt;sup&gt;E81&lt;/sup&gt;</td>
<td>Fed</td>
<td>26</td>
<td>70</td>
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<tr>
<td>Bruce &lt;sup&gt;E81/TM3&lt;/sup&gt;</td>
<td>Fed</td>
<td>12</td>
<td>69</td>
</tr>
<tr>
<td>Bruce &lt;sup&gt;E16&lt;/sup&gt;</td>
<td>Fed</td>
<td>20</td>
<td>54</td>
</tr>
<tr>
<td>Bruce &lt;sup&gt;E16/TM3&lt;/sup&gt;</td>
<td>Fed</td>
<td>14</td>
<td>80</td>
</tr>
<tr>
<td>Atg7&lt;sup&gt;d17&lt;/sup&gt;/Atg7&lt;sup&gt;d14&lt;/sup&gt;</td>
<td>Nutrient deprivation</td>
<td>21</td>
<td>77</td>
</tr>
<tr>
<td>CG5335&lt;sup&gt;d30&lt;/sup&gt;/Atg7&lt;sup&gt;d14&lt;/sup&gt;</td>
<td>Nutrient deprivation</td>
<td>95</td>
<td>202</td>
</tr>
<tr>
<td>Atg1 GLC</td>
<td>Nutrient deprivation</td>
<td>22</td>
<td>77</td>
</tr>
<tr>
<td>Atg1&lt;sup&gt;A3D&lt;/sup&gt;/TM3</td>
<td>Nutrient deprivation</td>
<td>53</td>
<td>64</td>
</tr>
</tbody>
</table>

Number in column 4 refers to the number of individual germarium scored in at least 7 different animals.
Table 2.6 Quantification of cell death in stage 8 degenerating egg chambers

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Nutritional Status</th>
<th>TUNEL positive</th>
<th>number</th>
<th>% of TUNEL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Atg7</em>&lt;sup&gt;d77&lt;/sup&gt;/<em>Atg7</em>&lt;sup&gt;d14&lt;/sup&gt;</td>
<td>Nutrient deprivation</td>
<td>17</td>
<td>69</td>
<td>25</td>
</tr>
<tr>
<td><em>CG5335</em>&lt;sup&gt;d30&lt;/sup&gt;/<em>Atg7</em>&lt;sup&gt;d14&lt;/sup&gt;</td>
<td>Nutrient deprivation</td>
<td>39</td>
<td>62</td>
<td>63</td>
</tr>
<tr>
<td><em>Atg1</em> GLC</td>
<td>Nutrient deprivation</td>
<td>3</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td><em>Atg1</em>&lt;sup&gt;Δ30&lt;/sup&gt;/TM3</td>
<td>Nutrient deprivation</td>
<td>19</td>
<td>37</td>
<td>51</td>
</tr>
</tbody>
</table>

Number in column 4 refers to the number of individual degenerating stage 8 egg chambers scored in at least 7 different animals.
2.5 References


Chapter 3 The effector caspase Dcp-1 catalytically regulates starvation-induced autophagy

3.1 Introduction

Autophagy is a lysosomal-mediated bulk degradation process that functions to provide the building blocks for ATP production or protein synthesis for cell survival in response to starvation. In addition, autophagy functions to eliminate damaged organelles such as mitochondria\(^1\) or aggregated proteins.\(^2-4\) Autophagy-related genes (\(Atg\) genes) are involved in the molecular machinery of autophagy, including autophagy induction, nucleation, membrane expansion and membrane retrieval, and many of the yeast \(Atg\) genes have orthologues in mammals and other higher eukaryotes.\(^5\) Model organism studies have provided evidence that autophagy is associated with several human diseases, including cancer, neurodegeneration, pathogenic infection and aging.

Recent findings show that autophagy not only plays a fundamental role in cell survival in response to starvation but also has a role in cell death in response to various stimuli in \(Drosophila\ melanogaster\). Autophagy is induced in response to starvation in the larval fat body to promote cell survival. \(Atg\) mutants were shown to have a shorter life span compared to wild type animals when they were under starvation.\(^6,7\) Mutations in \(Atg\) genes were shown to cause cell death defects, including incomplete degradation of larval salivary glands, reduced DNA fragmentation in larval midgut and mid-stage

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A version of this chapter will be submitted for publication. Hou YC, Moradian A, Morin G, Gorski SM. 2009. Effector caspase Dcp-1 catalytically regulates starvation-induced autophagy.
egg chambers, and suppression of amnioserosa cell death, suggesting autophagy can function as a cell death effector.\(^7\)\(^-\)\(^10\) One possibility that explains the mechanistic role of autophagy in cell death is that autophagy facilitates the degradation of specific cell survival factors. Such an example exists in mammalian cells where it was shown that autophagy assists in the degradation of the reactive oxygen species (ROS) scavenger catalase and triggers cell death.\(^11\) Cell death caused by autophagy mediated degradation of survival promoting proteins has not been demonstrated in \textit{Drosophila melanogaster}. An alternative explanation for the autophagy-related death promoting effects is that high levels of autophagy may deplete cellular components and organelles leading to a metabolic crisis and cell death. For example, overexpression of \textit{Drosophila melanogaster} Atg1 was shown to lead to reduced cell growth and caspase-dependent apoptotic death in the larval fat body.

We previously demonstrated that six cell death genes, \textit{Dcp-1}, \textit{hid}, \textit{Bruce}, \textit{Buffy}, \textit{debcl}, and \textit{p53} as well as \textit{Ras/Raf/MAPK} signaling pathway components have a role in autophagy regulation in \textit{Drosophila} cultured cells. We showed that \textit{Dcp-1} is required for cell death in germaria, and is also necessary for starvation-induced autophagy in both germaria and mid-stage egg chambers. Further, overexpression of Dcp-1 was sufficient to induce autophagy at these two stages even under well fed conditions. Loss-of-function mutations in \textit{Bruce} resulted in ectopic autophagy and cell death in both stages, regardless of nutrient status, indicating that Bruce acts normally to suppress both autophagy and cell death during \textit{Drosophila} oogenesis.\(^9\) Bruce is one of the inhibitor of apoptosis (IAP) family members, and IAPs are classified by the presence of baculoviral-IAP-repeat (BIR) domains that mediate protein-protein interactions and block the access of the catalytic
residue cysteine of caspases to their substrates. Thus, the presence of a BIR domain in Bruce suggests that Bruce may suppress Dcp-1 activity and epistasis analyses will be required to prove or disprove this hypothesis.

Dcp-1 is one of four effector caspases in *Drosophila melanogaster* and its role in apoptosis has been described. During *Drosophila melanogaster* development, apoptotic cell death is mostly mediated by the apical caspase Dronc that cleaves and activates downstream effector caspases Dcp-1 and drICE. Dcp-1 and drICE share high sequence similarity with each other and both of them share sequence similarity with mammalian effector caspase-3. However, genetic studies in flies indicate differences between the roles of the two prominent effector caspases Dcp-1 and drICE in apoptosis. Flies carrying mutations in *drICE* are pupal lethal and have reduced cell death in the embryonic nervous system, pupal retina, adult wing, and in response to stresses, including irradiation and the inhibition of protein synthesis. In contrast, null mutants of *Dcp-1* are viable and only show defects in starvation-induced cell death in germaria and mid-stage egg chambers. Double mutants of *drICE* and *Dcp-1* show more severe phenotypes in several *Drosophila melanogaster* tissues compared to those in flies lacking only drICE, indicating that Dcp-1 probably plays a partly redundant role with drICE and/or is able to compensate for its depletion. Biochemically, drICE and Dcp-1 have slightly different enzymatic specificities, since only Dcp-1 but not drICE was shown to be able to cleave human lamins. In addition, Dcp-1 can cleave itself or drICE *in vitro* but drICE could not cleave itself. These findings suggest that the mechanistic roles of Dcp-1 and drICE in developmental cell death might be different, and these two prominent
effector caspases may have at least some distinct enzymatic properties despite the fact that they share high sequence similarity with each other.

Numerous studies have focused on characterizing the mechanistic roles of caspases in apoptosis but the upstream regulatory pathways and substrates of caspases in non-apoptotic processes have been less well studied. Here I investigate the regulation of Dcp-1 and identify its candidate substrates during the process of autophagy. Since the presence of a BIR domain in Bruce suggests that Bruce may be a negative regulator of Dcp-1 mediated autophagy, I created Dcp-1<sup>prev</sup>; Bruce<sup>E81</sup> double mutants to examine the genetic relationship between Bruce and Dcp-1. In addition, I determined the roles of Bruce and Dcp-1 in starvation-induced autophagy in the larval fat body and midgut where the function of autophagy is to promote survival instead of death. I also examined the activation status of Dcp-1 and drICE, and requirement of Dcp-1 or drICE mediated proteolytic events in cells undergoing starvation-induced autophagy. Finally, to gain a better understanding of the molecular mechanisms of Dcp-1 in the autophagy process, I performed an immuno-affinity purification (IP) and tandem mass spectrometry (MS/MS) fragmentation based assay to identify potential substrates of Dcp-1 in autophagy inducing conditions.

3.2 Materials and methods

3.2.1 Cell culture and transfection

*Drosophila l(2)mbn* cells (provided by A. Dorn) were maintained in Schneider’s *Drosophila* medium (Invitrogen) supplemented with 10% FBS (nutrient full medium) in 25-cm<sup>2</sup> suspension flasks (Sarstedt) at 25°C. All experiments were performed 3 days
after passage, and cells were discarded after 25 passages. For cell transfection, 3μg of plasmid DNA and 18μL of Cellfectin (Invitrogen) were combined in 200μL Grace serum-free medium (Invitrogen), incubated at room temperature for 30 minutes and then added to 3x10^6 cells in 800μL Grace medium. Cells were incubated overnight (16 hours) at 25°C in one well of a 24 well suspension plate (Sarstedt). After incubation, cells were split and 2mL of 10% FBS Schneider’s medium were added.

3.2.2 Immunofluorescence

Transient-transfected cells were resuspended and 100μL of the suspension was added to each well of an 8-well CC2 coated chamber slide (Nunc) and incubated for an hour at room temperature. Nutrient-full medium was replaced with 2mg/mL glucose/PBS for 2 hours. Cells were fixed with 4% paraformaldehyde for 30 minutes, washed with PBS three times, permeablized with 0.2% Triton X-100 for 5 minutes and blocked with 1% BSA for 30 minutes. Anti-V5 (1:1000, Sigma) and Anti-LC3 (1:100, NanoTools) primary antibodies were incubated overnight at 4°C. Anti-mouse IgG conjugated to Alexa 488 (1:1000, Invitrogen) and anti-rabbit IgG conjugated to Cy3 (1:1000, Jackson Laboratories) secondary antibodies were incubated for 1 hour at room temperature. After immunostaining, cells were mounted with Slowfade Gold with DAPI (Invitrogen). Images were obtained using a 40X objective on a microscope (Axioplan2, Carl Zeiss, Inc.) and captured with a cooled mono 12-bit camera (QImaging) and Northern Eclipse image analysis software (Empix Imaging, Inc.). GFP-LC3 punctate dots were counted in cells expressing plasmids which were identified by positive V5 immuno-staining. A minimum of 50 cells per sample was counted for triplicate samples per condition.
3.2.3 LysoTracker Red (LTR) and DAPI staining

For larval fat body and midgut LTR analyses, approximately twenty second instar larvae 48 hr after hatching were transferred to a cornmeal/dextrose fly food agar plate supplemented with yeast paste. 24 hr later, larvae (fed) were immediately dissected or larvae (starved) were placed on a plate containing 20% sucrose for 1-4 hours prior to dissection.

For LTR staining in ovaries, flies were conditioned on yeast paste for 2 days (fed) or placed in a dry vial with access to a 10% sucrose solution for 4-5 days. Tissues, including the larval fat body, midgut, and ovary, were dissected in PBS and immediately transferred into PBS containing 0.8μM LTR (Invitrogen) for 2-5 minutes at room temperature in the dark. Tissues were then stained with 0.1mg/mL DAPI for 30 seconds, washed three times with PBS, and mounted with SlowFade (Invitrogen) at room temperature. Images were obtained with a 20 or 40X objective (Carl Zeiss Inc.) on an Axioplan 2 microscope and captured with a cooled mono 12-bit camera and Northern Eclipse image analysis software.

3.2.4 Immunoprecipitation (IP) and MS/MS analysis

For large-scale IP experiments, 96mL of V5-Dcp-1C196A or V5-vector control transfected l(2)mbn cell culture were combined into two 50mL polypropylene tubes and centrifuged at 800 rpm for 10 minutes. Nutrient full medium was replaced with either new 10% FBS/Schneider medium (Fed) or 2mg/mL glucose/PBS for 2 hour starvation treatment. Cells were centrifuged at 800 rpm for 10 minutes and crosslinked with 0.25% paraformaldehyde at 25°C for 40 minutes. 1.25M glycine (final 0.125M) was added and incubated for 5 minutes at room temperature to stop the cross-linking reaction. Samples
were then centrifuged at 800 rpm for 10 minutes. 10mL of lysis buffer (20mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 1% NP-40, 10mM β-glycerophosphate, 2mM sodium orthovanadate, 1mM AEBSF, 10μg/mL pepstatin A, 10μg/mL Leupeptin and 10μg/mL aprotinin) was added to samples. Cells were disrupted by passing through a 21G syringe five times and lysates were incubated at 4°C with agitation for 30 minutes. Cell extracts were centrifuged at 20,000g for 30 minutes and supernatants were stored at -80°C overnight. Supernatants were incubated with 100μL of a 50% slurry of Sepharose 4B (Sigma) and agitated gently for 1 hour at 4°C. Sepharose 4B was removed by centrifugation and supernatants were incubated with 40μL of a 50% anti-V5 affinity agarose resin for three hours at 4°C. Anti-V5 resins were recovered by centrifugation and washed 5X with cold lysis buffer and 2X lysis buffer with 500mM NaCl. Bound proteins were eluted by re-suspension in 0.5M formic acid for 30 minutes at 4°C. Eluates were boiled for 20 minutes at 95°C to reverse the formaldehyde cross-links. Eluates were then vacuum dried, re-suspended in protein sample buffer (Invitrogen) and separated by SDS-PAGE using a 10% NuPAGE gel (Invitrogen) and 1X MES buffer. Protein bands were visualized with the colloidal coomassie stain and each lane was cut into 16 equal sections. Gel slices were transferred into a 96 well plate, reduced with 10mM dithiothreitol (DTT), S-alkylated with 100mM iodoacetamide and then subjected to in-gel trypsin digestion with 20μL of 20ng/μL trypsin per well overnight at 37°C. Peptide mixtures were subjected to LC-MS/MS analysis on a Finnigan LCQ (PTRL-West) or a 4000QTRAP (Applied Biosystems) ion trap mass spectrometer via reversed phase HPLC nano-electrospray ionization. All MS-MS spectra were queried against Drosophila Ensembl sequence databases using Mascot (Matrix Sciences, London, UK) or X!tandem
algorithms. An in-house web-based database, SpecterWeb (Sun M, Kuzyk M, and Morin GM, unpublished), was employed to process raw mass spectrometric protein identifications. Non-specifically binding proteins that were identified in the IPs from the negative control (V5-vector only) were subtracted from identified proteins in the IPs from cells transfected with V5-Dcp-1 \( \text{C}^{196\text{A}} \) using SpecterWeb for each condition (fed or 2 hr starvation).

3.2.5 Western Blot

*Drosophila l(2)mhn* cells were subjected to different time periods of starvation treatment (2mg/mL glucose/PBS), ranging from 30 minutes to 8 hours. Protein lysates were loaded onto a 10% NuPAGE SDS-PAGE gel (Invitrogen) and separated with 1X MES buffer (Invitrogen). Proteins were transferred to a PVDF or nitrocellulose membrane (Invitrogen) and processed according to the standard protocol of Li-COR system. Anti-Dcp-1 (1:1000)\(^{22}\) or anti-drICE (1:1000)\(^{22}\) with anti-Actin JLA20 (1:1000; DSHB) were incubated overnight at 4\(^{\circ}\)C. Anti-mouse IR800 (Rockland Immunochemicals) and anti-guinea pig IR700 (Rockland Immunochemicals) were incubated for 1 hour at room temperature. Membranes were imaged using an Odyssey Infrared imaging System (Li-COR Biosciences). Odyssey software v.3.1 was employed to quantify the integrated intensity of each band. Anti-Actin antibody was used for evaluating loading controls.

3.2.6 Computational analyses of protein sequences

Protein sequences, corresponding to identified peptides, were obtained in FASTA format from Flybase. A subset of high confidence candidates (unique peptides \( >2 \) and \( \log(E) \)
(score>=0.3; statistical>0.75) based on scores of ten known mammalian caspase substrates predicted by the CasPredictor program. Mammalian orthologues of identified *Drosophila* proteins were obtained using the InParanoid database.

3.3 Results

### 3.3.1 Effector caspase Dcp-1 genetically interacts with IAP protein Bruce and functions downstream of Bruce

IAP family members have been shown to inhibit caspase activity by binding via a baculoviral IAP repeat (BIR) domain, and the presence of a BIR domain in Bruce suggests that it may have caspase inhibitory activity. Since Bruce and Dcp-1 loss-of-function mutants have opposite phenotypes in the *Drosophila melanogaster* ovary, it is possible that they interact genetically with each other. To test this possibility, we created *Dcp-1* ^Pre^/*Dcp-1* ^Pre^; *Bruce* ^E81^/*Bruce* ^E81^ (*Dcp-1* ^Pre^; *Bruce* ^E81^) double mutants. In the *Dcp-1* ^Pre^; *Bruce* ^E81^ double mutants, the ovarian atrophy phenotype of *Bruce* ^E81^ loss-of-function mutants was rescued (Figure 3.1A). The size of the ovaries in the *Dcp-1* ^Pre^; *Bruce* ^E81^ double mutants was larger compared to those in the *Bruce* ^E81^ mutants (Figure 3.1A) suggesting that loss of *Dcp-1* function might be able to rescue the *Bruce* phenotype. To understand if the rescue of the ovarian atrophy phenotype is due to suppression of cell death and/or autophagy, we placed *Dcp-1* ^Pre^; *Bruce* ^E81^ double mutants under well fed and nutrient deprivation conditions. Degenerating mid-stage egg
chambers in the well-fed $Dcp-1^{Prev/Cyo}$, $Bruce^{E81}/Bruce^{E81}$ mutants had condensed nuclei (Figure 3.1 B), as expected for the $Bruce^{E81}$ mutant phenotype. In contrast, in the well-fed $Dcp-1^{Prev/Dcp-1^{Prev}}$; $Bruce^{E81}/Bruce^{E81}$ double mutants, we observed no degenerating mid-stage egg chambers. Degenerating mid-stage egg chambers in the starved $Dcp-1^{Prev/Dcp-1^{Prev}}$; $Bruce^{E81}/Bruce^{E81}$ double mutants had a persistent nurse cell nuclei phenotype which was reported previously in the starved $Dcp-1^{Prev}$ mutants (Figure 3.1C). In addition, starved $Dcp-1^{Prev}$; $Bruce^{E81}$ double mutants showed the $Dcp-1^{Prev}$ mutant phenotype of no or low LTR staining in degenerating stage 8 egg chambers (Figure 3.1D). These findings place $Dcp-1$ downstream of $Bruce$, suggesting that Bruce might suppress cell death and autophagy in the ovary through restraining $Dcp-1$ activity.

### 3.3.2 IAP protein Bruce delays starvation induced autophagic responses in the larval fat body and midgut

Previously, I showed that loss of Bruce protein is sufficient to trigger starvation-induced autophagy using LysoTracker Red (LTR) staining in both germaria and degenerating midstage egg chambers during $Drosophila melanogaster$ oogenesis regardless of the nutrient availability status. To further investigate whether Bruce regulates starvation-induced autophagy in other tissues in $Drosophila melanogaster$, I employed LTR staining in fed $Bruce$ loss-of-function mutants. The larval fat body serves as a nutrient source and undergoes autophagy to provide energy for developing imaginal tissues during metamorphosis and facilitates survival in response to starvation. Studies done by Scott et al. showed that the fat body from third instar fed larvae displayed diffuse or faint LTR staining. In contrast, the fat body from nutrient deprived
third instar larvae displayed intense, punctate LTR staining and reached a maximal level of LTR staining after 4 hr starvation.\textsuperscript{6} The fat body of fed 3\textsuperscript{rd} instar larvae from two Bruce deletion mutants (Bruce\textsuperscript{E81} and Bruce\textsuperscript{E16}) had faint LTR staining similar to control animals (Figure 3.2 A-C). However, the fat body from both Bruce mutants reached intense LTR punctate staining in each cell after just 1 hr starvation, in contrast to control larvae (w\textsuperscript{1118}) under the same conditions (Figure 3.2 D-F). These data suggest that loss of Bruce function accelerates the autophagic response to nutrient deprived conditions in the larval fat body. Further, I analysed the autophagic response in the midgut of third instar larvae. The larval midgut dissected from well fed control animals had low or no LTR staining (Figure 3.2 G). When control larvae were subjected to 4 hours starvation, there was intense LTR punctate staining in each cell of the third instar larval midgut, suggesting that the larval midgut also has a robust autophagic response to nutrient deprivation (Figure 3.2 H). Similar to the fat body, I found the midgut from Bruce mutants reached a maximal level of LTR staining after 1 hr starvation in contrast to control animals that did not reach a comparable level until starved for at least 3-4 hours (Figure 3.2 I-J). Unlike in the ovary, loss of Bruce function is not sufficient to induce autophagy in the larval fat body and midgut but its absence results in an accelerated LTR response to starvation in these larval tissues.

\textbf{3.3.3 The effector caspase Dcp-1 accelerates the starvation-induced autophagic response but is not required for autophagy in the larval fat body}

Next I investigated the role of the effector caspase Dcp-1 in starvation induced autophagy in the larval fat body. Under nutrient rich conditions, I observed low or no LTR staining in the fat body cells that expressed full length Dcp-1 under the control of a
fat body *GAL4* driver (*CG-GAL4*) (Figure 3.3 A). When *CG-GAL4/UAS-fl-Dcp-1* larvae were transferred to 1 hour starvation, I observed an intense level of LTR staining in the fat body cells in contrast to control animals which still had faint LTR staining (Figure 3.3 B). Similar to *Bruce* loss-of-function mutants, expression of Dcp-1 was not sufficient to induce an autophagic response but instead accelerated the autophagic response to nutrient withdrawal in the larval fat body. To determine whether Dcp-1 is required for starvation induced autophagy in the larval fat body, I employed LTR staining in *Dcp-1* loss-of-function mutants. After four hours of nutrient deprivation, I still observed an abundance of LTR puncta in the larval fat body of *Dcp-1Prev* mutants indicating that Dcp-1 is not required for starvation-induced autophagy in the larval fat body (Figure 3.3 C and D). Similar results were observed in the larval midgut, in that after four hours of starvation, the larval midgut of *Dcp-1Prev* mutants had numerous LTR puncta similar to wild type (Figure 3.3 E and F).

### 3.3.4 Starvation has a rapid effect on activation of Dcp-1 but not drICE.

Next, I wanted to examine whether starvation had differential effects on activation of two effector caspases, Dcp-1 and drICE. To investigate this question, I starved *Drosophila l(2)mbn* cells in amino acid deprived medium (glucose/PBS) for different time periods, ranging from 30 minutes to 16 hours. Previous studies of Rpr-mediated apoptosis showed that Dcp-1 was cleaved at the inter-domain activation cleavage site Asp33 and Asp215, and drICE was cleaved at Asp28 and Asp230. Immunoblot analysis of endogenous Dcp-1 using an anti-Dcp-1 antibody that specifically recognizes the completely processed p20 subunit and full-length Dcp-1 showed that the inter-domain cleavage of Dcp-1 occurred even under the fed condition in *l(2)mbn* cells. Following 30
minutes of nutrient deprivation, I observed increased levels of p20 subunits of Dcp-1 (3 fold increase in expression compared to fed condition). Levels of p20 subunits of Dcp-1 continued to increase after 1 hour of starvation (6 fold increase in expression), and showed no differential expression compared to fed condition after 2 to 16 hours of starvation. In contrast, after 16 hours of starvation, inter-domain cleavage of drICE was not detectable as shown in the immunoblot analysis with an anti-drICE antibody that has been shown to recognize both full length and p20 subunit of drICE (Figure 3.4). These results demonstrate that nutrient deprivation has a rapid effect on proteolysis of Dcp-1 but not drICE.

3.3.5 The catalytic activity of Dcp-1 but not drICE is required for the induction of starvation-induced autophagy

To determine whether Dcp-1-mediated proteolytic events are required for the induction of starvation induced autophagy, a catalytically inactive Dcp-1 construct harboring a mutation of Cys to Ala was employed (Dcp-1\textsuperscript{C196A}). A fusion of the wild-type Dcp-1 with a V5 tag was expressed in \textit{l(2)mbr} cells stably transfected with a marker for autophagy, mammalian LC3 (Atg8 homologue) fused to GFP protein, and showed an increase in GFP-LC3 puncta in starved cells compared to cells transfected with the vector control (Figure 3.5 A and B). In contrast, expression of catalytically inactive Dcp-1\textsuperscript{C196A} failed to show a relative increase in GFP-LC3 puncta under the same conditions, indicating Dcp-1-mediated proteolytic events are required to regulate autophagy (Figure 3.5 A and B). Further I tested whether drICE mediated proteolytic events are required for autophagy. Expression of wild-type drICE or catalytically inactive drICE\textsuperscript{C211A} did not show an increase in GFP-LC3 puncta compared to the negative control (vector only)
These results rule out the possibility that cellular stress due to expression of the effector caspase drICE results in the induction of autophagy. Instead, these findings indicate that Dcp-1 mediated proteolytic events are essential for the induction of autophagy, suggesting that Dcp-1 cleaved substrates might play a role in autophagy regulation.

### 3.3.6 Potential regulators or substrates of Dcp-1 in cells undergoing autophagy

To identify potential substrates of Dcp-1 in autophagy inducing conditions, we performed an immuno-affinity purification (IP) and tandem mass spectrometry (MS/MS) fragmentation based assay. A study done by Kamada et al. used the yeast two-hybrid system and a modified caspase construct as the bait. They introduced a point mutation into caspase 3 which substituted serine for the active site cysteine and prevented proteolytic cleavage of substrates. They successfully identified gelsolin, an anti-apoptotic gene, as a substrate of caspase-3. We employed a similar modified strategy by expressing the N-terminally V5 tagged Dcp-1C196A as an IP bait protein in Drosophila l(2)mbn cells to prevent cleavage of substrates. In addition, we used the cross-linker, formaldehyde, to strengthen the transient association of Dcp-1 with potential substrates or interaction partners and then removed non-specifically bound proteins by subjecting complexes to extensive washing. V5 tagged Dcp-1C196A under the control of the actin promoter or a V5 vector-only negative control was transfected into l(2)mbn cells and then subjected to full medium (fed) or 2 hours starvation. Cell lysates were immunoprecipitated with the anti-V5 antibody and immunoprecipitates were analyzed by LC-MS/MS for protein detection and identification. A subset of high confidence candidates (unique peptides >2 and log(E) <-15) from both fed and 2 hour starved
conditions, and that were not detected in the V5 vector-only control, was selected for analyses of caspase cleavage site prediction (Table 3.1). We chose the CaSPredictor program which combines a PEST-like index and position-dependent amino acid matrices for prediction of potential caspase cleavage sites. Sixteen out of our twenty-one candidates that met the selection threshold contain potential caspase cleavage sites. We used the Inparanoid eukaryotic ortholog database to search for mammalian orthologs of our twenty-one Drosophila melanogaster candidate proteins. We then searched these mammalian orthologs to determine whether they are known caspase substrates based on data from two recent studies and the CASBAH database. The mammalian orthologs of seven of our candidate proteins are known caspase substrates, further suggesting that the Drosophila melanogaster counterparts of these seven proteins may also be caspase substrates. Overall, our IP-MS studies provide preliminary data that can be used to build a working model to help understand the molecular mechanism of Dcp-1 in autophagy regulation.

3.4 Discussion

The genetic relationship between Dcp-1 and Bruce was unknown previously, and we addressed this question by generating Dcp-1 \textit{Prev}; Bruce \textit{E81} double mutants. Our findings showed that loss-of-function of Dcp-1 could rescue the ovary atrophy phenotype of Bruce in the fed double mutants. In the starved Dcp-1 \textit{Prev}; Bruce \textit{E81} double mutants, degenerating mid-stage egg chambers had persistent cell nuclei and faint LTR staining which are phenotypes of Dcp-1\textit{Prev} mutants. Thus, the rescue of the ovary atrophy phenotype in Dcp-1 \textit{Prev}; Bruce \textit{E81} double mutants appears to result from the suppression of both cell death and autophagy. Our data suggest that Bruce acts to restrain Dcp-1
activity and suppress Dcp-1 mediated-cell death and autophagy in the ovary. We cannot rule out the possibility that other IAP proteins, such as DIAP1, play a similar function and also limit Dcp-1 activity during cell death or autophagy. In addition, it is possible that Bruce might suppress other effector caspases, such as drICE, to aid in the prevention of cell death and autophagy in the ovary.

Caspases are a family of proteases that are known to mediate the execution of apoptosis by cleaving cellular substrates. In addition to apoptosis, caspases function in non-apoptotic processes, including immunity, cell fate determination and compensatory proliferation. The molecular mechanisms by which Dcp-1 regulates starvation-induced autophagy are still unclear. Local activation or compartmentalization of active caspases is one regulatory mechanism by which high levels of active caspases could regulate non-apoptotic functions without triggering apoptosis. For example, immuno-reactivity for caspase activity using caspase 3 antibody is detected only in a cytoskeletal membrane complex, termed the individualization complex (IC), during the non-apoptotic process of Drosophila melanogaster spermatid individualization. During Drosophila metamorphosis, the larval brain undergoes a massive elimination of dendrites and axons without triggering cell death of the entire tissue, perhaps through localizing active caspases to dendrites only. Therefore, it is possible that Dcp-1 is localized to specific cellular organelles (e.g., autophagosomes) during its role in autophagy. However, I think that this might not be the case, since Dcp-1 immuno-staining did not seem to localize to specific cellular organelles but instead appeared to be distributed throughout the cytosol. In addition, my results showed that the catalytic activity of Dcp-1 but not drICE is essential for starvation-induced autophagy. This
observation suggests that expression of effector caspases do not in general induce cellular stress which in turn triggers an autophagy response indiscriminately. Instead, our results suggest that Dcp-1 might recognize and cleave substrates which mediate autophagy regulation distinctively. Caspases recognize at least four contiguous amino acids (P4-P3-P2-P1) in their substrates, and cleave after P1 which is usually an Asp residue.\(^{34}\) Dcp-1 has substrate specificity that is similar to mammalian caspase 3 and CED-3, the effector caspase in \textit{C.elegans}.\(^{21}\) Using positional scanning peptide libraries, it was shown that Dcp-1 has an absolute requirement for Asp in P4, a strong preference for Glu in P3 and is tolerant of several substitutions in P2.\(^{21}\) Thus, Dcp-1 has an optimal recognition motif of DEVD, which is the same as other caspases including caspase 2, 3, and 7 and CED-3.\(^{21}\) A scanning peptide library study has not been performed on drICE but it also has a clear preference for Asp in P1.\(^{35}\) A study from Song et al. showed that only Dcp-1 but not drICE was able to cleave human lamins, indicating that the specificities of Dcp-1 and drICE are not identical.\(^{21}\) Based on our findings and these biochemical studies of substrate specificity, we speculate that the two prominent \textit{Drosophila} effector caspases, Dcp-1 and drICE, might recognize different sets of substrates mediating apoptosis and autophagy independently or co-ordinately.

Caspases are synthesized as single-chain zymogens and exist as homo-dimers constitutively. After cleavage in the inter-domain cleavage site, the catalytic activity of caspases is increased significantly.\(^{36}\) The inter-domain cleavage causes conformation changes which allow caspases to expose the catalytic cysteine and allow subsequent substrate binding. Our data showed that cleavage of endogenous Dcp-1 in the inter-domain site already occurred in nutrient full medium and cleaved p20 subunits of Dcp-1.
were increased following 30 minutes of starvation treatment. These data suggest that nutrient deprivation might facilitate the inter-domain cleavage of Dcp-1 and thus help the subsequent binding/cleaving of Dcp-1 substrates for the execution of autophagy. It is possible that the timing of Dcp-1 catalytic activation could determine the sensitivity thresholds of autophagic and apoptotic responses. Nutrient deprivation might enhance Dcp-1 catalytic activities which promotes autophagy for cell survival, giving the cells a chance to recover. Prolonged starvation might result in activation of drICE that triggers apoptosis.

The discrepancies of Bruce and Dcp-1 mediated autophagic responses between larval tissues (fat body and midgut) and the ovary remain to be resolved. In the ovary, loss-of-function of Bruce is sufficient to trigger autophagy and cell death, and loss-of-function of Dcp-1 suppresses both processes. However, loss-of-function of Bruce or ectopic expression of Dcp-1 resulted in an acceleration of the autophagic response but was not sufficient to trigger autophagy in the absence of nutrition deprivation in the larval fat body and midgut. In addition, we were unable to identify an essential role for Dcp-1 in starvation-induced autophagy in both larval tissues. I speculate that insulin/PI3K/Akt signaling may play a role in determining the differences of Bruce and Dcp-1 mediated autophagic responses in these tissues. The larval fat body and midgut are terminally differentiated endoreplicating tissues (ERTs) that provide nutrients for undifferentiated imaginal cells during the larval stage. Cells in the ERTs do not directly respond to changes in dietary protein but instead use secondary humoral signals, most likely insulins, to continue cell growth before cells are severely depleted of amino acids. Protein starvation causes loss of PI3K activity in ERTs. I observed a low expression of
Bruce in the fed larval fat body and an increase in Bruce expression following just one hour of starvation, suggesting that activity of Bruce is also modulated in response to starvation (Figure 3.7). On the other hand, Akt kinase, which is activated by PI3K was shown to be expressed at a relatively low level in nurse cells of mid-stage egg chambers.\textsuperscript{38} Cell death in mid-stage egg chambers and salivary gland cells shares many similarities, and autophagy is induced following down-regulation of PI3K expression in salivary glands.\textsuperscript{8, 39} Based on these previous results, clearly there are differences in PI3K/Akt levels between tissues such as the fat body, where autophagy is important for survival in response to starvation, and tissues such as salivary glands and the ovary where autophagy plays a role in the cell death process. Thus, the delay in response to loss of Bruce or ectopic Dcp-1 expression in the larval fat body and midgut might be due to the time required for the generation of secondary humoral signals and the loss of PI3K activity. However, the reason why we did not observe an essential role for Dcp-1 in starvation induced autophagy in the larval fat body and midgut remains an outstanding question. Similarly, overexpression of a pro-apoptotic gene \textit{Hid} has been shown to induce autophagy but is not essential for starvation induced autophagy in the fat body. It is possible that a redundant pathway (e.g., ecdysone) can compensate for autophagy regulation in the larval fat body of \textit{Dcp-1} and \textit{Hid} mutants.

Our IP-MS studies provide a preliminary set of potential substrates and interacting proteins of Dcp-1 which can be used to generate a working model for how Dcp-1 executes autophagy (Table 3.1 and Figure 3.6). The clathrin-uncoating ATPase Hsc70-4 not only is a TOR interacting protein but is also involved in autophagy and endocytosis in the larval fat body.\textsuperscript{40} Loss-of-function Hsc70-4 mutants have decreased
TOR, Akt and phospho-Akt levels.\textsuperscript{40} Based on the CasPredictor program,\textsuperscript{23} we found a potential caspase cleavage site in the Hsc70-4 protein sequence and its mammalian homologue, HSP7C, is a known caspase substrate.\textsuperscript{24, 26} Inhibition of Hsp90, the mammalian homologue of \textit{Drosophila melanogaster} Hsp83, suppresses the Akt/TOR pathway and induces autophagy in murine embryonic fibroblasts.\textsuperscript{41} We also found a potential caspase cleavage site in the \textit{Drosophila melanogaster} Hsp83 protein sequence. Therefore, we hypothesize that Dcp-1 might cleave Hsc70-4 and/or Hsp83 and result in decreased Akt/TOR activity that then triggers autophagy.

We identified one of the \textit{Drosophila} peroxiredoxin family members, Jafrac1, as a potential Dcp-1 substrate.\textsuperscript{42} Purified recombinant Jafrac1 proteins are shown to reduce H$_2$O$_2$, and expression of Jafrac1 increases cell survival following H$_2$O$_2$ treatment in \textit{Drosophila} S2 cells.\textsuperscript{42} Starvation has been shown to trigger production of reactive oxygen species (ROS) such as H$_2$O$_2$, and HsAtg4, the mammalian homologue of yeast Atg4, is a direct target for oxidation by H$_2$O$_2$.\textsuperscript{43} Oxidation of HsAtg4 leads to induction of the autophagy process.\textsuperscript{43} Thus, it is possible that Dcp-1 cleaves Jafrac1, resulting in increased levels of H$_2$O$_2$ that could oxidize DmAtg4 and trigger autophagy. Our data demonstrated that prolonged starvation leads to increased Dcp-1 activity which could result in much higher levels of H$_2$O$_2$ leading to cell death. This speculative model is consistent with a previous study that showed autophagy degrades the ROS scavenger catalase, resulting in cell death in mammalian cells.\textsuperscript{11} We generated a putative model of Dcp-1 mediated autophagy based on some of our IP-MS data and previous observations (Figure 3.6). The important next steps will be to validate our interaction data, verify if candidate proteins with potential caspase cleavage sites are Dcp-1 substrates using
biochemical assays, and determine if the identified substrates have a functional significance in mediating autophagy. However, we cannot rule out the possibility that these candidate proteins might also function in apoptosis. Future studies that elucidate the roles of the candidate proteins in autophagy and/or apoptosis will help to establish the regulatory mechanisms of Dcp-1 governing the crosstalk between these two processes.
Figure 3.1 Ovarian atrophy phenotype resulting from *Bruce* mutations is rescued by *Dcp-1*

(A) The ovaries of *Bruce<sup>E81</sup>* mutants are poorly developed and atrophied even under well-fed conditions (left). In contrast, the ovaries of *Dcp-1<sup>Prev</sup>/Dcp-1<sup>Prev</sup>; *Bruce<sup>E81</sup>/Bruce<sup>E81</sup>* (*Dcp-1<sup>Prev</sup>; *Bruce<sup>E81</sup>* ) double mutants (right) are better developed compared to those of *Bruce* single mutants. (B) In well-fed *Dcp-1<sup>Prev</sup>/CyO, Bruce<sup>E81</sup>/Bruce<sup>E81</sup>* flies, ovaries had numerous dying stage 8 egg chambers (diamond arrows). (C) In nutrient-deprived *Dcp-1<sup>Prev</sup>; *Bruce<sup>E81</sup>* double mutants, ovaries had degenerating mid stage egg chambers (arrows) with a phenotype similar to *Dcp-1<sup>Prev</sup>* mutants, consisting of the persistent nurse cell nuclei and disappearance of follicle cells. n=3 animals. Scale bar: 100μm. (D) Degenerating mid-stage egg chambers (arrow) in nutrient-deprived *Dcp-1<sup>Prev</sup>; *Bruce<sup>E81</sup>* mutants showed no or low LTR staining that is characteristic of the *Dcp-1<sup>Prev</sup>* mutant phenotype. n=45 degenerating mid-stage egg chambers scored in three different animals. Percentage of LTR positive mid-stage egg chambers is 15.6%. (E) DAPI staining of the same degenerating mid-stage egg chamber shown in (D) in nutrient-deprived *Dcp-1<sup>Prev</sup>; *Bruce<sup>E81</sup>* mutants. Scale bar: 50μm. DAPI staining of nuclei is shown in white.
Figure 3.2 Bruce postpones starvation-induced autophagy in the larval fat body and midgut.

(A-C) The larval fat body from fed control \textit{w}^{1118}, \textit{Bruce}^E81 and \textit{Bruce}^E16 mutants showed low levels of LTR (red) staining. (D-F) Following 1hr starvation, fat body cells from control \textit{w}^{1118} animals showed low levels of LTR (red) staining, whereas the fat body cells from two different loss-of-function \textit{Bruce} mutants displayed intense, punctate LTR staining. (G) The larval midgut dissected from fed control \textit{w}^{1118} animals displayed faint LTR (white) staining. (H) In contrast, the midgut from 4hr starved control animals displayed intense LTR dots (white) in all cells. (I-J) Following 1hr starvation, the midgut from control animals showed low LTR (red) staining, whereas the midgut from \textit{Bruce} mutants displayed numerous LTR (red) puncta. Scale bar: 100\mu m.

At least six different animals were examined for (A-F) and at least three different animals were examined for (G-J). DAPI staining of nuclei is shown in blue.
Figure 3.2 continued
Figure 3.3 Expression of Dcp-1 accelerates autophagy in response to nutrient withdrawal but is not essential for autophagy in the fat body and midgut

(A) Following 1hr starvation, fat body cells from control animals (CG-GAL4) display faint LTR (white) staining.  (B) In contrast, expression of full length Dcp-1 in the fat body (CG-GAL4/UAS-fl-Dcp-1) resulted in intense LTR staining following 1hr starvation. Scale bar: 100μm.  (C-D) Following 4hr starvation treatment, fat body cells from control w^{1118} and Dcp-I^{Prev} mutants both displayed abundant LTR puncta. Scale bar: 20 μm.  (E-F) Following 4hr starvation treatment, midgut cells from control w^{1118} and Dcp-I^{Prev} mutants displayed intense LTR staining. Scale bar: 100 μm.

At least ten animals from each strain were examined in each condition.
Figure 3.3
Figure 3.4 Time course analyses of Dcp-1 and drICE cleavage at the inter-domain site during nutrient deprivation.

*Drosophila l(2)mbn* cells were subjected to different time periods of starvation treatment, ranging from 30 minutes to 16 hours. Cont = Control cells not subjected to starvation. Immunoblot analyses using anti-Dcp-1 and anti-drICE antibodies that specifically recognize the full length and the processed p20 subunit of Dcp-1 and drICE showed an increase in processed p20 subunits of Dcp-1 following 30 minutes starvation treatment. Inter-domain cleavage of drICE was not detectable even after 16 hours of starvation treatment. Actin was used as a loading control. The blot shown is representative of three independent experiments.

f.l Dcp-1= full length Dcp-1; ΔDcp-1= prodomain removed Dcp-1;
Dcp-1_p20=processed p20 subunit of Dcp-1; fldrICE=full length drICE.
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Figure 3.5 The catalytic activity of Dcp-1 but not drICE is required for starvation-induced autophagy

(A) Representative images of GFP-LC3 staining in l(2)mbn-GFP-LC3 cells transfected with V5-Dcp-1 and V5-Dcp-1C196A following 2hr starvation treatment. Arrows denote representative cells expressing indicated plasmid. (B) Fluorescent microscopic quantification of GFP-LC3 puncta in l(2)mbn-GFP-LC3 cells transfected with indicated plasmid following 2hr starvation treatment. Results shown represent mean ± SEM for combined data from three independent experiments. At least fifty V5 positive cells were examined in each of three independent experiments for each condition. Scale bar: 20μm.
Figure 3.5

A

B

![Figure 3.5 image](image-url)
In this model, the substrates of effector Dcp-1 are involved in the regulation of starvation induced autophagy in Drosophila. Dcp-1 mediates the cleavage of Hsc70-4 and Hsp83 which results in the loss of Akt/TOR activity and the induction of autophagy. Dcp-1 mediated cleavage of Jafrac1, a ROS scavenger, might result in increased levels of H$_2$O$_2$ that leads to DmAtg4 oxidation and autophagy induction.
Figure 3.7 Bruce expression increases in response to nutrient withdrawal

The larval fat body from a wild type (w1118) animal grown under well fed (top) or 1hr starvation (bottom) conditions was stained with anti-Bruce antibody. An increase in Bruce expression is evident following 1 hour starvation compared to the well fed condition. Scale bar=100μm. At least six animals were examined for each condition. DAPI staining of nuclei is shown in blue.
Figure 3.7

[Image of immunofluorescence images comparing fed vs. 1 hour starved conditions using α-Bruce and DAPI stains]
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Gene symbols and GO molecular functions are from Flybase. Predictions of caspase cleavage sites of identified proteins were determined by the CaSPredictor program and are listed in column 2. Mammalian orthologs of identified proteins are listed in column 4. An asterisk adjacent to the UniProt identifier indicates that the mammalian ortholog of our identified *Drosophila* protein is a known caspase substrate.
3.5 References


Chapter 4 Conclusions and future research

4.1 Overall summary and significance of the thesis research

Macroautophagy (autophagy hereafter) is a lysosome-mediated catabolic process involved in the degradation and recycling of intracellular components. The association of autophagy with cell death has attracted considerable attention and raised many unanswered questions. To contribute to a better understanding in this area, our approach was to conduct a systematic RNAi-based screen of Drosophila apoptosis-related genes to identify potential apoptosis-related modifiers of starvation-induced autophagy. Starvation or nutrient deprivation is a well characterized inducer of autophagy in Drosophila\(^1\) and many other organisms.\(^2\) \(^3\) We developed an efficient flow cytometer based Lysotracker Green (LTG) assay as a primary screen and coupled that to a secondary GFP-LC3 redistribution assay, both in Drosophila l(2)mbn cells, to provide readouts representing late and early stages of autophagy, respectively. As an initial validation of our screening strategy, we designed dsRNAs corresponding to autophagy genes and known autophagy regulators. Our findings showed that dsRNAs corresponding to 11 Drosophila Atg homologues were able to reduce LTG levels in starved l(2)mbn cells. Knockdown of known positive and negative autophagy regulators using RNAi also produced expected alterations in LTG and GFP-LC3. We next screened twenty apoptosis-

A version of this chapter has been published. Hou Y-C. C, Hannigan A.M, Gorski S.M. An executioner caspase regulates autophagy. Autophagy 2009; 5: 530-33. (Invited by the editorial board)
related and Ras pathway-related genes and found 9 that modified LTG and GFP-LC3 levels significantly. Knockdown of Dep-1, hid, debcl, buffy and p53 suppressed LTG and GFP-LC3 levels in starved cells, identifying these genes as positive regulators of autophagy. RNAi-mediated knockdown of Bruce, Ras, Raf, and MAPK enhanced LTG and GFP-LC3 levels in starved cells, identifying these genes as negative regulators of autophagy.

How might the identified gene products act to regulate or modulate the autophagic response in nutrient-deprived cells? Our data showed that the pro-apoptotic gene, hid, but not rpr, grim, or skl, acts to regulate starvation-induced autophagy in Drosophila l(2)mbn cells. Consistent with our findings, overexpression of hid was shown previously to induce autophagy in various Drosophila tissues including fat body, midgut, and salivary glands. Survival Ras/Raf/MAPK signaling has been shown to specifically inhibit the pro-apoptotic activity of Hid, and our observations indicate that the Ras/Raf/MAPK pathway also plays an inhibitory role in starvation-induced autophagy. The Hid protein contains five MAPK phosphorylation consensus sites; thus it is possible that survival signals regulate the crosstalk between autophagy and apoptosis through different threshold levels of MAPK-mediated phosphorylation on Hid. In addition, Hid is known to promote polyubiquitination of DIAP1 and antagonize its anti-apoptotic activity through proteosomal-dependent degradation. Surprisingly, we found that another IAP protein, Bruce, but not DIAP1 acts as a suppressor of autophagy suggesting that Bruce, instead of DIAP1, might be the downstream target of Hid and act to antagonize Hid-mediated autophagy. Bruce and its mammalian homologue, Apollon, share sequence conservation in the BIR (baculoviral-IAP-repeat) and UBC (ubiquitin-conjugating
enzyme) domains. Apollon has been shown to ubiquitinate and promote degradation of SMAC, the mammalian homologue of Hid. Perhaps *Drosophila* Bruce has a similar molecular function as Apollon and promotes degradation of Hid through ubiquitination, thereby acting to negatively regulate autophagy. However, Bruce is a large protein (530 kDa) and it is plausible that it could regulate autophagy through protein interactions with one of its other protein regions. Another candidate Bruce-interacting protein that we identified in our screen is the effector caspase Dcp-1. IAP family members can bind directly to caspases, and inhibit their activity. Thus, it is possible that Bruce suppresses Dcp-1 activity or promotes Dcp-1 degradation through its BIR and/or UBC domains. Such an interaction would be consistent with our identification of Dcp-1 as a positive regulator of autophagy. Based on our recent observations and these previous findings, we propose a hypothetical pathway for the regulation of starvation-induced autophagy in *Drosophila* (Figure 4.1). Clearly, epistasis analyses and protein interaction studies are required to prove or disprove this model, and understand how it integrates with other components (eg. Tor) already known to control the autophagic response to starvation.

To validate the autophagy modulating effects of some of the identified cell death-related genes *in vivo*, we used *Drosophila melanogaster* oogenesis as a model system. Nutrient deprivation triggers germline cell death at two specific stages during oogenesis, the germarium and mid-stage oogenesis. Using a GFP-LC3 transgenic *Drosophila* line as well as Lystotracker Red staining, we found that autophagy also occurs in response to nutrient deprivation at these two stages in oogenesis. An earlier study in *Drosophila virilis* similarly reported the presence of autophagic structures, as observed by TEM, in mid-stage (as well as late-stage) oogenesis. *Dcp-1* was shown previously to be required
for mid-stage egg chamber cell death.\textsuperscript{12} We further demonstrated that \textit{Dcp-1} is required for cell death in germaria, and is also necessary for starvation-induced autophagy in both germaria and mid-stage egg chambers. Further, overexpression of Dcp-1 was sufficient to induce autophagy at these two stages even under well fed conditions (Figure 4. 2). Loss-of-function mutations in \textit{Bruce} resulted in ectopic autophagy and cell death in both stages, regardless of nutrient status, indicating that Bruce acts normally to suppress both autophagy and cell death during \textit{Drosophila} oogenesis. Thus, our observations using RNAi targeting \textit{Dcp-1} and \textit{Bruce} in the \textit{l(2)mbn} cell line were confirmed \textit{in vivo} during \textit{Drosophila melanogaster} oogenesis. However, we observed that loss-of-function of Bruce or overexpression of Dcp-1 in the larval fat body or midgut resulted in a starvation-dependent acceleration of the autophagic response following starvation, rather than ectopic autophagy. Therefore, Bruce and Dcp-1-induced autophagy responses were different between the larval fat body, midgut and the ovary.

What are the factors that contribute to the discrepancies of Bruce and Dcp-1 mediated autophagic responses between larval tissues (fat body and midgut) and the ovary? Several reports have demonstrated that upstream signaling pathways such as TOR and PI3K can affect the mechanistic role of autophagy in cell death, cell growth and cell survival. In the larval fat body, an inverse relationship exists between autophagy and cell growth, and autophagy-defective cells had a growth advantage under physiological conditions.\textsuperscript{13} However, in cells which had defective TOR signaling, autophagy did not have an inhibitory effect on cell growth.\textsuperscript{1, 13} In the salivary gland, growth arrest and autophagy occur concurrently and autophagy contributes to cell death in the presence of apoptotic factors, suggesting that the levels of cell growth signaling might affect the
mechanistic role of autophagy. A recent report by Lu et al. showed that controlled expression of the tumor suppressor gene aplasia Ras homolog member I (ARHI) resulted in autophagic cell death in human ovarian cancer cells. However, multiple factors, including growth factors, inflammatory cytokines, and extracellular matrix proteins within xenograft tumors in mice switched ARHI-induced autophagy to promote tumor cell survival instead of cell death suggesting that levels of PI3K signaling might differentiate the role of autophagy in cell survival and cell death. In Drosophila, a relatively high level of PI3K signaling was observed in the larval fat body, and protein starvation resulted in loss of PI3K activity. In contrast, a relatively low level of expression of Akt expression, a PI3K substrate, was observed in the nurse cells of mid-stage egg chambers but not in the nurse cells of egg chambers of other stages. Thus, I speculate that upstream signaling pathways, such as the PI3K/Akt pathway, might be the factors that contribute to the discrepancies of Bruce and Dcp-1-mediated autophagic responses in different tissues. It will be important to determine the mechanistic roles of Bruce and Dcp-1-induced autophagy in cell death, growth and cell survival under conditions of competent or interrupted PI3K and TOR signaling in the future.

If an effector caspase is required for autophagy and apoptosis, what determines the balance between these two processes and what is the final cellular outcome? In the Drosophila ovary, the two cellular stress responses occurred together and it is possible that autophagy is part of the apoptotic response itself, an idea put forward already by Thorburn. Several cell death regulators have functions that are involved in the adaptation to stress. For example, EGL-1, a BH3-only protein, is required for metabolic stress, and AIF plays a role in redox stress. Hence, an alternative idea is that some
proteins involved in stress responses, such as autophagy, also evolved roles as cell death effectors. A previous biochemical study showed that the effector caspase Dcp-1 was able to auto-cleave/auto-activate and also cleave another effector caspase drICE.\(^{22}\) In contrast, drICE did not act to cleave itself.\(^{22}\) Our data showed that nutrient deprivation had different effects on Dcp-1 and drICE inter-domain cleavage, an indication of increased catalytic activity. Following 30 minutes starvation, we observed increased levels of p20 subunits of endogenous Dcp-1, suggesting that catalytic activity of Dcp-1 was increased shortly after starvation. In contrast, we did not observe p20 subunits of endogenous drICE even following 16 hours of starvation. It is possible that the timing of Dcp-1 activation could determine the sensitivity thresholds of autophagic and apoptotic responses. Starvation signals might initially induce Dcp-1 activation which promotes autophagy for cell survival, giving the cells a chance to recover and allow continued development. Prolonged starvation signals might result in activation of drICE and triggers apoptosis.

If activation of Dcp-1 determines the sensitivity thresholds of autophagic and apoptotic responses, what is the mechanism that regulates Dcp-1 activity? We discovered a genetic interaction between \textit{Dcp-1} and \textit{Bruce}, where a mutation in \textit{Dcp-1} blocked the ectopic autophagy and cell death observed in the ovaries of \textit{Bruce} mutants. Thus, our genetic data showed that IAP protein Bruce might restrain Dcp-1 activity. Other IAP protein family member such as DIAP1 could also play a role in the regulation of Dcp-1 activation. In mammalian cells, Cytochrome c (Cyt c) is released from mitochondria to the cytosol where it binds to Apaf-1 leading to caspase activation during an apoptotic stimulus.\(^{23}\) The release of Cyt c is negatively regulated by the anti-apoptotic
Bcl-2 family members such as Bcl-2 and Bcl-X\textsubscript{L}; thus activation of mammalian caspases is held in check by Bcl-2 proteins. Several mammalian Bcl-2 family proteins have been shown to also play an important role in autophagy regulation. Particularly, anti-apoptotic Bcl-2 negatively regulates autophagy through its interaction with autophagy protein Beclin 1 indicating that a threshold of autophagic response is also held in check by Bcl-2 proteins. Could activation of Dcp-1 be regulated by Bcl-2 family members or factors released from mitochondria? In \textit{Drosophila}, the role of mitochondria in cell death has been a subject of debate. Considerable evidence suggests that Cyt c does not play a clear role in \textit{Drosophila} apoptosis. The roles of two Bcl-2 family proteins, Debcl and Buffy, in apoptotic regulation have been studied in \textit{Drosophila}, and data clearly showed that Debcl has a pro-apoptotic function. The role of Buffy still appears to be somewhat elusive but most data suggest that it is anti-apoptotic. The role of the \textit{Drosophila} Bcl-2 proteins in the regulation of mitochondrial changes (i.e. the release of Cyt c or other mitochondria factors) during apoptosis is still unclear. Our RNAi screen showed that both Debcl and Buffy have a pro-autophagic function. Unlike mammalian cells, Bcl-2 family proteins do not seem to play a role in the regulation of caspase activation, and the autophagc response may not be held in check by Bcl-2 proteins in \textit{Drosophila}. However, we cannot rule out the possibility that additional Bcl-2 family members still await to be discovered in \textit{Drosophila}.

Caspases are known to mediate the execution of apoptosis by cleaving cellular substrates. Numerous studies have been conducted to identify specific substrates of caspases in apoptosis; however, substrates of caspases in non-apoptotic processes are less characterized. Our results demonstrated that catalytic activity of Dcp-1, but not drICE, is
required for starvation-induced autophagy suggesting that substrates of Dcp-1 mediate the execution of autophagy and that substrates of Dcp-1 in autophagy could be distinct from its substrates in apoptosis. An immunoprecipitation and mass spectrometry (IP-MS) based strategy was employed to identify substrates of Dcp-1 in cells undergoing autophagy. Future work is required to verify if identified proteins from our IP-MS studies are Dcp-1 substrates, and to determine if identified proteins have a functional significance in mediating autophagy. However, our initial IP-MS studies did provide a preliminary list of promising substrates which can be used to generate a working model for how Dcp-1 might act to regulate autophagy. We identified Hsc70-4, which genetically interacts with the nutrient sensor TOR\textsuperscript{32}, as a potential substrate of Dcp-1. Loss-of-function Hsc70-4 larvae showed decreased levels of Akt, phospho-Akt and TOR\textsuperscript{32}; thus our data are consistent with the possibility that Dcp-1 mediated-cleavage of Hsc70-4 leads to suppression of TOR activity and the subsequent induction of autophagy. Our data are also consistent with the possibility that Dcp-1 might mediate autophagy by regulating levels of reactive oxygen species (ROS), shown previously to cause oxidation of Atg4 and subsequent autophagy induction in mammals.\textsuperscript{33} We identified one of the \textit{Drosophila} peroxiredoxin family members, \textit{Jafrac1}, as a potential Dcp-1 substrate. \textit{Jafrac1} scavenges ROS and peroxides (H\textsubscript{2}O\textsubscript{2}), and expression of \textit{Jafrac1} promotes cell survival of S2 cells following H\textsubscript{2}O\textsubscript{2} treatment.\textsuperscript{34} Dcp-1 might cleave Jafrac-1 resulting in increased levels of H\textsubscript{2}O\textsubscript{2} that oxidize DmAtg4 and trigger autophagy.

The role of autophagy in cell death is still not well understood and appears to be context dependent. During developmental cell death, such as embryogenesis and insect metamorphosis, it has been proposed that autophagy acts to assist dead cell clearance
when insufficient phagocytes are available for corpse removal.\textsuperscript{35, 36} Three recent studies have demonstrated that autophagy is involved in developmental cell death processes.\textsuperscript{14, 37, 38} In a mouse embryoid body cavitation model\textsuperscript{37} and in a mouse neuroepithelium model,\textsuperscript{38} autophagy was shown to be essential for the clearance of dying cells by generating engulfment signals, including lysophosphatidylcholine secretion (come-get-me signal) and phosphatidylserine exposure (eat-me signal). During \textit{Drosophila} metamorphosis, autophagy genes were demonstrated to be required for complete salivary gland cell degradation.\textsuperscript{14} In the \textit{Drosophila} ovary, nutrient deprivation signals trigger gerarium (region 2A) and mid-oogenesis cell death to remove defective egg chambers before the investment of energy into them,\textsuperscript{9, 39} and our results showed that nutrient deprivation also triggers autophagy at these two stages.

To investigate the role of autophagy during gerarium and mid-oogenesis cell death, we analyzed the phenotype of \textit{DmAtg1} germline clones and \textit{DmAtg7} mutant ovaries. Although chromatin condensation appeared normal, TUNEL staining, an indicator of DNA fragmentation, appeared reduced in gerarium cells and degenerating mid-stage egg chambers in \textit{DmAtg1} and \textit{DmAtg7} mutants. How might autophagy be involved in DNA degradation during \textit{Drosophila} gerarium and mid-stage nurse cell death? DNA degradation is mediated by multiple nucleases\textsuperscript{40}, including cell-autonomous and waste-management nucleases. Most cell autonomous nucleases generate TUNEL-reactive DNA fragments.\textsuperscript{41} Autophagy might positively regulate the activity of cell-autonomous nucleases and thus be involved directly in the regulation of DNA fragmentation. Of the numerous cell-autonomous nucleases identified so far, apoptosis-inducing factor (AIF) might be a promising target of autophagy. Following an apoptotic
stimulus, AIF is released from the mitochondrial inter membrane space and translocates to the nucleus resulting in DNA fragmentation.\textsuperscript{42} Recent publications show that defective mitochondria are selectively targeted by autophagy for degradation.\textsuperscript{43-45} Inhibition of autophagy might prevent the release of AIF from mitochondria resulting in decreased DNA fragmentation. Alternatively, autophagy could modulate the activity of the lysosomal nuclease, DNAseII, which generates 5’-hydroxyl and 3-phosphate ends that are unrecognizable substrates for TdT of the TUNEL assay. In this scenario, autophagy might act normally to delay or suppress DNAse II-mediated DNA degradation,\textsuperscript{41} and thus autophagy inhibition would result in accelerated DNAseII activity and a concomitant decrease in TUNEL positive DNA. Electron microscopy analyses have shown that nurse cell debris is engulfed by surrounding follicle cells in dying mid-stage egg chambers.\textsuperscript{46} Therefore, the TUNEL negative nurse cell nuclei in the DmAtg1 and DmAtg7 mutants could also be accumulated cell corpse DNA which could not be recognized by engulfing cells because they failed to display engulfment signals. However, in mouse embryoid body and retinal neuroepithelium models, dying cells in Atg gene mutants failed to display engulfment signals but still showed TUNEL staining,\textsuperscript{37, 38} a result that differs from our observations in the Drosophila ovary. The possible role(s) of autophagy in DNA degradation, as illustrated in Figure 4.3, remain to be further investigated. It is interesting to note that cell-autonomous DNA degradation is not essential for cell death but appears to affect the efficiency or extent of death at least in some systems. In contrast, removal of dead cell debris can be required for sustained organism survival.\textsuperscript{40} Thus, we propose that at least one of the functions of autophagy in the Drosophila ovary is to
enhance or suppress the efficiency of cell degradation and/or promote corpse clearance associated with cell death.

4.2 Current limitations and summary of future research directions

Our RNAi screen identified nine cell death related genes that are required for starvation-induced autophagy. Additional cell death genes might function in autophagy regulation but were either not tested in our screen or not detected because of insufficient knockdown by RNAi, a long half-life of the corresponding proteins, and/or functional redundancy. Future validation of the remaining identified genes in relation to autophagy during *Drosophila* oogenesis or other in vivo systems will be valuable in the future. In addition, epistasis analyses of these genes can be achieved either by RNAi or *Drosophila* genetic approaches and can be used to establish the pathway of starvation-induced autophagy in *Drosophila*. The role of autophagy in oogenesis cell death, especially DNA degradation, remains an outstanding question to be investigated. Our data suggest that discrepancies exist for Bruce and Dcp-1 mediated autophagic responses between larval tissues (fat body and midgut) and the ovary. Increased numbers of animals are required to validate the results observed in larval fat body and midgut. Further, immunostaining analyses of PI3K or Akt expression levels may provide insights to resolve the discrepancies. Our initial IP-MS studies provide a preliminary list of potential Dcp-1 substrates that may mediate autophagy and this data will be strengthened by performing biological replicates. A weakness of our approach is that unlike other methods that directly analyze cleavage events of caspase, our method utilized a binding approach that is indirect. Thus, our approach could not distinguish the actual substrates of Dcp-1 from interacting proteins that bind to Dcp-1. An in vitro cleavage assay need to be employed
to carefully examine whether identified proteins are cleaved by Dcp-1. The most important next step will be to verify and determine if these putative Dcp-1 substrates have any functional significance in mediating the execution of autophagy, and our RNAi screen is one method that can be employed to test for their involvement in autophagy.

4.3 Potential applications of the research findings

Mammalian homologues of *Drosophila* Bruce, Hid and Dcp-1 are Apollon, Smac and Caspase-3, respectively. It remains to be tested whether the autophagy regulating functions of Bruce, Hid and Dcp-1 are conserved in these mammalian counterparts. Interestingly, overexpression of Apollon can suppress apoptosis, and recent evidence suggests that this occurs indirectly via p53.\(^{47}\) As noted above, Apollon can also ubiquitinate the pro-apoptotic protein Smac, as well as Caspase-9.\(^{7}\) Smac/DIABLO is released from the mitochondria to antagonize IAPs, namely XIAP, cIAP-1 and -2, survivin and Apollon.\(^{8}\) In this way, Smac promotes the activation of Caspase-3 and is pro-apoptotic. Perhaps it has a similar pro-autophagy mode of action. Low levels of Smac\(^{48}\) and Caspase-3\(^{49}\) have been associated with chemotherapy resistance, and based on our model in Figure 4.1, low level activation would be consistent with induction of autophagy. While genetic studies showed that autophagy may act as a tumor suppressor mechanism,\(^{50,51}\) it has been demonstrated also that autophagy can play a protective role during chemotherapy and radiation treatment.\(^{52-56}\) Since Smac mimetics and suppression of IAP proteins are under active investigation as anti-cancer treatments,\(^{57}\) it may be worthwhile to investigate a therapeutic strategy that combines Smac mimetics with autophagy inhibition. If Smac, Apollon and Caspase-3 do function in autophagy regulation, it will be important to understand their effects in both normal and cancer cells.
And given the complexity of apoptotic signaling pathways, it is likely that additional cell
dearth-related genes with a link to autophagy will be discovered.
Based on the known apoptosis-related interactions of the gene products identified in our study, we propose a putative pathway involved in the regulation of starvation-induced autophagy in Drosophila. In this model, the effector caspase Dcp-1 plays a key role in defining the balance between autophagic and apoptotic responses. Starvation facilitates activation of Dcp-1 that leads to induction of autophagy resulting in cell survival or cell death depending on cellular context. Dcp-1 activation perhaps leads to subsequent cleavage of drICE and eventually cell death.
Figure 4.1

Survival Signals
↓
↓
Ras/Raf/MAPK
↓
Hid
↓
Bruce
↓
Dcp-1

early
autophagy
↓
Survival

late
cleavage of
drICE
↓
Cell death

?
Figure 4.2 The effector caspase Dcp-1 is sufficient for the induction of autophagy during *Drosophila* oogenesis.

Expression of activated Dcp-1 in the germline (*UASp-GFP-LC3/+; nanos-GAL4/nanos-GAL4 UASp-tDcp-1*)\(^{58}\) resulted in nurse cell death during mid-oogenesis (arrow) and dying nurse cells had numerous GFP-LC3 puncta (green). DAPI staining of nuclei is shown in white. Scale Bar: 50μm
Figure 4.3 Possible relationships between autophagy and DNA degradation in *Drosophila oogenesis*

This diagram depicts how autophagy might play a role in the DNA degradation process based on the reduced TUNEL staining phenotype observed in dying mid-stage egg chambers of *DmAtg1* and *DmAtg7* mutants. Autophagy could positively regulate the activities or sub-cellular localization of cell-autonomous nucleases (that generate TUNEL-reactive fragments) and thereby enhance DNA degradation. Alternatively, autophagy may negatively regulate the activity of lysosomal nuclease DNaseII (that generates TUNEL non-reactive DNA ends), and thereby suppress or delay DNase-II mediated DNA degradation. Finally, autophagy could sustain the high ATP levels that are required for display of engulfment signals, including lysophosphatidylcholine (come-get-me) and/or phosphatidylserine (eat-me).
Figure 4.3

Autophagy

?→

Apoptotic DNA degradation

Cell-autonomous Nucleases (TUNEL +)

DNAse II (TUNEL -)

Come-get-me and Eat-me signals
4.4 References


Appendices

Appendix A: abbreviations list

CT, cycle threshold

dsRNA, double stranded ribonucleic acid

{l(2)mbn}, lethal (2) malignant blood neoplasm

LTG, lysotracker green

LTR, lysotracker red

IP, immuno-affinity purification

MS, mass spectrometry

PBS, phosphate buffer saline

QRT-PCR, quantitative reverse transcription PCR

UAS, Upstream Activating Sequence

CMA, Chaperone-mediated autophagy

ER, endoplasmic reticulum

CARD caspase recruitment domains

DED death effector domains

MEFs, mouse embryonic fibroblasts

zVAD, pan caspase inhibitor

ROS, reactive oxygen species

TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling

FBS, fetal bovine serum

3MA, 3 methyladenine

Baf, Bafilomycin
PI, propidium iodide
DAPI, 4',6-diamidino-2-phenylindole
BIR, baculoviral IAP repeat
GLCs, germline clones
IC, individualization complex
ERTs, endoreplicating tissues
UBC, ubiquitin-conjugating
TEM, transmission electron microscopy
Atg, autophagy-related
AIF, Apoptosis-inducing factor
TRAIL, TNF-related apoptosis-inducing ligand
FADD, Fas-associated protein with death domain
DAPK, death-associated protein kinase
TOR, target of rapamycin
Nc, Dronc (initiator caspase)
Dcp-1, death caspase-1 (effector caspase)
Ice, drICE (effector caspase)
rpr, Reaper (core cell death effector)
Skl, Sickle (core cell death effector)
DIAP1 (th), Drosophila inhibitor of apoptosis protein-1
Raf (phl), MAP kinase kinase kinase
MAPK (rl), (mitogen activated protein kinase)
SMAC, Second mitochondria-derived activator of caspase
XIAP, X-linked inhibitor of apoptosis

cIAP-1 (BIR2), Baculoviral IAP repeat-containing protein 2

cIAP2 (BIR3), Baculoviral IAP repeat-containing protein 3

DFCP1, double FYVE domain containing protein

Lamp2a, lysosome associated membrane protein type 2a

Class III PI3K, Class III phosphatidylinositol 3 kinase

PI3P, phosphatidylinositol 3-phosphate

UVRAG, Ultraviolet irradiation resistance associated tumour suppressor gene

PE, phosphatidylethanolamine

MAP1LC3B or LC3, microtubule associated protein 1 light chain 3B