Characterization of the *Drosophila* HtrA2/OMI Ortholog, a Mitochondrial Protein of Pro-Apoptotic Function

by

Robert Michael Flick

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Biochemistry
University of Toronto

©Copyright by Robert Michael Flick 2008
Characterization of the *Drosophila* HtrA2/OMI Ortholog, a Mitochondrial Protein of Pro-Apoptotic Function

Master of Science, 2008

Robert Michael Flick

Department of Biochemistry, University of Toronto

**Abstract**

While mitochondria are traditionally associated with energy production, recent studies identified its function in controlling the onset of apoptosis. Mitochondrial apoptotic control results from sequestering pro-apoptotic proteins, which are secreted following cellular stress. HtrA2/OMI is secreted from mitochondria to the cytosol following apoptotic induction, binds and degrades the inhibitor of apoptosis protein, XIAP in mammals, activating the caspase cascade. This study characterizes the expression of *Drosophila* HtrA2/OMI, a mitochondrial protein, its processing by Rhomboid-7 and demonstrates its pro-apoptotic function. Following exposure to apoptotic stress, dOMI is secreted from mitochondria and its expression profile displays an increase in a cleaved form consistent with Rhomboid-7 processing. dOMI expression resulted in sensitization of cells to apoptotic stress, observed through an increase in caspase activity. These data further validate the use of *Drosophila* in the study of mitochondrial driven apoptosis while implicating a potential role for Rhomboid-7 in apoptosis through proteolytic cleavage of dOMI.
Acknowledgments

It seems that no matter what, whenever you begin to acknowledge those who have made an impact on the outcome of any endeavor you will inevitably forget someone. As such I would like to start by thanking anyone that I may have overlooked while writing this acknowledgement.

Well, first and foremost I would like to thank my parents, Roy and Bernadette Flick. Without their support throughout the years none of this would have been possible. They have always been there for me, through the good times as well as the bad. They never gave up on me and have continued to be supportive throughout my life in all of my endeavors especially in my pursuit of academia. To my sister Deanna Flick, thanks for always being there when I needed someone to talk to and willing to lend a hand if need be. Although you have gotten me into just as much trouble as you have gotten me out of, I couldn’t have asked for a better sister.

To all my friends, thanks for always being there to remind me that there is always a way to make room for fun and pleasure in your life. It is through my friends that I have been exposed to a variety of experiences that I would never have imagined. You guys and gals showed me how to take what seemed to be the ordinary and turn it into the extraordinary.

I would like to thank my supervisor, Dr. Angus McQuibban for providing the opportunity for me to pursue my Masters degree in his laboratory at the University of Toronto. I would also like to thank my committee members, Dr. Craig Smibert and Dr. Walid Houry for providing guidance throughout the course of this project. I would like to thank all the members of the McQuibban lab, each of them has helped influence the body of work presented here in one way or another, especially Dr. Jeffrey Lee whose mentorship helped make this possible.

To all those mentioned and those who I have missed, thanks, your influences have greatly impacted the outcome of the work presented here and have shaped me into the person I am today.

Cheers,

Robert Michael Flick
# Table of Contents

**TITLE PAGE** .................................................................................................................. i
**ABSTRACT** ...................................................................................................................... ii
**ACKNOWLEDGMENTS** .................................................................................................... iii
**TABLE OF CONTENTS** ................................................................................................... iv
**LIST OF FIGURES** .......................................................................................................... vi
**ABBREVIATIONS** ........................................................................................................... vii

**CHAPTER 1: GENERAL INTRODUCTION** ....................................................................... 1

- THE PROBLEM AT HAND .................................................................................................. 2
- DROSOPHILA AS A MODEL ORGANISM ....................................................................... 3
- THE MITOCHONDRION .................................................................................................. 4
  - General Composition .................................................................................................... 4
  - Mitochondrial Energy Production ............................................................................... 7
    - Krebs Cycle ............................................................................................................... 7
    - Electron Transport Chain ....................................................................................... 9
  - Mitochondrial Dynamics .......................................................................................... 11
  - Mitochondrial Control of Apoptosis ........................................................................ 13
    - Mitochondrial Outer Membrane Permeabilization (MOMP) .................................. 13
    - Formation and Function of the Apoptosome ......................................................... 15
    - The Caspase Cascade: Activation and Inhibition .................................................. 18
    - Conservation and Variation of Canonical Apoptosis between Mammals and Drosophila 21

- HTRA2/OMI .................................................................................................................. 25
- RHOMBOD-Z ................................................................................................................ 26
  - Identification of the Rhomboid Family of Proteins ................................................ 26
  - Rhomboids and Mitochondrial Morphology ............................................................. 28

**CHAPTER 2: MATERIALS AND METHODS** .................................................................... 31

- CELL LINES AND MEDIA .............................................................................................. 32
- PLASMIDS AND CONSTRUCTS ..................................................................................... 32
- dsRNAi GENERATION .................................................................................................. 33
- APOPTOTIC INDUCTION ............................................................................................. 33
- TRANSIENT TRANSFECTION ...................................................................................... 34
- RNAi MEDIATED KNOCKDOWN ................................................................................ 35
- IMMUNOFLUORESCENCE ........................................................................................... 35
- IMMUNOPRECIPITATION AND CO-IMMUNOPRECIPITATION .................................. 36
# Table of Contents

**SUB-CELLULAR FRACTIONATION** ................................................................. 37
**IMMUNOBLOTTING** ...................................................................................... 37
**ASSAY FOR CASPASE ACTIVITY** .................................................................. 38

## CHAPTER 3: CHARACTERIZATION OF EXPRESSION PATTERNS/LEVELS OF DOMI, A PREDICTED MITOCHONDRIAL PROTEIN OF PRO-APOPTOTIC FUNCTION .......... 40

**INTRODUCTION** ............................................................................................ 41
**RESULTS AND DISCUSSION** ....................................................................... 49
  - Cloning of dOMI into Gateway System ......................................................... 49
  - Exogenous Expression of dOMI in Drosophila S2 Cell Line Reveals Several Distinct Forms Suggesting a Series of Processing Events .......................................................................................................................... 53
  - Knockdown of dOMI By RNAi ........................................................................ 57
  - Exposure to Apoptotic Stimuli Results in Altered dOMI Expression .............. 59
  - dOMI is Released from the Mitochondria Following Exposure to Apoptotic Stress .......... 62
  - Identifying dOMI as a Substrate for the Mitochondrial Serine Protease Rhomboid 7 ........ 71

## CHAPTER 4: ACTIVATION AND MODULATION OF APOPTOSIS IN DROSOPHILA VIA THE CASPASE CASCADE .............................................................................. 75

**INTRODUCTION** ............................................................................................ 76
**RESULTS AND DISCUSSION** ....................................................................... 80
  - Induction of the Caspases Cascade in Response to Cellular Stress .................. 80
  - Inhibition of the Apoptosis Achieved Through a Broad Spectrum Caspase Inhibitor .......... 84
  - Alterations in Modulators of the Caspase Cascade Resulting in Sensitization and Desensitization to Apoptotic Stimuli .......................................................................................................................... 86
  - Modulation of the Caspase Cascade through Expression of dOMI, Elucidating a Pro-apoptotic Role in Drosophila ......................................................................................................................... 89

## CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS ........................................ 97

**SUMMARY OF RESULTS** ............................................................................... 98
**FUTURE DIRECTIONS** ................................................................................... 99
**CONCLUDING REMARKS** ............................................................................ 103

**REFERENCES** ............................................................................................. 105
List of Figures

Fig.1. General structure of an individual mitochondrion............................................................. 6
Fig.2. Schematic of the Krebs Cycle.......................................................................................... 8
Fig.3. Schematic of the Electron Transport Chain...................................................................... 10
Fig.4. Reticular Mitochondrial Network.................................................................................... 12
Fig.5. Formation of the Apoptosome......................................................................................... 17
Fig.6. Comparison of canonical apoptotic signaling in mammals and *Drosophila*.................... 24
Fig.7. General topology of the human HtrA2 protein................................................................. 43
Fig.8. Alignment of HtrA2 and the predicted *Drosophila* homolog, dOMI.............................. 46
Fig.9. Topology prediction for the dOMI protein....................................................................... 47
Fig.10. Gateway cloning of dOMI............................................................................................. 52
Fig.11. Exogenous expression of wild-type and mutant dOMI in S2 cells................................. 54
Fig.12. Assay of overall dOMI transfection in S2 cells using a GFP reporter.............................. 56
Fig.13. RNAi mediated knockdown of dOMI in S2 cells........................................................... 58
Fig.14. Effect of H$_2$O$_2$ induced apoptosis upon dOMI expression....................................... 61
Fig.15. Subcellular fractionation of S2 cells transfected with wild-type or mutant dOMI in the presence and absence of apoptotic stress............................................................ 64
Fig.16. Mitochondrial localization of exogenous HA tagged wild-type and S266A mutant dOMI in transiently transfected S2 cells as observed by immunofluorescence...................... 67
Fig.17. Mitochondrial localization of exogenous HA tagged wild-type and S266A mutant dOMI in transiently transfected S2r+ cells as observed by immunofluorescence.................... 68
Fig.18. Mitochondrial release of exogenously expressed HA tagged wild-type and S266A mutant dOMI in S2r+ cells following UV induced apoptosis.............................................. 69
Fig.19. Mitochondrial release of exogenously expressed HtrA2 following STS induced apoptosis in HeLa cells........................................................................................................ 70
Fig.20. Rhomboid-7 mediated processing of dOMI S266A.......................................................... 72
Fig.21. Observed protein-protein interaction between dOMI S266A and Rhomboid-7............. 74
Fig.22. Schematic of DEVD assay principle............................................................................... 79
Fig.23. Dose dependant activation of the caspase cascade in S2 cells in response to H$_2$O$_2$ Treatment......................................................................................................................... 81
Fig.24. Alternate activation of the caspase cascade..................................................................... 83
Fig.25. Inhibition of caspase activity through the general caspase inhibitor zVAD-fmk............. 85
Fig.26. Effect of RNAi mediated knockdown upon activation of the caspase cascade.............. 88
Fig.27. Lack of dOMI mediated modulation of the caspase cascade following exposure to H$_2$O$_2$................................................................................................................................. 91
Fig.28. Lack of dOMI mediated modulation of the caspase cascade following exposure to STS................................................................................................................................. 93
Fig.29. Inducible dOMI expression resulting in sensitization of S2 cells to STS driven apoptosis........................................................................................................................................ 96
Abbreviations

ΔΨm (change in membrane potential)
Ac-DEVD-AFC (Acetyl DEVD aminofluoro-coumarin)
Ac-DEVD-pNA (Acetyl DEVD p-nitroanilide)
ActD (Actinomycin D)
AIF (Apoptosis Inducing Factor)
ANT (Adenine nucleotide translocator)
Apaf-1 (Apoptotic protease activating factor 1)
ATP (Adenosine triphosphate)
Bak (Bcl-2 homologous antagonist killer)
Bax (Bcl-2-associated X protein)
Bcl-2 (B-cell CLL/lymphoma 2)
BH3 (Bcl-2 homology domain 3)
BIR (Baculoviral inhibition of apoptosis protein repeat)
CAD (Caspase activated DNAse)
CARD (Caspase recruitment domain)
ccdB (controller of cell division or death B)
CDD (Conserved domain database)
CDMC (Canadian Drosophila Microarray Center)
CF (Cytosolic fraction)
CHX (Cycloheximide)
CVo (Complex Vo)
CypD (Cyclophilin D)
dARK (Drosophila Apaf-1 related killer)
dATP (Deoxyadenosine triphosphate)
DGRC (Drosophila Genomics Resource Center)
DMEM (Dulbecco’s modified eagle’s medium)
DNA (Deoxyribonucleic acid)
drICE (Drosophila Interleukin-1 beta-converting enzyme)
Dronc (Drosophila Nedd2-like caspase)
dsRNAi (Double stranded ribonucleic acid interference)
DTT (Dithiothreitol)
ECL (Enhanced chemiluminescence)
EDTA (Ethylene diamine tetraacetic acid)
eGFP (Enhanced green fluorescent protein)
EGFR (Epidermal growth factor receptor)
ER (endoplasmic reticulum)
FADH (Flavin adenine dinucleotide dihydride)
FBS (Fetal bovine serum)
Fzo (Fuzzy Onions)
GFP (Green fluorescent protein)
GTP (Guanosine triphosphate)
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HID (Head involution defective)
HtrA2 (High temperature requirement protein A2)
IAP (Inhibitor of apoptosis binding protein)
IBM (IAP binding motif)
iCAD (Inhibitor of caspase activated DNAse)
IMS (Intermembrane space)
IP (Immunoprecipitation)
Mdn2 (Motor neuron degeneration 2)
MF (Mitochondrial fraction)
Mgm1 (deletion of Mitochondrial genome maintenance protein 1)
MOMP (Mitochondrial outer membrane permeabilization)
MTS (Mitochondrial targeting sequence)
NADH (Nicotinamide adenine dinucleotide hydride)
NBD (N-terminal binding domain)
NCBI (National Center for Biotechnology Information)
OPA1 (Optic atrophy 1)
PARL (Presenilin-associated rhomboid-like)
PARP (Poly adenosine diphosphate ribose polymerase)
PBS (Phosphate buffered saline)
PCD (Programmed Cell Death)
PCR (Polymerase chain reaction)
PTP (Permeability transition pore)
RBD1 (Rhomboid 1)
RING (Really interesting new gene)
RNAi (Ribonucleic acid interference)
SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis)
STS (Staurosporine)
TBST (Tris buffered saline with Tween 20)
TCA (Trichloroacetic acid)
TM (Transmembrane)
TOPO (Topoisomerase)
UV (Ultraviolet)
VDAC (Voltage-dependant anion channel)
w/t (Wild-type)
XIAP (X-linked inhibitor of apoptosis protein)
Chapter 1: General Introduction
The Problem at Hand

Apoptosis, a form of programmed cellular death, is required for normal cellular development. This conserved pathway plays a role in embryonic development\textsuperscript{1,2}, in addition to maintaining cellular homeostasis in the adult organism\textsuperscript{1-4}. Misregulation of this process result in either a decreased sensitivity to apoptotic stimuli leading to increased cellular proliferation, or an increase in apoptotic signaling and subsequent cellular death\textsuperscript{1-4}. While diseases such as cancer and autoimmunity can be linked to desensitization to apoptotic stimuli\textsuperscript{1,2}, increases in apoptotic signaling can contribute to immunodeficiencies as well as neurodegenerative diseases such as dominant optic atrophy or Parkinson’s disease\textsuperscript{1-4}. The pathways involved in apoptosis can be split into two distinct subsets, one involving activation of downstream effector molecules known as caspases through mitochondrial regulation, referred to as canonical signaling\textsuperscript{3-6}. The other involves a variety of signaling pathways resulting in direct caspase activation known as non-canonical signaling\textsuperscript{7,8}. Activation of downstream capase effector molecules in canonical apoptotic signaling is achieved through secretion of pro-apoptotic factors from the mitochondria to the cytosol\textsuperscript{3,4,9-11}. Secreted pro-apoptotic proteins such as HtrA2/OMI trigger sequential activation of members of the caspase cascade, resulting in cellular death\textsuperscript{12-16}. While the effect of mutations or modifications in the apoptotic program can be readily identified through tissue culture approaches, the effects upon the organism as a whole become difficult to ascertain within higher-level organisms such as
mammals due to longer gestation periods, fewer progeny produced and complex genetic inheritance\textsuperscript{17}. In order to combat such limitations it becomes necessary to identify a model organism that functions as an acceptable compromise between cellular function and simplicity of cultivation. For the purpose of this study, I assessed the function of HtrA2/OMI in the complex metazoan \textit{Drosophila melanogaster} in order to further validate the use of this organism as a model for the study of canonical apoptotic signaling.

\textbf{Drosophila as a Model Organism}

The complex metazoan, \textit{Drosophila melanogaster}, lends itself as a viable model organism for the study of a large range of cellular processes, stemming from several key features of this organism. Firstly, the cultivation of \textit{Drosophila} in a laboratory environment is relatively simple, resulting in the production of a large number of progeny over a short, 10-day generation period\textsuperscript{17}, while model organisms such as the mouse require a longer, 21-day gestation period and produce a smaller number of progeny. For the purpose of genetic screens, \textit{Drosophila} provides two benefits in that its genome is comprised of only 4 chromosomes, as opposed to 16 in the yeast strain \textit{Saccharomyces cerevisiae}, thus simplifying genetic inheritance amongst progeny, and that mutational analysis can be conducted on a large scale to determine the effect upon a given process\textsuperscript{17}. Key features of the \textit{Drosophila} exoskeleton, such as the compound eye, provide unique methods for studying mutational and genetic interactions simply by visual observation of the resultant phenotype of the affected feature\textsuperscript{17}. Another feature of \textit{Drosophila} contributing to its use as a model organism is
conservation of cellular processes, such as DNA replication, protein synthesis and mitochondrial cellular respiration as well as components and functions involved in the apoptotic program between flies and mammals\textsuperscript{17}. These apparent evolutionary conservations of cellular processes have resulted in the identification of \textit{Drosophila} homologs for the majority of known human disease genes\textsuperscript{17}. A distinct drawback to the use of \textit{Drosophila} as a model organism is the requirement for constant maintenance since laboratory stocks cannot be frozen and subsequently revived\textsuperscript{17}, in addition to the ease of contamination of stocks by a single fly. Through these features, \textit{Drosophila melanogaster} provides an excellent compromise between simplicity of cultivation, genetics and phenotypic scoring while maintaining functional conservation of pertinent cellular processes.

**The Mitochondrion**

**General Composition**

The mitochondrion is a subcellular organelle found within eukaryotic organisms with the exception of amitochondrial eukaryotic organisms such as \textit{Giardia intestinalis} and \textit{Trachipleistophora hominis}\textsuperscript{18,19}. Mitochondria contain four distinct compartments, the outer and inner membranes (each composed of a phospholipid bilayer), the intermembrane space (IMS) and the matrix (Fig. 1)\textsuperscript{20,21}. The inner membrane is arranged into a series of involutions forming the cristae, while the outer membrane is devoid of such structures. The mitochondrion possesses its own genome consisting of a circular DNA molecule located within the matrix\textsuperscript{20,21}. Despite possessing its own genome, the majority of proteins required for mitochondrial function are encoded by the nuclear genome, requiring
import into the mitochondria following translation\textsuperscript{22,23}. In addition to accommodating the mitochondrial genome as well as the components required for its maintenance, replication and transcription into functional proteins, the matrix functions as the site in which the Krebs cycle (a key metabolic process involved in ATP production) is conducted\textsuperscript{21,24}. The inner membrane contains a variety of embedded proteins, including those involved in ATP generation via the electron transport chain\textsuperscript{21,24,25}. Another result of the electron transport chain is the generation of a membrane potential, $\Delta \Psi_m$, between the matrix and the IMS, achieved through pumping protons from the former compartment to that of the latter\textsuperscript{21,24,25}. This $\Delta \Psi_m$, which is further harnessed to facilitate ATP production, is required for proper mitochondrial function and cell survival\textsuperscript{21,24,25}. Recently, the IMS has been identified as storage site for a variety of pro-apoptotic factors that are released from the mitochondria following exposure to apoptotic stimuli\textsuperscript{3,4,9-11}. 
Fig. 1. General Structure of an individual mitochondrion.
Mitochondrial Energy Production

Typically, mitochondria are regarded as the “powerhouses” of the cell, responsible for the generation of useable energy for the cell in the form of ATP. Within the mitochondria, energy production is regulated by two distinct and sequential components of the same pathway, which are known as the Krebs cycle\textsuperscript{21,24} and Electron Transport chain\textsuperscript{21,24,25}.

Krebs Cycle

The Krebs cycle occurs in the mitochondrial matrix and involves the oxidation of Acetyl CoA into CO$_2$, with the energy produced being transferred to a variety of metabolic processes in the form of GTP or as electrons in NADH and FADH$_2$ (Fig. 2)\textsuperscript{21,24}. The initial input of Acetyl CoA is provided through the process of glycolysis (conversion of glucose to pyruvate) and subsequent generation of Acetyl CoA from pyruvate, or through fatty acid oxidation liberating activated fatty acids from triacylglycerols stored within adipose tissue\textsuperscript{21,24}. Both NADH and FADH$_2$ molecules generated in this process are subsequently processed through the electron transport chain, resulting in the production of ATP\textsuperscript{21,24}. 


Fig. 2. Schematic of the Krebs Cycle.
**Electron Transport Chain**

The electron transport chain is a metabolic pathway in which protons are pumped out of the matrix into the IMS generating the previously described $\Delta \Psi_m$. This is achieved using the NADH and FADH$_2$ molecules produced in the Krebs cycle as electron donors for a sequence of redox reactions carried out by the complexes I-IV as well as Coenzyme Q and cytochrome C of the electron transport chain (Fig 3)$^{21,24,25}$. Transfer of electrons between the proteins involved in this pathway is coupled to proton translocation to the IMS producing a $\Delta \Psi_m$ across the inner membrane$^{21,24,25}$. This generated $\Delta \Psi_m$, is then responsible for the import of protons back into the matrix, facilitated by the F$_1$F$_0$ ATP synthase, complex V, resulting in the production of useable energy in the form of ATP$^{21,24,25}$. 
Fig. 3. Schematic of the Electron Transport Chain. Blue line indicates the flow of electrons from NAD$^+$ to O$_2$. Membrane potential ($\Delta \Psi_m$) is denoted by relative charges on either side of the membrane (+ in the IMS and - in the Matrix).
Mitochondrial Dynamics

Traditionally, the mitochondria were depicted as static, kidney shaped organelles\textsuperscript{20,21}. Recent studies have revealed that contrary to dogma, mitochondria typically exist as a network of interconnected organelles (Fig. 4), continually undergoing a series of fusion and fission processes resulting in the constant movement and remodeling of this network\textsuperscript{26,27}. To maintain normal cellular function, proper regulation of these fusion and fission events is essential, with misregulation impairing cellular processes, which can be detrimental to the viability of the organism. For example, mitochondrial fusion plays a pivotal role in \textit{Drosophila} spermatogenesis. During spermatid maturation, mitochondria aggregate near the nucleus and undergo massive membrane fusion producing two distinct mitochondrial aggregates which intertwine with one another to form a spherical nebenkern. Following elongation of the flagellum, the nebenkern unfolds and elongates along the axoneme, providing locomotive energy for the resulting sperm\textsuperscript{28-30}. In \textit{Drosophila}, Fuzzy onions (Fzo)\textsuperscript{28,30} and Rhomboid-7\textsuperscript{29}, are identified as essential for proper nebenkern formation, where mutations result in a loss of fusion of the mitochondria and male sterility. In humans, dominant optic atrophy is identified by early onset blindness and has been linked to mutation in the mitochondrial protein OPA1, a protein involved in mitochondrial fusion with mutations resulting in increased mitochondrial fragmentation\textsuperscript{31,32}.
Fig. 4. Reticular Mitochondrial Network. Mitochondria visualized by Confocal Microscopy using mouse anti-CV/a antibody for detection of mitochondria and a secondary anti-mouse antibody coupled to AlexaFluor 546. (A) COS-7 (Cercopithecus aethiops) cells displaying interconnected network of mitochondria. (B) S2 (Drosophila melanogaster) cells displaying interconnected network of mitochondria.
Mitochondrial Control of Apoptosis

While functioning as the “powerhouse” of the cell, mitochondria also play a key role in the process of apoptosis. Apoptosis, or programmed cellular death (PCD), occurs through a series of biological processes whose final outcome results in the destruction of the cell. As described previously, the apoptotic pathway can be divided into two subsets, one involving the direct activation of caspases through a series of adapter proteins (non-canonical)\(^7,8\), the other involving activation of the caspase cascade facilitated by mitochondrial regulation (canonical)\(^3-6\). In the canonical pathway, mitochondria function as storage for pro-apoptotic factors including cytochrome C, Diablo, AIF and HtrA2. Following exposure to an apoptotic stimulus, mitochondria undergo a series of morphological changes resulting in the release of these pro-apoptotic factors into the cytosol and the subsequent activation of the caspase cascade\(^3,4,9-11\). While the two apoptotic pathways are activated through different mechanisms, both result in cell shrinkage, membrane blebbing, chromatin condensation followed by DNA fragmentation and eventually the destruction of the cell via the formation of vesicles referred to as apoptotic bodies\(^1-11\).

Mitochondrial Outer Membrane Permeabilization (MOMP)

The first stage of the canonical apoptotic pathway involves permeabilization of the mitochondrial outer membrane. This permeabilization leads to the release of pro-apoptotic factors from the IMS into the cytosol resulting in activation of the caspase cascade\(^3,11,33,34\). This occurs via two
different mechanisms, the first involving the opening of the permeability transition pore (PTP)\textsuperscript{33-35}, while the second involves the formation of the Bax/Bak complex\textsuperscript{33,34,36-39}. The PTP is proposed to be composed primarily of the voltage-depandannt anion channel (VDAC or Porin), the adenine nucleotide translocator (ANT) and cyclophilin D (CypD)\textsuperscript{33-35}. Opening of the PTP is stimulated by a variety of signals and is capable of allowing passive transport of molecules up to approximately 1.5kDa in size through the inner mitochondrial membrane\textsuperscript{39}. This opening is capable of allowing both ions and water to pass between the IMS and the matrix. Disruption of the normal ionic gradient between the matrix and the IMS results in a loss of membrane potential (ΔΨm) an indication of the onset of apoptosis, while an influx of water into the matrix is capable of leading to its subsequent swelling. This swelling results in the rupture of the outer membrane leading to MOMP and the subsequent release of pro-apoptotic factors from the IMS to the cytosol\textsuperscript{33,34,36-39}. Most commonly, MOMP takes place through the formation of the Bax/Bak complex. Primarily the anti-apoptotic Bcl-2 protein family members and the pro-apoptotic BH3 protein family members regulate the formation of this complex. The Bcl-2 family members function through the prevention of the Bax/Bak complex formation, which is accomplished by sequestering pro-apoptotic activators\textsuperscript{38-41}. To overcome this, a series of BH3 proteins either directly activate the formation of this complex or inhibit the Bcl-2 family members, thus alleviating their inhibition of the Bax/Bak complex formation\textsuperscript{37-41}. Following formation of this complex, MOMP is achieved causing the release of pro-apoptotic factors into the cytosol. Such released proteins
include Cytochrome C, Diablo, HtrA2, AIF as well as Endonuclease G, each of which plays a functional role in the onset of apoptosis, ranging from the initiation of the caspase cascade, alleviation of caspase inhibition, to triggering DNA condensation and fragmentation.

While proteins such as Cytochrome C, Diablo and HtrA2 elicit their effects in the cytosol following MOMP, both Endonuclease G and AIF undergo further translocation to the nucleus. Following nuclear import, each of these proteins plays a role in the degradation of the nuclear genome. Endonuclease G is a DNAse, capable of degrading the nuclear genome into large fragments ranging between 50 and 300 kb. Conversely, the function of AIF remains uncharacterized although it has been shown to cause chromatin condensation followed by fragmentation of DNA. Both Endonuclease G and AIF are capable of functioning independent of caspase activation, while still requiring release from the mitochondria to function.

**Formation and Function of the Apoptosome**

The apoptosome is a large multimeric complex formed by the association of seven subunits into a heptamer of approximately 1.0 MDa. The subunits of the active apoptosome are comprised of three different proteins, cytochrome C, apoptosis protease activating factor-1 (Apaf-1) and Procaspase-9. In addition to its known role in cellular respiration via the electron transport chain, cytochrome C also plays a pivotal role in the onset of apoptosis. Following exposure to apoptotic stress resulting in MOMP, cytochrome C is released from
pools believed to reside in the IMS of the cristae into the cytosol\textsuperscript{33}. Following mitochondria release, cytochrome C binds Apaf-1 via WD40 repeats of Apaf-1 causing a conformation change allowing for the binding of ATP and formation of an Apaf-1 heptamer. This Apaf-1 heptamer then recruits a single Pro-caspase-9 monomer via its caspase recruitment domain (CARD) and subsequently Pro-caspase-9 recruits a second Pro-caspase-9 monomer forming an asymmetrical dimer, believed to contain only one active site (Fig 5)\textsuperscript{5,9}. The completed apoptosome containing the active caspase-9 dimer then cleaves the effector caspases from immature to mature forms thus initiating the caspase cascade\textsuperscript{5,9,11,43}. 
Fig. 5. Formation of the Apoptosome. (A) Apoptosome formation facilitated by binding of cytochrome C to the WD40 repeats of Apaf-1 and subsequent formation of the Apaf-1 heptamer. (B) Formation of the asymmetrical caspase-9 dimer.
The Caspase Cascade: Activation and Inhibition

Apoptosis occurs through an amplification cycle of the initial stimulus leading to the eventual death of the cell. This amplification occurs via the caspase cascade, a sequential activation of cysteine proteases capable of degrading cytoplasmic proteins as well as activating proteins involved in nuclear control of apoptosis. Between 12 and 13 caspases are encoded by the human genome. The key proteases involved in the canonical pathway are the already discussed initiator caspase, caspase-9, as well as two effector caspases, caspase 3 and 7. While caspase-9 is activated in response to an asymmetrical dimerization event, caspase-3 and -7 are each expressed in an inactive form that requires proteolytic cleavage to generate the active caspases. The activated (cleaved) caspase-3 recognizes a peptide sequence, DEVD, and cleaves substrates containing this sequence at the second (C-terminal) aspartic acid residue. Substrates containing the DEVD signal peptide sequences include Actin and iCAD. Cleavage of Actin results in the disassembly of the actin cytoskeleton, while cleavage of iCAD results in the release of the CAD protein, a DNAse that is then imported to the nucleus where it participates in one of the hallmarks of apoptosis, the fragmentation and degradation of the nuclear genome. Caspase-3 also cleaves the nuclear substrate PARP that contains the signal peptide sequence DEVD. Since PARP normally participates in the repair of single stranded DNA nicks, a process counterproductive to the genomic fragmentation typically associated with apoptosis, cleavage and deactivation by
caspase-3 further amplifies this fragmentation by shutting down part of the DNA repair pathway\(^4^8\).

Since activation of the caspase cascade is detrimental to cell survival, the pathway is further regulated utilizing a specialized set of proteins capable of inhibiting the cascade. These proteins collectively referred to as IAPs, bind to caspases thereby preventing the association and interaction with additional factors involved in the onset of apoptosis\(^4^9\)-\(^5^1\). Interactions between IAPs and caspases occur through the three IAP BIR domains (BIR1, BIR2, BIR3)\(^5^2\), wherein proteins possessing the consensus sequence AVPF (or IAP binding motif (IBM)) recognize and bind to the BIR domains of IAPs\(^1^1\),\(^4^9\)-\(^5^2\). The three common IAPs are IAP1, IAP2, and XIAP (X-linked inhibitor of apoptosis protein), with IAP1 and IAP2 being primarily responsible for the inhibition of caspase 8, a caspase activated upstream of MOMP, XIAP on the other hand binds to caspases 3 and 7 through interactions with its BIR2 domain and binds caspase 9 via its BIR3 domain, effectively inhibiting their roles in the caspase cascade\(^1^1\),\(^4^9\)-\(^5^2\). To overcome this caspase inhibition, Diablo and HtrA2 are released from the mitochondria into the cytosol following MOMP at which point bind to and/or cleave XIAP, thus removing XIAP inhibition of caspase 3, 7 and 9\(^3\),\(^4\),\(^9\),\(^1^2\)-\(^1^6\),\(^5^2\)-\(^5^4\).

Diablo is initially expressed as an immature protein containing an N-terminal mitochondrial targeting sequence (MTS) and following mitochondrial import undergoes catalytic cleavage producing a mature Diablo molecule\(^5^4\),\(^5^5\). Removal of its N-terminal pre-sequence occurs through an interaction with the inner membrane peptidase complex located on the mitochondrial inner
membrane and results in the exposure of Diablo’s IBM AVP\textsuperscript{55}. This mature Diablo, now present within the IMS is released to the cytoplasm following MOMP where it binds XIAP via the BIR2 and BIR3 domains while maintaining a greater affinity for the latter\textsuperscript{51-55}. Binding of Diablo to XIAP antagonizes its interaction with the caspases and alleviates XIAP inhibition of the caspase cascade. One feature to note is that while Diablo binds to and antagonizes XIAP, this interaction can be overcome through proteolytic degradation of Diablo facilitated by the RING domain of XIAP. The RING domain of XIAP (as well as IAP1 and IAP2) functions as an E3 ubiquitin ligase with specificity for Diablo and as such causes ubiquitination of Diablo, targeting it for degradation\textsuperscript{56}.

While the pro-apoptotic effect of Diablo occurs through antagonizing XIAP-caspase interaction\textsuperscript{52-56}, the serine protease HtrA2 both antagonizes XIAP-caspase interaction\textsuperscript{12-16} as well as degrades XIAP\textsuperscript{57-59}. HtrA2 is initially expressed as an immature protein comprised of 4 distinct domains, an N-terminal MTS, TM, serine protease and PDZ domains as well as possessing an IBM, AVPS, located just C-terminal to its TM domain. Immature HtrA2 is imported to the mitochondria via its MTS while the presence of its TM domain is predicted to cause localization to the mitochondrial inner membrane. Following import, HtrA2 undergoes proteolytic cleavage of its N-terminus generating the mature protein with an exposed 4 aa IBM, AVPS. This cleavage event is shown to occur through a self-processing event facilitated by its own serine protease domain. Following MOMP, mature HtrA2 is released to the cytosol where it competitively binds the BIR2 and BIR3 domains of XIAP, removing the inhibition of caspase-3,
-7 and -9. In addition to binding XIAP, HtrA2 cleaves this IAP molecule, causing inactivation and resulting in amplification of the signal produced by the caspase cascade\textsuperscript{12-16,57-59}.

Conservation and Variation of Canonical Apoptosis between Mammals and Drosophila

As previously described, the mammalian canonical apoptotic pathway is regulated by a series of mitochondrial proteins whose release following MOMP is required for caspase activation (Fig. 6A)\textsuperscript{3,4,9,10,34}. Therefore mitochondria are not only a cellular “powerhouse” responsible for ATP production, but also checkpoints in the cell death machinery. The model organism \textit{Drosophila melanogaster} undergoes apoptosis in a similar manner involving the conservation of several components in this pathway while having several distinct variations (Fig 6B)\textsuperscript{49,50}. While homologs of Apaf-1, casapases-3 and 9, as well as XIAP are found in \textit{Drosophila} as the proteins dARK\textsuperscript{49,50,60-63}, Dronc\textsuperscript{49,50,61,64,65}, drICE\textsuperscript{49,50,61,64-67}, and IAP1\textsuperscript{49,50,60,61,68}, respectively, regulation of this pathway in \textit{Drosophila}, particularly the involvement of mitochondrial factors, remains uncertain. This uncertainty stems from conflicting data regarding cytochrome C function\textsuperscript{69-74}, the presence of cytosolic IAP antagonists\textsuperscript{49,50,75-77} and until recently an unidentified role for an HtrA2 homolog, dOMI\textsuperscript{78,79}.

Cytochrome C release is a hallmark of apoptotic induction in mammals, where following MOMP, cytochrome C is released from the mitochondria to the cytosol where it is responsible for formation of the apoptosome and subsequent
activation of the caspase cascade. In *Drosophila*, cytochrome C involvement remains a point of contention wherein conflicting evidence has been presented regarding its capability to trigger apoptosis. Several studies have shown evidence for cytochrome C requirement in spermatid differentiation through the activation of caspases in a process resembling apoptosis, while it has also been shown that cytochrome C displays an altered conformation in apoptotic cells. Despite these findings, most studies elude to a failure to detect cytochrome C release to the cytosol following exposure to apoptotic stress in *Drosophila* cells and subsequently the inability to bind the Apaf-1 homolog dARK in the formation of the apoptosome, a hallmark of canonical mammalian apoptosis.

In mammals, the IAP, XIAP, inhibits the activity of caspases-3, 7, and 9 through BIR mediated protein-protein interactions and this inhibition is alleviated by the mitochondrial release of pro-apoptotic IAP antagonists. Two previously described mammalian mitochondrial IAP antagonists are Diablo and HtrA2. In *Drosophila*, three IAP antagonists, Reaper, HID and Grim were initially identified to competitively bind IAP1 thus removing inhibition of the caspase cascade. While these proteins were shown to function in a manner similar to that of mammalian Diablo, each lack the presence of a MTS or TM domain and as such each protein is expressed and localized to the cytosol, thus providing further evidence for a lack of mitochondrial involvement in apoptosis in *Drosophila*. While Diablo antagonizes XIAP solely through competitive binding, the mitochondrial serine protease HtrA2 also elicits its
pro-apoptotic effect through proteolytic cleavage of of XIAP$^{12-16,57-59}$. Through this method, mitochondrial HtrA2 release in mammals represents a checkpoint signifying a commitment to the apoptotic program.
Fig. 5. Comparison of Canonical Apoptotic Signalling in Mammals and Drosophila.
HtrA2/OMI

HtrA2 has been widely studied in mammalian models, resulting in its identification as a pro-apoptotic molecule. Initial studies indicate that following exposure to apoptotic stimuli, HtrA2 is released from the mitochondria to the cytosol eliciting a pro-apoptotic effect\textsuperscript{12-16,57-59}. The pro-apoptotic effect of HtrA2 is described to result from an interaction with\textsuperscript{12-16,57} and degradation of the IAP protein XIAP\textsuperscript{58,59,80} and by doing so removing its inhibition of the caspase cascade. It was further shown that the function of HtrA2 was dependant on a catalytic serine residue at position 306, responsible for both autocatalytic processing of HtrA2 producing the mature protein\textsuperscript{81} and degradation of XIAP\textsuperscript{13,14,57,80}. X-ray crystallography revealed that formation of an HtrA2 homotrimeric structure was essential for proteolytic activity and that a monomeric HtrA2 was incapable of autoproteolysis or proteolytic degradation of XIAP\textsuperscript{82}.

While HtrA2 has been identified to function directly in the activation of the caspase cascade through its interaction with XIAP resulting in apoptosis, HtrA2 has also been shown to play a role in neuromuscular disorders such as Parkinson’s disease in humans\textsuperscript{83} as well as the Parkinson’s like phenotype in mnd\textsuperscript{2} mutant mice\textsuperscript{84,85,86}. In either neuromuscular disorder, identification of a mutation in or deletion of HtrA2 resulted in the loss of catalytic function, suggesting a neuroprotective role of the wild-type protein. In mnd\textsuperscript{2} mutant mice, loss of HtrA2 function was shown to correspond to increased mitochondrial permeabilization denoted by a loss of $\Delta \Psi m$ and increased susceptibility to apoptotic stimuli\textsuperscript{86}. It is proposed that while the pro-apoptotic function of HtrA2 is
due to degradation of XIAP following mitochondrial release, the observed
neuroprotective role of HtrA2 is the result of maintenance of normal mitochondrial
morphology, specifically membrane permeability.

It was the goal of this study to identify the Drosophila ortholog of the
mammalian HtrA2 molecule to further validate the use of Drosophila as a model
organism in the study of canonical apoptotic signaling. While characterization of
the mammalian HtrA2 has been conducted, studies identifying an ortholog in the
model organism Drosophila had not previously been conducted. In addition to
the data presented here, two recent papers identified the Drosophila gene
CG8464 as the ortholog to the mammalian HtrA2, denoted dOMI\textsuperscript{78,79}. In both
studies, in vitro analysis using recombinant dOMI and IAP1 proteins identified
interaction of dOMI with IAP1 and its proteolytic degradation, similar to the
degradation of XIAP by HtrA2.

**Rhomboid-7**

*Identification of the Rhomboid Family of Proteins*

The Rhomboid family of proteins represents a highly conserved family,
present within all kingdoms of life\textsuperscript{87-90}. Initially a *rhomboid* mutation was
identified for its requirement in *Drosophila* wing vein development and was
dubbed *veinlet*. The *rhomboid* gene was subsequently identified for its
requirement in *Drosophila* embryonic patterning and the resulting embryonic
phenotype placed it in the *spitz* group of genes\textsuperscript{91}. Spitz functions as the ligand
for the Epidermal growth factor receptor (EGFR), a component of a broad
signaling pathway involved in the development of nearly every tissue in *Drosophila*\(^91\). Expression of Rhomboid parallels activation of the EGFR signaling pathway\(^92\), with ectopic expression being sufficient for activation of EGFR signaling\(^93,94\) while removal of Rhomboid was capable of mimicking a Spitz null phenotype\(^95\), suggesting activity of Spitz is dependent on Rhomboid. Contrary to initial assumptions, neither Rhomboid nor Spitz are localized to the cell surface, instead Spitz was shown to be confined to the endoplasmic reticulum (ER) while Rhomboid is localized to the Golgi apparatus\(^96\). Spitz undergoes translocation to the Golgi apparatus via Star, where it undergoes cleavage of its transmembrane (TM) anchor in a Rhomboid dependent manner, allowing for secretion of the Spitz ligand and initiation of the EGFR signaling pathway\(^96,97\). Sequence analysis of the Rhomboid protein revealed no resemblance to any known protease while identifying Rhomboid as a trans-membrane protein\(^92\). In order to identify the mechanism for Spitz catalysis, mutational analysis of Rhomboid was conducted upon conserved residues revealing a serine protease-like catalytic triad within the membrane bilayer (spread across three TM domains)\(^98\). Mutational analysis revealed a series of seven amino acid residues located within the Spitz TM domain responsible for proteolysis by Rhomboid\(^99\). Most Rhomboid proteins from bacterium, invertebrates and vertebrates were capable of specifically cleaving the Spitz motif\(^100\).

In *Drosophila*, seven distinct *rhomboid*-like genes have been identified, with the original rhomboid gene renamed as *rhomboid*-1, and the subsequent six *rhomboid*-2 through *rhomboid*-7\(^90\). Rhomboid-2 is identified to promote cell
differentiation in germline stem cells\textsuperscript{101} while Rhomboid-3 plays a role in Drosophila eye development\textsuperscript{90}, embryogenesis\textsuperscript{102}, and protection from apoptosis in epidermal cells\textsuperscript{103}. Recently, Rhomboid-7 has been revealed to function in mitochondrial dynamics\textsuperscript{29}, while roles for Rhomboid-4 through Rhomboid-6 remain undetermined.

**Rhomboids and Mitochondrial Morphology**

Rhomboid proteins have been shown to be essential for the maintenance of normal mitochondrial morphology. Initial studies involving the deletion of the *Saccharomyces cerevisiae* protein Rbd1 resulted in altered mitochondrial morphology observed as small fragmented mitochondria as well as aggregates\textsuperscript{104,105}. Additionally, ∆rbd1 yeast strains displayed a slow growth phenotype that was further linked to an inability to grow on a non-fermentable carbon source, glycerol, indicating a respiration deficiency\textsuperscript{104,105}. In a search for potential substrates of Rbd1, the yeast protein Mgm1 was identified using the following set of criteria; mitochondrial localization, and a predicted single TM domain\textsuperscript{104}. ∆mgm1 strains were observed to display the same slow growth phenotype and respiratory deficiency as well as mitochondrial dysfunction, observed with ∆rbd1\textsuperscript{104,105}. Further studies identified the requirement for Rbd1 in the processing of Mgm1\textsuperscript{104,105}.

Conservation of Rbd1 is observed in *Drosophila* (Rhomboid-7)\textsuperscript{29} as well as in humans (PARL)\textsuperscript{106}, and the Rbd1 substrate Mgm1 also shows conservation as OPA1-like\textsuperscript{104} and OPA1\textsuperscript{107,108} in *Drosophila* and humans respectively. PARL
knockout mice reveal no apparent fragmentation of the reticular mitochondrial network but result in changes in cristae structure. This change in cristae structure is attributed to a requirement for cleaved OPA1 in tethering of the cristae, while lack of cleaved OPA1 results in an increase in apoptosis due to release of cytochrome C\textsuperscript{109,110}. This suggests that while PARL is not responsible for mitochondrial fusion, it is required for tethering of the cristae through cleavage of OPA1 and subsequently functions as an anti-apoptotic factor through the sequestration of cytochrome C in the cristae. Loss of OPA1 in humans results in dominant optic atrophy, a mitochondrial disease responsible for childhood onset blindness\textsuperscript{107}.

\textit{rhomboid}-7 mutant flies are characterized by reduced viability, where ~90% die prior to pupariation. Of those flies reaching pupariation, ~10% die during eclosion while the remaining adults have a lifespan of ~3 days following eclosion. \textit{rhomboid}-7 mutants are also identified by male sterility due to irregular formation of the nebenkern as an aggregate of mitochondria which have failed to fuse. These \textit{rhomboid}-7 mutant flies also display muscular defects, resulting from small irregular mitochondria located between myofibrils. In \textit{Drosophila} S2 cells, RNAi mediated knockdown of Rhomboid-7 causes fragmentation of the reticular mitochondrial network, suggesting that it functions in mitochondrial fusion\textsuperscript{29}. While Rhomboid-7 has been identified to function in the maintenance of the reticular mitochondrial network by promoting mitochondrial fusion, similar to that of the yeast homolog Rbd\textsuperscript{104,105}, there is currently no evidence to support a
potential anti-apoptotic role for Rhomboid-7 as observed with the mammalian PARL\textsuperscript{106,109,110}.

Using previously described criteria for identification of Rbd1 substrates, the \textit{Drosophila} proteome was probed in order to identify potential Rhomboid-7 substrates. These criteria consisted of the presence of a MTS, a TM domain in addition to the presence of a glycine alanine (GA) motif located within the TM domain of the target protein\textsuperscript{98,99,104}. Among potential substrates identified in this manner was dOMI, an ortholog of the mammalian pro-apoptotic protein HtrA2. It was the goal of this study to characterize and validate any interaction between Rhomboid-7 and dOMI in an attempt to identify a potential role of Rhomboid-7 in apoptosis.
Chapter 2: Materials and Methods
Cell lines and media

*Drosophila* S2 and S2R+ cell lines were obtained from the DGRC. These lines were maintained in M3 media supplemented with 10% FBS (Sigma) at 26°C. Human HeLa cells were obtained from ATCC and were maintained in DMEM (Sigma) supplemented with 50mg/mL of both Penicillin (Sigma) and Streptomycin (Sigma) in addition to 10% FBS (Sigma) at 37°C.

Plasmids and Constructs

pENTR/D TOPO and LR clonase kits were obtained from Invitrogen. Drosophila destination vectors, pAWH, pAWF, pAWG, were obtained from the DGRC. PCR amplified dOMI cDNA (forward primer CACCATGGCTTTGCGCG- GTTCCCA, reverse primer GGGATCTTCTGGCGTAATGG) was TOPO ligated into the pENTR/D TOPO vector according to Invitrogen’s guidelines yielding the pENTR dOMI vector. pENTR dOMI was subjected to site directed mutagenesis as per the Stratagene QuikChange protocol, generating a single amino acid substitution in the coding region of dOMI at position 266 from serine to alanine. Both wild-type and mutant pENTR dOMI were subjected to LR clonase reactions with either pAWH or pAWF according to Invitrogen’s guidelines yielding pAWH dOMI and pAWF dOMI constructs. pAWF Rhomboid-7 wild-type and mutant constructs were provided by Dr. Jeffrey Lee. Wild-type and mutant pRm dOMI constructs were obtained from Challa et. al.⁷⁸
dsRNAi Generation

Forward and reverse primers for dsRNAi constructs were designed to include a 5' T7 RNA polymerase promoter (GAATTAATACGACTCACTATAGG-GAGA). dOMI forward primer GCGCGGTTCCCACCGCTT, reverse primer GAAAGTCGCCAGATCGGA. IAP1 forward primer ATGGCATCTGTAGCTGATCTCTCTCT, reverse primer TCGACGCATGGTGGCGACCGTTACCCTCG. IAP2 forward primer ATGACGGAGCTGGTCATGGAGCTGGAGAGC, reverse primer GAACTGACACTTTGGCGACCACTTGGCGTG. Grim forward primer CCTATTTACCCCGACC, reverse primer GTTCTCCTTGGAGGTGGC. Reaper forward primer ATGGCAGTGGCATTCTAC, reverse primer TTGCGATGGCTTGCGATA. Using the appropriate primers, RNAi templates for dOMI, IAP1, IAP2, Grim and Reaper were amplified by PCR using the respective cDNA molecules (CDMC). dsRNAi was generated using the Promega RiboMAX Large Scale RNA Production System – T7, following the included protocol.

Apoptotic Induction

Induction of apoptosis in S2 cells was conducted using either H_2O_2, Cycloheximide (CHX), Staurosporine (STS), Actinomycin D (Act D) or UV irradiation. Induction with H_2O_2, CHX, STS and Act D was conducted by direct addition of the reagent to S2 cell cultures followed by 20 hr (H_2O_2) or 8 hr (CHX, STS, Act D) incubation at 26ºC. UV induced apoptosis was achieved by irradiation of cells in PBS with 100mJ of UV light followed by recovery in complete M3 media for 4 hours.
Transient Transfection

S2 cells were seeded 1x10^6 cells/mL in 2 mL of complete M3 media into six-well plates 30 min prior to transfection. Cells were transfected using Fugene 6 transfection reagent with modification to the manufacturer’s protocols (Roche). 15µL of Fugene 6 was diluted in 200µL of serum free DMEM (Sigma), vortexed briefly and incubated for 5 min. 2.5µg of total DNA (1.25µg of each vector) was diluted to 50µL in serum free DMEM, added to the diluted Fugene 6, vortexed briefly and incubated for 30 min. The reaction mixture was added dropwise to six-well plates seeded with S2 cells and incubated at 26ºC for two days. For expression of wild-type or mutant dOMI, 1.25µg of the respective pAWH dOMI vector was co-transfected with 1.25µg of an empty pAWH vector. For co-expression experiments with Rhomboid-7, 1.25µg of mutant pAWH dOMI was co-transfected with 1.25µg of either pAWF, wild-type or mutant pAWF Rhomboid-7 constructs. Additionally, 1.25µg of wild-type and mutant pAWF Rhomboid-7 were co-transfected with 1.25µg of pAWH. For calculation of transfection efficiency, 1.25µg of a pAWG (GFP reporter) vector was co-transfected with either 1.25µg of pAWH, wild-type or mutant pAWH dOMI. For cell death experiments involving CuSO₄ inducible promoters, either 2.5µg of empty pRm vector transfected, or 1.25µg of pRM was co-transfected with 1.25µg of wild-type or mutant pRM dOMI. Protein expression was induced using 0.7mM CuSO₄ (Bioshop), 24 hours following transfection and allowed to incubate for an additional 24 hours. For S2R+ transfections, cells were seeded at 15-20%
confluency the day prior to transfection and followed the same protocol as previously mentioned.

**RNAi mediated knockdown**

*Drosophila* S2 cells were diluted to a concentration of 1x10^6 cells/mL in M3 media supplemented with 10% FBS and 2mL were plated per well of a six-well plate. 15µg of dsRNAi targeting the desired molecule was added directly to the media and incubated for three days to permit turnover of pre-existing protein.

**Immunofluorescence**

For Immunofluorescence studies involving S2 cells, coverslips were pre-treated with 500µg of the lectin protein, concanavalin A (Sigma) for 20 min, aspirated and allowed to dry. Following a 1mL rinse with complete M3 media, 500µL of S2 cells were added to 1.5mL of complete M3 media and incubated for 2 h on treated coverslips. S2R+ cells were cultured in six-well plates containing coverslips for Immunofluorescence studies. Coverslips with either S2 or S2R+ cells were prepared according to a modified Mitosciences protocol. Culture media was aspirated from each well and coverslips were rinsed gently three times in PBS. Cells were fixed to coverslips in 4% paraformaldehyde (in PBS) for 10 min and rinsed three times in PBS. Cells were permeabilized with 0.1% of the detergent, Triton X-100 in PBS for 10 min at room temperature and rinsed three times in PBS. Cells were blocked overnight at 4ºC in 10% Goat serum (Invitrogen).
S2R+ cells were incubated for 2 hours at room temperature with primary antibodies (2µg/mL mouse anti-CVα (Mitosciences, MS502), 10µg/mL rabbit anti-HA (Sigma, H6908)) in 10% Goat serum, followed by three 10min rinses in 1% Goat serum. Cells were then incubated for 2 h in fluorophore-conjugated secondary antibodies (2µg/mL of Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 546 goat anti-mouse (Invitrogen, A-11070 and A-11030 respectively)) in 10% Goat serum at room temperature, away from light. Cells were rinsed three times 10 min in 1% Goat serum, away from light, and mounted on coverslips using Fluoromount-G (Southern Biotech). Cells were visualized using a Zeiss LSM 510 confocal microscope.

**Immunoprecipitation and Co-Immunoprecipitation**

Transfected S2 cells were harvested by centrifugation (600x g), rinsed in 1x PBS and suspended in 500µL of IP Buffer (150mM NaCl, 10mM Tris•HCl pH 7.4, 1mM EDTA, 1% TX-100, 0.5% NP-40), gently vortexed to suspend and placed at 4°C for 25min with shaking. Samples were spun at 16,000x g for 5min at 4°C and the supernatants were incubated with an anti-HA antibody (2µg/mL Sigma H6908) overnight at 4°C with shaking. 20µL of Protein-G Sepharose beads (Sigma, P3296) were added to each sample and incubated for 2 h at 4°C with shaking. Each sample was spun down at 600x g for 30 sec and 25µL of 2x SDS-PAGE sample buffer was added to the pellet. Samples were boiled for 2min prior to loading on SDS-PAGE gel.
Sub-cellular Fractionation

Sub-cellular fractionation was performed as described previously by Means et al. S2 cells were seeded at 3x10^6 cells per well (6 well plate) in triplicate, two days prior to fractionation. Cells were pooled and harvested by centrifugation at 1000 x g for 10 min. Pellets were resuspended in 500μL of Mitochondrial Isolation Buffer (20mM HEPES pH 7.5, 50mM KCl, 1.5mM MgCl₂, 1mM EDTA, 250mM Sucrose and 1mM DTT supplemented with Bioshop Protease Inhibitor Cocktail) and lysed by 25 strokes of a dounce homogenizer. Lysates were centrifuged at 1000 x g for 10 min at 4ºC to remove nuclei and unlysed cells. The supernatant was then centrifuged at 10,000 x g for 15 min at 4ºC, yielding the mitochondrial enriched pellet (MF) and aqueous cytosolic extract (CF). Protein precipitation of the CF was conducted by adding 50μL of 100% trichloroacetic acid (TCA) followed by 10 min incubation on ice. The CF was then centrifuged at 16,000 x g for 10 min at 4ºC and the supernatant was aspirated. Residual TCA was removed from the CF pellet by rinsing with cold acetone three times (centrifuging at 16,000 x g for 10 min at 4ºC between each rinse). Pellets were stored at -20ºC.

Immunoblotting

Cell lysates were run on 13% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked overnight at 4ºC in TBST (20mM Tris•HCl pH 7.6, 150mM NaCl, 0.05% Tween 20) containing 5% milk powder. Membranes were rinsed for 10 min in TBST then incubated for 2 h with the appropriate primary antibody (0.4μg/mL rabbit anti-HA (Sigma, H6908),
0.5µg/mL mouse anti-HA (Sigma, H3663), 1µg/mL mouse anti-CVα (Mitosciences, MS502), 0.2µg/mL mouse anti-FLAG M2 (Sigma, F1804)) in TBST containing 1% milk powder. Membranes were rinsed three times 5 min in TBST and incubated for 2 h with the appropriate secondary antibody (1:10,000 Peroxidase-conjugated Goat anti-Rabbit, 1:10,000 Peroxidase-conjugated Goat anti-Mouse (Jackson ImmunoResearch Laboratories Inc., 111-035-045 and 115-035-174 respectively)) in TBST containing 1% milk powder. Membranes were washed 3 times 5 min in TBST and developed using ECL Plus Western Blotting Detection Reagents (Amersham).

**Assay for Caspase Activity**

Caspase activity in S2 cells was determined using modified protocols for either the DEVD-pNA\textsuperscript{6} or DEVD-AFC\textsuperscript{75} substrates. S2 cells were centrifuged at 1000 x g for 5 min and washed with 500µL of PBS. Whole cell lysates were prepared by vortexing S2 cell pellets in Caspase buffer (50 mM HEPES pH 7.6, 5mM EDTA, 1% Triton X-100) followed by incubation for 10 min at 4°C with shaking. Lysates were clarified by centrifugation for 5 min at 16,000 x g and 4°C to remove nuclei and unlysed cells. Protein concentration of the supernatants was determined with a BCA Protein Assay Kit (Pierce). 50µg of each whole cell lysate was diluted to 100µL in Caspase buffer and incubated with 100µL Caspase buffer supplemented with 4mM DTT and either 200µM DEVD-pNA or 20µM DEVD-AFC (final concentration of 2mM DTT and 100µM DEVD-pNA or 10µM DEVD-AFC) in the dark for 2 h at 26°C. The extent of caspase activation was monitored on a SpectraMax M5 by absorbance (405nM) for
DEVD-pNA or by fluorescence (excitation: 400nm, emission: 505nm) for DEVD-AFC.
Chapter 3: Characterization of Expression

Patterns/Levels of dOMI, a Predicted Mitochondrial Protein of Pro-apoptototic Function
Introduction

Mammalian HtrA2 (also known as OMI) is identified as a mitochondrial protein involved in the onset of caspase dependant apoptosis. HtrA2 is initially expressed as a 458 aa immature protein that is post-translationally targeted to mitochondria by an N-terminal MTS. In addition to an N-terminal MTS, HtrA2 is comprised of three additional protein domains, a TM domain (aa 105-121), a trypsin-like protease domain (aa 182-330) and a protein binding PDZ domain (aa 390-445). HtrA2 also possesses several additional features including a 4 aa IBM, AVPS, located C-terminal to the TM domain at position 135\(^{12-16}\). A graphical depiction of HtrA2 architecture can be seen in figure 7A. Following import to the mitochondria, HtrA2 undergoes an autocatalytic processing event dependent upon the presence of a catalytic serine residue at position 306\(^{81}\). The end result is the generation of a mature HtrA2 protein with an exposed N-terminal IBM, AVPS (Fig 7B). This autocatalytic processing, in addition to the general proteolytic activity of HtrA2, relies upon the formation of an HtrA2 homotrimer. This homotrimer is formed through the trimerization motif, QYNFIA, located at residue 146. Mutation of F149 blocks trimerization and the protease activity of HtrA2\(^{82}\). The mature HtrA2 protein functions in a pro-apoptotic manner, activating the caspase cascade. Following exposure to an apoptotic stimulus, mature HtrA2 is released from the mitochondria into the cytosol following Bax/Bak mediated MOMP\(^{12-16}\). Cytosolic HtrA2 binds the caspase inhibitor XIAP via its BIR2 and BIR3 domains\(^{13,14}\) in a similar manner to the pro-apoptotic protein Diablo\(^{51-55}\). While Diablo functions through competitive binding of XIAP
over caspases 3, 7 and 9\textsuperscript{51-55}, HtrA2 also triggers its depletion via proteolytic cleavage\textsuperscript{57-59,80}. Despite the ability of HtrA2 to bind XIAP, its pro-apoptotic function is dependent upon the presence of the catalytic serine, 306, responsible for the degradation of XIAP\textsuperscript{13,14,57,80}. Therefore, HtrA2 amplifies the apoptotic stimuli through the removal of the caspase inhibitor XIAP.
Fig. 7. Domain Arrangement of human HtrA2 protein. (A) Graphical depiction of the immature HtrA2 protein consisting of an N-terminal MTS (aa 1-32), TM domain (aa 105-121), IBM (aa 135-138), trimerization motif (aa 146-151), serine protease domain (aa 182-330) containing a catalytic serine residue at position 306, and a PDZ domain (aa 390-445). (B) Graphical depiction of HtrA2 following autocatalytic processing resulting in the exposure of an N-terminal IBM, AVPS, at position 135.
While mitochondrial control of apoptosis has been widely studied in mammalian systems, the extent of its conservation, specifically within the model organism *Drosophila melanogaster* remains unclear. This is primarily due to conflicting evidence regarding the role of cytochrome C in the apoptotic program in *Drosophila*\(^{69-74}\). To study the potential involvement of the mitochondria upon the onset of apoptosis in *Drosophila*, an ortholog to the mammalian HtrA2 was identified as CG8464 or dOMI. This ortholog was identified from the *Drosophila* proteome through a blastp query using the human HtrA2 isoform 1 protein sequence obtained from the NCBI database as a reference. Following a Clustal W sequence alignment between human HtrA2 and the predicted *Drosophila* ortholog dOMI, a sequence identity of ~43\% was observed (Fig 8). The protease domain of human HtrA2, particularly the region containing the known catalytic serine 306\(^{13,14,57,80}\) is conserved in dOMI and predicts the presence of a catalytic serine at residue 266 (Fig 8). The dOMI protein sequence was analyzed by a series of web accessible domain prediction software programs including MitoProt (for prediction of N-terminal MTS) (Fig 9A), TMHMM (for TM domain prediction) (Fig 9B), and the NCBI CDD (alignment against known domains) (Fig 9C). In addition to general domain predictions, the dOMI protein sequence was analyzed for the presence of the IBM, AVPS\(^{12-16}\) as well as the known trimerization motif, QYNFIA\(^{82}\). A sequence resembling the HtrA2 IBM was identified within the predicted TM domain of dOMI with a sequence of AVVS at residue 75, while a sequence similar to the HtrA2 trimerization motif was found at residue 102 with the sequence DFNFIA. This data is summarized in figure 9D, as a graphical
representation of these predicted features of dOMI. While dOMI maintains high sequence conservation as well as notable similarities in predicted topology with that of its human ortholog HtrA2, its actual function within *Drosophila* is unknown. Recent publications have demonstrated a potential role for dOMI in programmed cellular death, specifically apoptosis\(^7,^8\), while mechanisms leading up to its pro-apoptotic function remain to be characterized.
**Fig. 8. Alignment of HtrA2 and the predicted Drosophila homolog, dOMI.**

Using the predicted protein sequence of CG6464 (dOMI) and the known human HtrA2 sequence, an alignment was conducted using ClustalV. The resultant alignment depicted an overall sequence conservation of approximately 43%. An aligned trimerization motif is shown boxed in blue while a region encompassing the HtrA2 catalytic serine residue is boxed in red. The resultant highly conserved region encompassing the catalytic serine residue in human HtrA2 (S366), places a predicted catalytic serine for dOMI at S266.
Fig. 9. Domain prediction for the dOMI protein. (A) The protein sequence of dOMI was entered into the MTS prediction software MitoProt, resulting in the prediction of an N-terminal MTS spanning the first 31 aa of dOMI. (B) Running dOMI through the prediction software TMHMM resulted in the identification of a probable TM helix between aa 63 and 83, potentially responsible for insertion into the mitochondrial inner membrane. (C) Schematic representation of the domains predicted to be present within dOMI as determined from a search of the Conserved Domain Databases (CDD) available through NCBI. This identified the presence of a trypsin-like serine protease domain between aa 142 and 299 as well as a general protein binding, PDZ, domain from aa 323 to 400. (D) Amalgamation of the predicted features of dOMI, including a predicted IEM motif within the TM domain (AVVS) from aa 75 to 78, as well as a trimerization motif (DFNFIA) found between aa 102 and 107.
In addition to being selected for its potential role in mitochondrial driven apoptosis, dOMI was also identified as a potential substrate candidate for the serine protease Rhomboid 7. Rhomboid 7, a 351 aa, seven pass transmembrane serine protease of the mitochondrial inner membrane functions in the process of mitochondrial fusion. Previously reported data has shown a requirement of Rhomboid 7 for regulation of mitochondrial dynamics, particularly that knockdown of this protein results in an increase in fragmented mitochondria suggesting the requirement of Rhomboid 7 in mitochondrial fusion\textsuperscript{29}. Mutations in \textit{Drosophila} Rhomboid 7 are linked to mitochondrial dysfunction leading to adverse effects upon neuronal function, fertility and viability\textsuperscript{29}. Rhomboid proteins function as intramembrane proteases, cleaving target molecules within the transmembrane domain\textsuperscript{98}. Specificity of intramembrane cleavage by Rhomboid proteins is achieved by recognition of a small GA motif located within the TM domain of the substrate molecule proposed to result in the disorder of typical helical structure due to its high conformational flexibility\textsuperscript{99}. Utilizing the known features of Rhomboid 7 as well as known functional data regarding Rhomboid mediated proteolysis, a set of theoretical criteria were determined for identification of potential Rhomboid 7 substrates. These criteria included; import of the substrate to the mitochondria denoted by the presence of a MTS, localization to the mitochondrial inner-membrane through the possession of a TM domain as well as the presence of a GA motif and subsequent predicted cleavage site located within the TM domain\textsuperscript{98-100,104}. The predicted \textit{Drosophila} HtrA2 ortholog, dOMI was determined to fit all of the aforementioned criteria,
containing an N-terminal MTS, a TM domain predicted to insert within the mitochondrial inner membrane as well as a predicted cleavage site containing the GA motif incorporated into the IBM AVVS, yielding a sequence of GAVVS, within the identified TM domain.

**Results and Discussion**

*Cloning of dOMI into Gateway System*

In order to study the role of the *Drosophila* HtrA2 ortholog, dOMI, a tissue culture approach was performed. This approach required the generation of DNA vectors containing the cDNA coding sequence of dOMI linked to an epitope tag. dOMI cDNA was PCR amplified to include a 5’ CACC sequence preceding the ATG start codon to allow for directional cloning into the pENTR/D vector. Additionally the amplified dOMI fragment was designed to lack a stop codon at its 3’ to allow for later addition of epitope tags via gateway destination vectors. To ensure directional ligation into the pENTR/D vector, a blunt-end producing DNA polymerase, Pfu Turbo, was used for amplification of dOMI. Pfu Turbo was also selected for its built in proofreading function in order to prevent the introduction of any mutations within the dOMI coding sequence. The resultant 1270bp amplified dOMI fragment, was ligated into the pENTR/D vector through Topoisomerase I mediated DNA ligation producing the 3850bp pENTR dOMI vector (Fig. 10A) which was confirmed by DNA sequencing.

To study the function of dOMI, a proposed catalytically inactive mutant was developed by site directed mutagenesis of the pENTR dOMI vector. This
was achieved by mutating the DNA codon of the proposed catalytic serine 266 to that of alanine. By conducting the mutagenesis upon the pENTR dOMI vector, generating a pENTR dOMI S266A vector, it was possible to conduct parallel cloning of both the wild-type and mutant dOMI, minimizing the possibility of variations between different constructs.

To produce transfectable dOMI vectors, the pENTR clones were subjected to LR clonase reactions with either the destination vectors pAWH or pAWF. This LR clonase reaction occurred through direct recombination of the attL1 and attL2 sites within the entry (pENTR) vector and the attR1 and attR2 sites of the destination (pAWH or pAWF) vectors. This recombination resulted in the transfer of either the dOMI or dOMI S266A coding sequence into either pAWH or pAWF from pENTR, while replacing it with a copy of the ccdB gene, originally found in the pAWH and pAWF vectors (Fig. 10B). ccdB is a gene that inhibits the growth of E.coli through inhibition of DNA gyrase\textsuperscript{111,112}, allowing it to be used as a means by which to remove unwanted by-products of the LR clonase reaction. Following transformation of the LR clonase reaction mixture into E.coli cells, cells transformed with either pAWH dOMI or pAWF dOMI are capable of producing colonies while those transformed with the pENTR ccdB vector do not form colonies due to ccdB mediated inhibition. pAWH and pAWF were chosen as destination vectors for dOMI since they both utilize the Actin 5C promoter for control of protein synthesis, allowing for expression within insect cells, specifically *Drosophila*. Additionally, these destination vectors provided the addition of C-terminal epitope tags as opposed to N-terminal tags since dOMI
contains a predicted, cleavable N-terminal MTS, thus allowing detection of the mature protein. While pAWH provides a 3xHA epitope tag, pAWF provides a 3xFLAG epitope tag, both of which are maintained in-frame with dOMI allowing for the expression and detection of dOMI fusion protein within *Drosophila* cells. The DNA sequence of each vector was confirmed through DNA sequence analysis indicating the in-frame insertion of the wild-type or mutant dOMI into pENTR, pAWH and pAWF.
Fig. 10. Gateway cloning of dOMI. (A) PCR amplified dOMI cDNA is ligated into pENTR/D TOPO via Topoisomerase I, yielding the pENTR dOMI vector. Directional cloning is facilitated by a 3' CACC overhang on the dOMI cDNA. (B) pENTR dOMI is subjected to an LR clonase reaction with the pAWH destination vector, resulting in the generation of the pAWH dOMI vector. This causes direct recombination of the attL1 and attL2 sites of pENTR dOMI with the attR1 and attR2 sites of pAWH, producing recombinant pAWH dOMI (viable in E. coli) and pENTR codB (not viable in E. coli) vectors.
Exogenous Expression of dOMI in Drosophila S2 Cell Line Reveals Several Distinct Forms Suggesting a Series of Processing Events

In order to test the validity of the generated constructs, *Drosophila* S2 cells were transfected with tagged dOMI constructs. Expression of both the wild-type and mutant dOMI constructs resulted in several distinct forms, indicating the possibility of a series of processing events (fig 11). These distinct forms are predicted to account for the full length protein (~53kDa), a MTS cleaved form (~50kDa), a TM cleaved form (~47kDa) and two additional, as of yet unidentified forms (~58kDa and ~45kDa). Expression of the catalytically inactivated mutant dOMI identified two main differences between that of the wild-type dOMI. First, the overall expression levels of the mutant are approximately 25x higher than that of the wild-type as determined by comparison of the CVα loading controls (fig 11), suggesting an increased transfection efficiency for that of the mutant or that the stability of the mutant protein is greater than that of the wild-type. The second difference in figure 11 is the overall pattern of expression between the wild-type and mutant dOMI proteins. While the wild-type protein predominantly expresses the full length protein, the predominant mutant form corresponds to the MTS cleaved protein. Additionally, the mutant dOMI displays two additional forms not observed in cells expressing the wild-type, which may represent additional processing/modification.
Fig. 11. Exogenous expression of wild-type and mutant dOMI in S2 cells. S2 cells were transiently transfected with HA tagged dOMI or HA tagged dOMI S266A mutant and whole cell lysates were assayed by western using an HA antibody for detection of dOMI and a CVα antibody as a loading control. Banding pattern variations between the wild-type and mutant protein can be seen with the mutant maintaining two additional bands not present in the wild-type sample. The identifiable bands correspond to molecular weights of approximately 58kDa, 53kDa, 50kDa, 47kDa and 45kDa, with the latter two only present within mutant samples. The mutant dOMI sample was loaded at a 25x dilution in comparison to that of the wild-type dOMI sample, indicating roughly a 25x higher expression of the mutant protein that of the wild-type.
To determine the possible cause for the difference in expression levels, S2 cells were transiently transfected with either an empty vector, the wt or mutant dOMI constructs combined with a plasmid containing eGFP under the control of the Actin 5C promoter allowing for constitutive expression of this reporter. S2 cells harvested from this series of co-transfections were observed by fluorescence microscopy to quantify cells expressing GFP (Fig 12). Constitutive over-expression of wild-type dOMI resulted in a significant reduction in the both the total number of cells expressing the GFP reporter in comparison to both the null transfection (using an empty pAWH vector) as well as to that of the mutant dOMI construct. This suggests that the wild-type dOMI may function in a pro-apoptotic manner resulting in the loss of the cell population transfected with dOMI. Additionally the presence of the catalytic serine to alanine point mutation did not significantly alter transfection efficiency of the reporter construct. Taken together, these data indicate that expression of dOMI involves several processing events in Drosophila cells leading to the formation of the pro-apoptotic mature protein whose function is dependent on a catalytic serine at position 266.
Fig. 12. Assay of overall dOMI transfection in S2 cells using a GFP reporter. S2 cell cultures co-transfected with a GFP reporter plasmid as well as either wild-type or the S266A mutant dOMI constructs were scored for cells expressing the GFP reporter. Those cells expressing GFP were identified by fluorescence microscopy as cells positively transfected and values were compared to the total number of cells visible by light microscopy. It was observed that cultures transfected with the wild-type dOMI construct resulted in a lower overall transfected cell population as opposed to both the empty vector as well as the mutant dOMI. This was taken as an initial indication that transfection of dOMI in S2 cells results in a loss of cells dependent upon the presence of a catalytic serine residue at S266, presumably a result of apoptosis.
Knockdown of dOMI By RNAi

In addition to observing the effect of exogenously expressed dOMI in Drosophila cells, dsRNAi mediated knockdown provides another means by which to observe the function of dOMI. Since an antibody targeting the endogenous dOMI was initially unavailable, the extent of dOMI knockdown by dsRNAi was monitored using an exogenously expressed, tagged dOMI. Treatment of S2 cells with 15µg of dOMI dsRNAi followed by transfection of the HA tagged dOMI displayed complete knockdown of exogenous dOMI expression (fig 13). Despite using an exogenous dOMI construct, the target sequence for the RNAi remains conserved between both the genomic dOMI sequence (the first exon) as well as the transfected cDNA sequence. Due to this sequence conservation, it can be assumed the observed knockdown (fig 13), is indicative of endogenous knockdown within S2 cells. Knockdown of dOMI was recently confirmed by Challa et al using dsRNAi targeting the same region of the dOMI coding sequence as used in these studies\textsuperscript{78}. RNAi mediated dOMI knockdown was further used for assaying caspase activation in the absence of dOMI.
Fig. 13. RNAi mediated knockdown of dOMI in S2 cells. S2 cells transiently transfected with wild-type HA tagged dOMI were subjected to treatment with 15μg of dOMI RNAi and lysates were assayed by western. Presence of dOMI was assayed using a rabbit HA antibody, while mouse Actin antibody was used as a loading control. Treatment with dOMI RNAi was shown to cause complete knockdown of exogenously expressed dOMI, inferring its capability to cause knockdown of endogenous dOMI.
Exposure to Apoptotic Stimuli Results in Altered dOMI Expression

It has been proposed that dOMI functions in a similar pro-apoptotic manner to that of its mammalian counterpart HtrA2\textsuperscript{12-16,57-59}, therefore, the expression of dOMI was observed under apoptotic stress. A series of apoptotic stimuli were tested to identify the optimal conditions for apoptotic induction resulting in the use of 10mM H\textsubscript{2}O\textsubscript{2}, responsible for the generation of ROS resulting in the onset of apoptosis (Refer to Chapter 4). S2 cells were transfected with both HA tagged wild-type and mutant dOMI prior to treatment with H\textsubscript{2}O\textsubscript{2}. Following western blotting, an alteration in the processing of both the wild-type and mutant dOMI was observed following apoptotic stress (fig 14). With wild-type dOMI the proposed MTS cleaved band (~50kDa) was reduced while the proposed full-length protein band (~53kDa) appeared unaffected. This alteration in dOMI expression indicates either a degradation or subsequent processing event occurs in response to the onset of apoptosis. Conversely, the mutant dOMI exhibited a shift from the predominant MTS cleaved form (~50kDa), to that of one of the shorter proposed TM cleaved form (~47kDa), not visible in the wild type banding pattern. The processed form of the mutant dOMI corresponds to the mature form of the protein, rendered inactive through mutation of its catalytic serine residue. These findings suggest that exposure to an apoptotic stimulus results in the further processing of dOMI from its ~50kDa MTS cleaved form to the ~47kD, TM cleaved active form responsible for sensitizing cells to apoptosis, and that this active form is not detectable in cells expressing the wild-type protein due to its detrimental effect upon cell viability.
This hypothesis is further supported by the evidence observed in figure 12, where expression of wt dOMI and not the mutant, was responsible for a decrease in the population of transfected cells.
Fig. 14. Effect of H₂O₂ induced apoptosis upon dOMI expression. S2 cells transfected with dOMI wild-type or S266A mutant constructs were treated with 10 mM H₂O₂ and assayed for dOMI expression by western. In either case an observable modification in dOMI expression was apparent wherein exposure of the wild-type dOMI to an apoptotic stimulus resulted in the loss of its ~50kDa band, while the mutant showed a transition from the ~50kDa band to that of its ~47kDa band, indicating the presence of a distinct processing event.
To monitor the subcellular localization of dOMI under normal and apoptotic conditions in S2 cells, two separate approaches were applied using exogenously expressed dOMI. First, S2 cells were transiently transfected with either wild-type or mutant dOMI, lysed under mechanical pressure and subjected to differential centrifugation. This was conducted in both the presence and absence of apoptotic induction with 10mM H$_2$O$_2$ to observe any changes in localization following the onset of apoptosis. Expression of wild-type dOMI, was observed only in mitochondrial enriched fractions, regardless of treatment (Fig 15). Cells treated with H$_2$O$_2$, displayed drastically reduced expression levels of dOMI within the mitochondrial enriched fraction without any subsequent detection within the cytosolic extract. This dramatic loss of detectable wild-type dOMI indicated that the loss of dOMI may be the result of the death of the small population of cells transfected with dOMI following apoptotic induction. S2 cells transfected with a mutant dOMI subjected to the same treatment exhibited the same localization pattern with dOMI being detectable only in mitochondrial enriched fractions, despite treatment with an apoptosis inducing reagent (fig 15). In contrast to the wild-type, mutant dOMI was not subject to a loss in total signal, instead, the pattern of expression is notably altered, resulting in the accumulation of the smaller ~47kDa band, with the depletion of the larger ~50kDa, coinciding with previously described data (fig 14). In either case, the lack of detectable dOMI within the cytosolic extract is not indicative of its inability to be released
from the mitochondria but may indicate that dOMI is contained in some other subcellular compartment following the onset of apoptosis.
Fig. 15. Subcellular fractionation of S2 cells transfected with wild-type or mutant dOMI in the presence and absence of apoptotic stress. Transfected S2 cells were cultured in either the presence or absence of 10 mM H$_2$O$_2$ in order to induce apoptosis. Harvested samples were subjected to mechanical lysis of the plasma membrane followed by differential centrifugation to obtain fractions representative of mitochondrial enrichment (MF) or of the cytosol (CF). Purity of the fractions was assessed using the mitochondrial marker CVa and the cytosolic marker Actin. Both wild-type and mutant dOMI were observed to maintain a mitochondrial localization in both the presence or absence of apoptotic stimuli while maintaining the profiles observed in figure 14.
To further evaluate the subcellular localization of dOMI in S2 cells, cells were transfected with either wild-type or mutant dOMI then subjected to immunohistochemistry and confocal microscopy to determine dOMI localization. Under normal conditions, both wild-type and mutant dOMI co-localized with the mitochondrial protein CVα, indicating the presence of dOMI within the mitochondria (fig 16). Exposure of transfected S2 cells to a variety of apoptotic stimuli including H₂O₂, UV and STS resulted in an inability for the S2 cells to adhere to coverslips prior to immunohistochemistry and as such an inability to assess the localization of dOMI under these conditions. To overcome this caveat, a second cell line, S2R+, was used which is derived from the Drosophila S2 cell line. The S2R+ line propagates as an adherent layer, requiring no additional treatment of slides prior to immunohistochemical analysis, unlike its parental S2 line. S2R+ cells transfected with either wild-type or mutant dOMI showed co-localization with CVα (fig 17), indicating mitochondrial localization similar to that observed in S2 cells (fig 16). Following exposure to the apoptotic inducers, H₂O₂ or STS, S2R+ cells lost adherence to the coverslips, while exposure to UV irradiation had no such effect. S2R+ cells were transfected with either wild-type or mutant dOMI then subjected to UV irradiation to induce apoptosis. In S2R+ cells exposed to UV light, exogenous wt dOMI did not entirely co-localize with the mitochondrial marker CVα (fig 18), indicating release from the mitochondria following UV induced apoptosis. While dOMI does not entirely co-localize with a mitochondrial marker, it fails to display the typical diffuse cytoplasmic pattern as seen in mammals (fig 19)¹²,¹⁶, instead displaying a
punctate pattern that is non-mitochondrial. S2R+ cells transfected with the mutant dOMI also displayed a punctate expression pattern following UV irradiation, albeit more pronounced than that of the wild-type, that did not appear to co-localize with CVα (fig 18). The notable difference between the punctate expression of wild-type and mutant dOMI post UV irradiation remains uncharacterized and may be an artifact of over-expression. While dOMI is seen to localize to the mitochondria under normal conditions (fig 16, 17), a punctate, non-mitochondrial pattern observed for both the wild-type and mutant dOMI following exposure to apoptotic stress (fig 18) may indicate translocation to another subcellular compartment following mitochondrial release.
Fig. 16. Mitochondrial localization of exogenous HA tagged wild-type and S266A mutant dOMI in transiently transfected S2 cells as observed by immunofluorescence. (A) Wild-type dOMI as detected by a rabbit antibody specific to the HA tag (shown in green). (B) Detection of the mitochondrial marker CVa in using a mouse derived antibody (shown in red). (C) Merged image displaying co-localization of wild-type dOMI and CVa in S2 cells (shown in yellow). (D) Mutant dOMI as detected by a rabbit antibody specific to the HA tag (shown in green). (E) Detection of the mitochondrial marker CVa in using a mouse derived antibody (shown in red). (F) Merged image displaying co-localization of mutant dOMI and CVa in S2 cells (shown in yellow).
Fig. 17. Mitochondrial localization of exogenous HA tagged wild-type and S266A mutant dOMI in transiently transfected S2r+ cells as observed by immunofluorescence. (A) Wild-type dOMI as detected by a rabbit antibody specific to the HA tag (shown in green). (B) Detection of the mitochondrial marker CVa in using a mouse derived antibody (shown in red). (C) Merged image displaying co-localization of wild-type dOMI and CVa in S2r+ cells (shown in yellow). (D) Mutant dOMI as detected by a rabbit antibody specific to the HA tag (shown in green). (E) Detection of the mitochondrial marker CVa in using a mouse derived antibody (shown in red). (F) Merged image displaying co-localization of mutant dOMI and CVa in S2r+ cells (shown in yellow).
Fig. 18. Mitochondrial release of exogenously expressed HA tagged wild-type and S266A mutant dOMI in S2r+ cells following UV induced apoptosis. Transfected cultures were treated with 100mJ of UV in phosphate buffered saline and allowed to recover for 4 hours in complete M3 media. (A) Wild-type dOMI as detected by a rabbit antibody specific to the HA tag (shown in green). (B) Detection of the mitochondrial marker CValpha in using a mouse derived antibody (shown in red). (C) Merged image displaying punctate, non-localized wild-type dOMI (shown in green) and CValpha (shown in red) in S2r+ cells. (D) Mutant dOMI as detected by a rabbit antibody specific to the HA tag (shown in green). (E) Detection of the mitochondrial marker CValpha in using a mouse derived antibody (shown in red). (F) Merged image displaying punctate, non-localized mutant dOMI (shown in green) and CValpha (shown in red) in S2r+ cells.
Fig. 19. Mitochondrial release of exogenously expressed HtrA2 following STS induced apoptosis in HeLa cells. HtrA2-myc transfected HeLa cells were visualized by confocal microscopy using fluorophore conjugated secondary antibodies. Apoptosis was induced in transfected cells using 1μM STS (D-F). (A,D) HtrA2 as detected by rabbit anti-MYC antibody is shown in green. (B,E) Mitochondria as detected by mouse anti-CVa antibody is shown in red. (C, F) Co-localization of HtrA2 and the CVa mitochondrial marker is shown in yellow.
Identifying dOMI as a Substrate for the Mitochondrial Serine Protease Rhomboid 7

With dOMI meeting the requirements for Rhomboid 7 substrates\textsuperscript{98-100,104}, the interaction between these two proteins was assessed through a series of co-expression experiments. FLAG tagged Rhomboid 7 wild-type and catalytically inactive mutant constructs were designed by Dr. Jeffrey Lee using the Gateway system and Rhomboid 7 cDNA obtained from the DGRC. S2 cells co-transfected with wild-type dOMI and either wild-type or mutant Rhomboid 7 resulted in loss of signal for both proteins in each case (data not shown), the cause of which remains unknown. To overcome this, mutant dOMI was used for co-expression with Rhomboid 7. Following co-expression with wild-type Rhomboid 7, mutant dOMI was observed to undergo a shift in its expression resulting in depletion of the ~50kDa band and accumulation of the ~47kDa band, not apparent in co-expression with the catalytically inactive Rhomboid 7 S256A (fig 20). Despite Rhomboid 7 mediated accumulation of the ~47kDa dOMI band, subsequent accumulation of the ~45kDa band was not observed, identifying another processing event independent of Rhomboid 7. Alteration in mutant dOMI banding pattern mimics the effect observed following exposure of cells to apoptotic stress (fig 14), suggesting a potential role for Rhomboid 7 in the catalytic processing of dOMI into its ~47kDa form following the onset of apoptosis.
Fig. 20. Rhomboid 7 mediated processing of OMI S266A. HA tagged dOMI S266A mutant and FLAG tagged dRho7 wild type or S256A mutant were expressed both separately and in conjunction within S2 cells. Lysates were immunoblotted using the respective antibodies while an antibody targeting CVA was used as a loading control. Co-expression of mutant dOMI with wild-type Rhomboid 7 and not the mutant displayed a transition from the ~56kDa band to that of the ~47kDa band of dOMI indicating the necessity of catalytically active Rhomboid 7 for this transition.
To further evaluate the validity of this potential processing of dOMI by Rhomboid 7, a series of IPs were conducted to identify any direct interaction between the two proteins. Transfected S2 cell lysates were subjected to IP via dOMI and assayed for the presence of both dOMI and Rhomboid 7. In the presence of mutant dOMI, both wild-type and mutant Rhomboid 7 proteins were observed to be pulled down by IP (fig 21), indicating that dOMI and Rhomboid 7 likely form a direct interaction of high affinity. In addition to the observed protein-protein interaction of Rhomboid 7 and dOMI by IP, the banding pattern remained consistent with previous co-expression experiments, supporting Rhomboid 7 mediated processing of dOMI.
Fig. 21. Observed protein-protein interaction between dOMI S266A and Rhomboid 7. HA tagged dOMI S266A mutant and FLAG tagged dRho7 wild type or S258A mutant were expressed both separately and in conjunction within S2 cells. Cells were lysed and subjected to immunoprecipitation using a rabbit anti-HA antibody to pull down dOMI S266A followed by immunoblotting for both dOMI S266A (mouse anti-HA) and FLAG Rho7 wt or S266A mutant (iM2 FLAG). Rhomboid 7 was shown to be pulled down in the presence of dOMI indicating a potential, direct interaction between the two proteins, independent Rhomboid 7's catalytic activity.
Chapter 4: Activation and Modulation of Apoptosis in *Drosophila* via the Caspase Cascade
Introduction

The caspase cascade represents an evolutionary conserved component of the programmed cell death machinery which is commonly referred to as apoptosis. Normal function of this pathway is involved in the establishment of cellular homeostasis as well as embryonic development. Apoptosis allows for the removal of damaged or unnecessary tissue such as soft tissue present between digits of humans during early gestation, while modifications or alterations in the normal function of this pathway can have detrimental effects upon the organism such as Syndactyly (the formation of fused digits in humans). Mutations resulting in stimulation of this pathway lead to increased cellular death resulting in tissue degradation, observed in many neurodegenerative diseases such as Parkinson’s disease. Conversely, mutations causing desensitization to death stimuli result in uncontrollable cellular proliferation as observed in cancer. A key checkpoint in the apoptotic program is achieved through the regulation of the caspase cascade. Activation of this cascade is responsible for a variety of downstream detrimental processes including general proteolysis, disassembly of the actin cytoskeleton, as well as chromosomal condensation and subsequent DNA degradation. Regulation of the caspase cascade is achieved in a two-fold manner involving expression of inactive pro-caspase molecules as well as inhibition via interaction with IAPs. In Drosophila, the caspase cascade functions in a manner comparable with that of the known mammalian pathway, with functional homologs of APAF-1, Caspase-9, Caspase-3 and XIAP being represented by
the proteins dARK$_{49,50,60-63}$, Dronc$_{49,50,61,64,65}$, drICE$_{49,50,61,64-67}$ and IAP1$_{49,50,60,61,68}$ respectively. Following exposure to apoptotic stimuli, dARK activates the initiator caspase Dronc through the formation of the apoptosome. This active Dronc cleaves the pro-caspase-3 homolog drICE, activating it. Activation of drICE is also regulated by interaction with an anti-apoptotic molecule, IAP1$_{49,50}$. IAP1 binds Dronc and drICE via interaction with its BIR repeats inhibiting interaction with downstream effectors of the apoptotic pathway$_{49,50,61,64-68}$. Alleviation of caspase inhibition in mammals is achieved by the release of mitochondrial pro-apoptotic factors$_{2-6,10,11}$ such as Diablo$_{52-55}$ and HtrA2$_{12-16,57-59}$ to the cytosol, recently published data has indicated that a similar mechanism may be employed in Drosophila through the mitochondrial serine protease dOMI$_{78,79}$. Apoptosis in Drosophila, can be monitored experimentally through activation of the effector caspase drICE. drICE recognizes the peptide sequence DEVD and proteolytically cleaves proteins containing this sequence C-terminal to the second aspartic acid residue$_{6,66,67,69,75}$. The requirement of this sequence for drICE mediated proteolysis allows for the use of synthetic DEVD substrates containing reporter molecules covalently bonded to the C-terminal aspartic acid residue. Two such molecules are the colorimetric substrate Ac-DEVD-pNA$_6$ and the fluorometric substrate Ac-DEVD-AFC$_{75}$. Exposure of the synthetic substrates to active drICE results in the release of the reporter molecule through proteolytic cleavage, allowing for detection of the free reporter (fig 22). Using this assay, the extent of apoptosis can be assessed in Drosophila cytosolic extracts via the
capability of the effector caspase drICE to cleave the synthetic DEVD reporter substrate.
Fig. 22. Schematic of DEVD assay principle. (A) Synthetic peptides Ac-DEVD-pNA or Ac-DEVD-AFC, are recognized and cleaved by Caspase-3 (mammals)/drICE (Drosophila), thus resulting in the release of the covalently bonded reporter molecule pNA or AFC respectively. The free reporter molecules are detected by either colorimetric assay at 405nm (pNA) or through a fluorometric assay with an excitation of 400nm and emission of 505nm (AFC). (B) Representative chemical cleavage of the synthetic peptide substrate Ac-DEVD-pNA showing the release of free pNA.
Results and Discussion

*Induction of the Caspases Cascade in Response to Cellular Stress*

Conditions for apoptotic induction were determined using a variety of reagents by monitoring activation of the caspase cascade, more specifically the activity of the effector caspase drICE\(^{6,66,67,69,75}\). S2 cell lysates were tested for their capability to recognize and cleave the peptide sequence DEVD, using either the colormetric or fluorometric substrates DEVD-pNA\(^6\) and DEVD-AFC\(^75\) respectively. Initially, H\(_2\)O\(_2\) was tested for its ability to activate this pathway and was shown to have a dose dependant activation of drICE (fig 23). This response occurred optimally following 20 h of treatment with 10mM of H\(_2\)O\(_2\), while increased concentrations resulted in a decrease in detectable caspase activity, either due to complete cell death or to the onset of necrosis in treated cells.
Fig. 23. Dose dependent activation of the caspase cascade in S2 cells in response to H$_2$O$_2$ treatment. S2 cells were treated with a range of H$_2$O$_2$ concentrations for 20hrs, and then lysates were assayed for the ability to cleave the synthetic dTrICE substrate Ac-DEVD-AFC. DEVDase activity was shown to have a maximal activity following treatment with 10mM H$_2$O$_2$, followed by a drastic drop in caspase activity.
Several additional reagents were employed to induce apoptosis, including the protein synthesis inhibitor CHX, which binds the 80S ribosomal subunit, inhibiting translation\textsuperscript{117}, the RNA synthesis inhibitor Actinomycin D, which forms complexes with single-stranded DNA, inhibiting RNA polymerase function\textsuperscript{118,119}, the phospholipid/calcium dependent protein kinase inhibitor STS\textsuperscript{120}, as well as through DNA damage via UV irradiation\textsuperscript{121}. For CHX, Actinomycin D and STS, caspase activity was assessed following exposure to the respective reagent for 8 h (fig 24) using previously identified concentrations. The use of an altered time frame for apoptotic induction with these reagents was necessary since longer exposure to both CHX and STS resulted in complete cell death (data not shown). Figure 24 displays the extent of caspase activation for each of these reagents indicating the ability for both 10\mu M CHX and 1uM STS to induce apoptosis, while 1\mu M Actinomycin D had no significant effect upon caspase activity. Despite CHX’s ability to induce caspase activation, it was not used for further studies due to its method of action (inhibition of protein synthesis)\textsuperscript{117} being counterproductive to the expression of exogenous proteins.
Fig. 24. Alternate activation of the caspase cascade. S2 cells were treated with 10μM CHX (*), 1μM Actinomycin D (*) or 1μM STS (†) for 6hrs then assayed for caspase activity using the synthetic substrate Ac-DEVD-pNA. Both CHX and STS were shown to cause activation of the caspase cascade while treatment with Actinomycin D showed no discernable effect.
Inhibition of the Apoptosis Achieved Through a Broad Spectrum Caspase Inhibitor

The broad-spectrum, competitive caspase inhibitor zVAD-fmk inhibits activation of the caspase cascade, preventing the onset of apoptosis\textsuperscript{122,123}. While previous experiments established the conditions for activation of the caspase cascade (fig. 23, 24), it was necessary to show that the previous signals were in fact a result of caspase activation and not experimental artifacts. This was achieved by treating S2 cells to zVAD-fmk prior to apoptotic stress, thus inhibiting the activation of the caspase cascade while lysates were assayed for DEVDase activity (fig 25). zVAD-fmk completely inhibited DEVDase activity in S2 cells, indicating the previously observed DEVDase activities (fig 23, 24) were the result of activation of the caspase cascade, more specifically drICE. The use of zVAD-fmk validated the DEVDase assay in S2 cells, providing a valuable tool for studying the effects of exogenous factors upon the onset of apoptosis and activation of the caspase cascade.
Fig. 25. Inhibition of caspase activity through the general caspase inhibitor zVAD-fmk. S2 cells were treated with 10mM H₂O₂ for 20hrs in the presence or absence of the known caspase inhibitor zVAD-fmk and assayed for caspase activity using Ac-DEVD-pNA as a synthetic substrate. zVAD-fmk was shown to reduce caspase activity to that of baseline levels apparent in untreated S2 cells even following H₂O₂ treatment.
To further validate the use of the DEVDase assay for effectors of the caspase cascade, RNAi mediated protein knockdown was conducted on S2 cells targeting a variety of known pro and anti-apoptotic factors\textsuperscript{60,75}. To assess the ability for pro-apoptotic effectors to affect the activation of the caspase cascade, dsRNAi targeting IAP1 inhibitors, Reaper and Grim proteins were selected\textsuperscript{75}. Conversely, the inhibitor of apoptosis proteins IAP1 and IAP2 were selected for RNAi mediated knockdown to assess the ability of anti-apoptotic effectors to modulate the activation of the caspase cascade\textsuperscript{60}. In either case, RNAi constructs were generated according to previously published reports identifying specific DNA sequences necessary for knockdown of the target proteins\textsuperscript{60,75}. S2 cells were subjected to RNAi treatment prior to chemically driven induction of apoptosis, then tested for caspase activity via the DEVD assay (fig 26). S2 cells treated with IAP1 dsRNAi displayed increased sensitization to apoptotic stress when subjected to the apoptosis-inducing agent H\textsubscript{2}O\textsubscript{2}, and in addition underwent spontaneous apoptosis in cells not treated with H\textsubscript{2}O\textsubscript{2}. While IAP1 knockdown was observed to trigger increased caspase activation, RNAi targeting IAP2 had no significant effect upon caspase activity. Treatment of S2 cells with RNAi targeting the pro-apoptotic protein Reaper caused a protective effect upon cells treated with H\textsubscript{2}O\textsubscript{2}. This was observed through the significant decrease in DEVDase activity of cells pre-treated with Reaper RNAi as compared to control S2 cells (n=3, p-value=0.0019). S2 cells treated with Grim dsRNAi displayed no
significant effect on caspase activation following \( \text{H}_2\text{O}_2 \) treatment. To assess the potential role of dOMI in caspase dependant apoptotic signaling, RNAi mediated dOMI knockdown in S2 cells was conducted under normal and pro-apoptotic conditions (fig 26). S2 cells treated with RNAi targeting dOMI displayed no alteration in DEVDase activity in either the presence or absence of the apoptotic reagent \( \text{H}_2\text{O}_2 \). This indicates that canonical apoptotic signaling in \textit{Drosophila} occurs independently of dOMI, contrary to previously observed results (fig 12), or the presence of redundant regulation of caspase activation.
Fig. 26. Effect of RNAi mediated knockdown upon activation of the caspase cascade. (A) S2 cells were treated with either 15μg of IAP1 or dOMI RNAi for 3 days followed by treatment with 10mM H2O2 for 20hrs. Caspase activity was assayed via cleavage of the Ac-DEVD-AFC substrate. IAP1 RNAi was shown to cause an increase in caspase activity while dOMI RNAi showed no significant effect. (B) S2 cells were subjected to RNAi mediated knockdown of IAP1, IAP2, Grim, Reaper or dOMI followed by treatment with 4mM H2O2 for 20hrs. Assay of caspase activity using the substrate Ac-DEVD-AFC revealed an IAP1 RNAi mediated increase in caspase activity and a Reaper RNAi mediated decrease in caspase activity, while IAP2, Grim and dOMI RNAi showed no significant effects upon caspase activation following H2O2 treatment.
The experiments shown in figure 26 determined that both sensitization and desensitization of S2 cells to apoptotic stimuli could be achieved through modulation of pro-apoptotic (Reaper) and anti-apoptotic (IAP1) effectors of the caspase cascade, while depletion of a proposed mitochondrial effector of apoptosis, dOMI had no effect. While the inability of dOMI knockdown to alter caspase dependant apoptotic apoptosis suggests a lack dOMI involvement in apoptosis, it may indicate the presence of a redundant apoptotic pathway, active in the absence of dOMI.

Modulation of the Caspase Cascade through Expression of dOMI,
Elucidating a Pro-apoptotic Role in Drosophila

Previous studies determined that HtrA2 was responsible for a pro-apoptotic effect in mammalian cells resulting in activation of the caspase cascade\textsuperscript{12-16,57-59,80}, while the Drosophila ortholog, dOMI, remained uncharacterized. While it has been experimentally shown that dOMI processing is altered following exposure to apoptotic stress (fig 14), it was necessary to determine if dOMI was in turn was capable of altering the onset of apoptosis. To test the effect of dOMI upon apoptosis, exogenous wild-type and mutant dOMI were constitutively expressed in S2 cells followed by treatment with \( \text{H}_2\text{O}_2 \), while the extent of apoptosis was monitored via DEVDase activity (fig 27). S2 cells transfected with both wild-type and mutant dOMI showed no alteration in
caspase activation following treatment with H$_2$O$_2$, suggesting a lack of dOMI involvement in modulation of the caspase cascade.
Fig. 27. Lack of dOMI mediated modulation of the caspase cascade following exposure to H$_2$O$_2$. S2 cells transiently transfected with the constitutive wild-type or S266A mutant dOMI constructs were treated with 10mM H$_2$O$_2$ for 20hrs prior to assay of caspase activity using Ac-DEVD-pNA. Neither wild-type or mutant dOMI showed any significant effect upon caspase activity, nor did the vector control pAWH, while S2 cells treated with IAP$^1$ RNAi displayed an increased caspase activity following H$_2$O$_2$ treatment.
To validate this finding, apoptosis was induced through STS treatment of cells, expressing the wild-type or mutant dOMI and DEVDase activity was observed (fig 28). In agreement with previous data, cells expressing either the wild-type or mutant dOMI showed no modification of caspase activity, indicating a lack of dOMI involvement in caspase driven apoptosis.
Fig. 28. Lack of dOMI mediated modulation of the caspase cascade following exposure to STS. S2 cells transiently transfected with the constitutive wild-type or S265A mutant dOMI constructs were treated with 1mM STS for 12hrs prior to assay of caspase activity using Ac-DEVD-AFC. Neither wild-type or mutant dOMI showed any significant effect upon caspase activity, nor did the vector control pAWH following STS treatment.
Since these findings were contrary to both previous indications of pro-apoptotic dOMI function (fig 12) as well as studies indicating the function of its mammalian homolog, HtrA2^{12-16,57-59,80}, it became necessary to identify caveats of the current assay. As previously identified (fig 12), maximum transfection efficiency was occurring at approximately 5% of all cells in culture while cells expressing the wild-type dOMI construct only accounted for roughly 1% of the total population. This indicated the possibility that while an effect may be occurring in those cells expressing dOMI following apoptotic induction, any modification to cell death may be masked by the signal derived from the population of cells that remain un-transfected. This led to the identification of a potential drawback involving the use of constructs employing constitutive over-expression via the Actin 5C promoter. By constitutively over-expressing dOMI, those cells transfected with the wild-type construct may undergo apoptosis prematurely resulting in the loss of the transfected population (as seen in fig 12) prior to treatment with apoptosis inducing agents. Constructs with dOMI expression under the control of an inducible promoter would allieviate this problem, allowing for protein expression just prior to induction of apoptosis. A publication by Challa et al, conducted a series of experiments using such a construct, wherein dOMI expression was placed under the control of the CuSO₄ promoter. Their data indicated a pro-apoptotic role of wild-type following treatment with the apoptotic agent STS, resulting in activation of the caspase cascade^{78}. The constructs used by Challa et. al. were obtained, and tested using the previously described DEVDase assay. Transfected S2 cells were treated
with CuSO$_4$ to induce expression of either wild-type or mutant dOMI then exposed to STS prior to assaying DEVDase activity (fig 29). Induced expression of the wild-type dOMI displayed a significant increase in caspase activity (n=2, p-value=0.002) in accordance with previously published data, indicating a pro-apoptotic function for dOMI involving activation of the caspase cascade. Further, induced expression of mutant dOMI had no significant effect upon caspase activity, suggesting the pro-apoptotic function of dOMI is dependent upon its proposed catalytic serine at residue 266. Since the inducible wild-type dOMI was shown to elicit a pro-apoptotic effect upon S2 cells, it is reasonable to conclude that use of a constitutively expressed dOMI construct led to false negative DEVDase activity results caused by premature apoptosis and/or reduction of the transfected population of cells.
Fig. 29. Inducible dOMI expression resulting in sensitization of S2 cells to STS driven apoptosis. dOMI expression was induced using 700mM CuSO4 for 24hrs in S2 cells transiently transfected with pRm wild-type or S266A mutant dOMI (obtained from Challa et al. 78). Cells were then treated for 12hrs with STS and assayed for caspase activity using Ac-DEVD-AFC. Cells transfected with pRm dOMI showed increased caspase activity which was dependent on the presence of the proposed catalytically active serine at residue 266.
Chapter 5: Summary and Future Directions
Summary of Results

Models of apoptosis in mammals have identified the requirement for the release of pro-apoptotic mitochondrial factors following exposure to cellular stress\textsuperscript{2-6,9,10}. Mammalian HtrA2 is identified as an IAP antagonist, released from the mitochondria to the cytosol under apoptotic conditions, where it binds and degrades XIAP, resulting in caspase activation\textsuperscript{12-16,57-59,80}. Using human HtrA2 as a reference point, this study identified a \textit{Drosophila} ortholog, dOMI, possessing both a sequence conservation of \textasciitilde{}43\% (fig 8), as well as a general conservation of structural elements, including that of an IBM and serine protease domain (fig 9D). Exogenous expression of wild-type and a proposed catalytic mutant dOMI in \textit{Drosophila} S2 cells identified several distinct isoforms indicating the requirement for a series of processing events in dOMI protein maturation (Fig 12). These isoforms of dOMI are suggested to correspond to the full-length protein at \textasciitilde{}53kDa, a MTS cleaved form at \textasciitilde{}50kDa, a TM cleaved form at \textasciitilde{}46kDa, as well as two as of yet unidentified isoforms at \textasciitilde{}58kDa and \textasciitilde{}45kDa (Fig 12). The expression pattern of these isoforms is modified under apoptotic stress, resulting in a transition between the proposed MTS cleaved form to that of a TM cleaved form, believed to correspond to the active dOMI molecule (fig 16). Additionally, this same processing modification was observed in co-expression experiments with Rhomboid-7, indicating the strong possibility that Rhomboid-7 directly processes dOMI from the MTS cleaved form (\textasciitilde{}50kDa) and the proposed active TM cleaved form (\textasciitilde{}46kDa) (fig 22, fig 23). While dOMI localized to the mitochondria under normal conditions (fig 16,17), exposure to apoptotic stress
resulted in a release of dOMI from the mitochondria. Unlike the mammalian ortholog, dOMI did not display the typical diffuse cytoplasmic pattern seen with HtrA2 following the onset of apoptosis (fig 21)\textsuperscript{12,16}, instead displayed a punctate pattern (fig 20), consistent with recently published data\textsuperscript{79}. This indicates the possibility of translocalization of dOMI from the mitochondria to an as of yet undetermined sub-cellular compartment, such as lysosomes, peroxisomes or golgi for subsequent degradation following the onset of apoptosis. Expression of wild-type dOMI in S2 cells resulted in an apparent loss of transfected cells (fig 14) indicative of cell death. This in conjunction with lowered expression levels (fig 13) suggests that unaltered caspase activity (fig 29,30) is the result of untransfected cells masking the signal of the small population of dOMI transfected cells. This is further supported by recently published work by Challa et al., where expression of dOMI under control of an inducible promoter resulted in increased caspase activity following exposure to apoptotic stress\textsuperscript{78}. Using this inducible dOMI construct, wild-type dOMI was shown to function in a pro-apoptotic manner, since cells transfected with wild-type dOMI were sensitized to apoptotic stress as indicated by an assay of caspase activity (fig 31), consistent with the previously published data\textsuperscript{78,79}.

**Future Directions**

While the data obtained in this study as well as that presented in recent publications\textsuperscript{78,79} supports the involvement of dOMI in the onset of apoptosis, several issues still remain to be addressed. The expression of dOMI in *Drosophila* cells has identified a processing requirement in the generation of the
mature protein (fig 11). While the identity of each of these isoforms has been previously been proposed, the actual sequence of each form remains undetermined. To further gain insight into the function of dOMI and the events leading to the formation of the mature protein, the N-terminal sequence of each form should be identified. Such N-terminal sequencing of dOMI requires the production of a suitable quantity of purified protein corresponding to each identified form. Under current conditions, the quantity of dOMI obtained via transfection (fig 11) remains inadequate for purification and subsequent N-terminal sequencing by Edman Degradation. This issue can be overcome by the generation of a stable cell line expressing a tagged dOMI under control of an inducible promoter. The use of an inducible promoter for dOMI expression is necessary to prevent premature dOMI mediated cell death as previously described (fig 12, 27, 28, 29). Expression of dOMI would be induced prior to harvesting the cells, while purification of dOMI from such cells would require affinity chromatography targeting an epitope tag present at the C-terminal of the exogenously expressed protein. Further separation of the multiple isoforms of dOMI can be achieved through SDS-PAGE, allowing visualization by Coomassie blue staining and subsequent excision of each distinct band allowing N-terminal sequencing of each distinct form.

Evidence for mitochondrial dOMI release following apoptotic induction was observed via microscopy (fig 18), while such release was not confirmed by Western of fractionated cells expressing tagged dOMI (fig 15). The publication by Igaki et al confirmed mitochondrial release of dOMI by microscopy using an
antibody to the endogenous protein, displaying the same punctate pattern as observed in figure 18, while showing no cytosolic release of endogenous dOMI by fractionation. Contrary to the evidence provided in this study as well as that of Igaki et al.\textsuperscript{79}, the release of dOMI to the cytosol following apoptotic induction was displayed by Challa et al. using an exogenously expressed myc tagged dOMI\textsuperscript{78}. Despite identifying the mitochondrial release of dOMI during apoptosis by fractionation, the work by Challa et al shows simultaneous release of cytochrome C (contrary to the majority of published data) and fails to confirm their findings via microscopy\textsuperscript{78}. Therefore, the effect of apoptotic stimuli on dOMI subcellular localization must be unambiguously identified through microscopy as well as subcellular fractionation using the endogenously expressed protein. Since previously described expression efficiency of dOMI in S2 cells (fig 11, 12) may have resulted in restriction of loss of dOMI signal following apoptosis (fig 14, 15), it would be necessary to use stable dOMI cell lines (as previously described) for validation of dOMI subcellular localization. While fractionation of treated dOMI cells would be conducted according to previous conditions, detection of dOMI by immunofluorescence could be further expanded through the use of probes for additional subcellular compartments such as lysosomes and golgi to identify the nature of the observed punctate dOMI expression following the onset of apoptosis.

While dOMI functions in a pro-apoptotic manner in \textit{Drosophila} (fig 29)\textsuperscript{78,79}, the structural elements required as well as the active form of dOMI in apoptosis remains uncharacterized. To determine the requirements for proper function of
dOMI, a series of truncation and deletion mutants are required. Design of these mutants would be based upon the known sequences of each dOMI isoform (previously described) and involve the removal of each identified structural element (MTS, TM domain, Protease domain, PDZ domain) (fig 9) and any combination thereof. Using inducible constructs for each of the proposed mutants in both wild-type and catalytically inactive dOMI, stable cell lines would be generated and assessed for the ability to induce caspase activation. Caspase activity would be monitored as previously described, where exposure to apoptotic stress would be conducted following induction of exogenous protein expression. The resultant data would identify the pro-apoptotic isoform of dOMI as well as the structural elements required for its proper function.

This study identified dOMI as a possible substrate for the mitochondrial serine protease Rhomboid-7. Co-expression of the mutant dOMI with Rhomboid-7 displayed Rhomboid-7 dependant processing leading to the accumulation of the TM cleaved form of dOMI (fig 22), while direct interaction between these two proteins was identified by immunoprecipitation (fig 23). Accumulation of the TM cleaved dOMI facilitated by Rhomboid-7 (fig 22), was similar to the accumulation observed following the onset of apoptosis (fig 14). As such, apoptosis driven dOMI processing is likely the result of Rhomboid-7 cleavage of dOMI, indicating the potential involvement of Rhomboid-7 in the apoptotic program. The potential role of Rhomboid-7 involvement in apoptosis and its interaction with dOMI is supported by unpublished data by McQuibban et al. showing a neurodegenerative effect of co-expression of dOMI and Rhomboid-7 resulting in
complete loss of the *Drosophila* ommatidia. To further evaluate the proposed pro-apoptotic role of Rhomboid-7 in *Drosophila*, it is necessary to assess its effect upon the caspase cascade. This requires a series of co-expression experiments with both the wild-type and mutant dOMI followed by subsequent monitoring of caspase activation in S2 cells. Due to previously described limitations in transfection and expression of exogenous proteins in S2 cells, stable cell lines corresponding to the required co-expression conditions would be required. As previously mentioned, inducible expression of exogenous proteins would be required to prevent premature cell death due to the proposed pro-apoptotic natures of both dOMI and Rhomboid-7. To unambiguously determine the role of Rhomboid-7 and its interaction with dOMI in the apoptotic program, the following cell lines would be required; Rhomboid-7, Rhomboid-7 S256A, dOMI, dOMI S266A, Rhomboid-7 + dOMI, Rhomboid-7 + dOMI S266A, Rhomboid-7 S256A + dOMI, and Rhomboid-7 S256A + dOMI S266A. Prior to stimulation of apoptosis, exogenous protein expression would be induced and caspase activity assessed as previously described following the completion of the apoptotic treatment.

**Concluding Remarks**

The work conducted in this study as well as recent publications\textsuperscript{78,79} has provided further evidence for the involvement of mitochondrial factors in canonical apoptotic signaling in *Drosophila melanogaster* through examination of dOMI. dOMI functions in a pro-apoptotic manner, involving release from the mitochondria (fig 18) and subsequent activation of the caspase cascade...
dependant on a catalytic serine residue at position 266 within its protease domain (fig 29). This work provided evidence for Rhomboid-7 mediated dOMI processing (fig 20, 21) resulting in the accumulation of the TM cleaved dOMI (~47kDa) similar to that observed under apoptotic stress (fig 14). Since both Rhomboid-7 expression and apoptotic induction result in the accumulation of the ~47kDa TM cleaved dOMI, it is proposed that Rhomboid-7 maintains a pro-apoptotic function in *Drosophila*. It is proposed that following exposure to apoptotic stimuli, Rhomboid-7 recognizes the GA motif in the TM domain of dOMI, cleaves this motif between aa 74 and 75 resulting in the exposure of the IBM AVVS. The cleaved dOMI is then exported from the mitochondria where it binds IAP1 via its N-terminal IBM and degrades IAP1, facilitated by an active serine residue at position 266. By binding and degrading IAP1, dOMI alleviates the inhibition of the caspase cascade, triggering the onset of apoptosis. While dOMI function requires further investigation, it appears to represent conservation in the canonical apoptotic program between *Drosophila* and humans. Further exploration of dOMI function may result in the validation of *Drosophila melanogaster* as a model organism for the study of canonical apoptosis in humans.
References


31) Ishihara, N, Fujita, Y, Oka, T, Mihara, K. Regulation of mitochondrial morphology through proteolytic cleavage of OPA1. EMBO J 2006;25(13):2966-77


43) Twiddy, D, Cohen, GM, Macfarlane, M, Cain, K. Caspase-7 is directly activated by the approximately 700-kDa apoptosome complex and is released as a stable XIAP-caspase-7 approximately 200-kDa complex. J Biol Chem 2006;281(7):3876-88.

61) Kiessling, S, Green, DR. Cell survival and proliferation in Drosophila S2 cells following apoptotic stress in the absence of the APAF-1 homolog, ARK, or downstream caspases. Apoptosis 2006;11(4):497-507.

67) Fraser, AG, McCarthy, NJ, Evan, GI. drICE is an essential caspase required for apoptotic activity in Drosophila cells. EMBO J 1997;16(20):6192-9.


71) Arama, E, Bader, M, Srivastava, M, Bergmann, A, Steller, H. The two Drosophila cytochrome C proteins can function in both respiration and caspase activation. EMBO J 2006;25(1):232-43.


118) Glynn JM, Cotter TG, Green DR. Apoptosis induced by Actinomycin D, Camptotheacin or Aphidicolin can occur in all phases of the cell cycle. Biochem Soc Trans. 1992;20(1):84


