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The Role of the Novel Lupus Antigen, Acheron, in Moderating Life and Death Decisions

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THE ROLE OF THE NOVEL LUPUS ANTIGEN, *ACHERON*, IN MODERATING LIFE AND DEATH DECISIONS

A Thesis Presented

By

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ABSTRACT

THE ROLE OF THE NOVEL LUPUS ANTIGEN, ACHERON, IN MODERATING LIFE AND DEATH DECISIONS

May 2014

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Programmed cell death (PCD) is a major regulatory mechanism employed during development and homeostasis. The term PCD was coined to describe the death of the intersegmental muscles (ISMs) of moths at the end of metamorphosis. The timing of ISM death in the Tobacco Hawkmoth, *Manduca sexta,* is regulated by a fall in the titer of the steroid molting hormone 20-hydroxyecdysone (20E) late on day 17 of pupal-adult development. This triggers the release of the peptide hormone, Eclosion Hormone (EH), which mediates its effects via the secondary messenger cGMP. It has been previously demonstrated that ISM death requires *de novo* gene expression. One induced gene in the ISMs encodes the novel protein Acheron. However, Acheron's role in PCD is unknown.

Acheron is a novel member of the Lupus-Antigen family of RNA binding proteins. In humans, Acheron is expressed in many tissues including the myoepithelial cells in mammary ducts. Analysis of the mammary gland revealed that Acheron mRNA levels were elevated in some basal-like breast cancers in women. Ectopic expression of Acheron in human MDA-MB-231 breast cancer cells results in dramatic elevations in proliferation, angiogenesis and metastasis. Moreover, Acheron expressing MDA-MB-231 cells in mouse xenographs resulted in tumors that were five times larger than control cell

tumors. These data suggests that Acheron enhances the growth of some human breast cancers.

This thesis describes two primary studies. The first tested the hypothesis that Acheron functions as a survival protein for cells *in vitro*. MDA-MB-231 cells engineered to express Acheron were challenged with various death-inducing treatments, which act via different signaling pathways, to determine if Acheron expression confers survival. Acheron protects cells from apoptosis induced by nutrient withdrawal, proteosome inhibition, heat stress, mitochondrial toxins, inhibiting cellular respiration, DNA damage, and oxidative stress. The second study tested the hypothesis that Acheron is phosphorylated by a cGMP-dependent kinase in the ISMs when the cells initiate death following adult eclosion. Using a non-radioactive *in-vitro* kinase assay I observed that Acheron is phosphorylated via a cGMP-dependent kinase, presumed via kinase binding motif predictions to be Protein Kinase G. Furthermore I show that phosphorylation is coupled to Acheron degradation.

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CHAPTER I

INTRODUCTION

A Brief History of Developmental Biology

Scientists have sought answers to questions pertaining to embryonic development since times of antiquity. During the fourth century BC, Aristotle recorded his observations of embryos and stages during embryonic development to determine if development was the result of preformationism or epigensis (National Research Council, 2000). As advances in technology gave rise to techniques such as microscopy, these observational studies became more refined. It was not until the late 19th century that the field of development really expanded. During 1880-1885, the first atlas of human embryos titled "*Anatomie Menschlicher Embryonen*" was published by Wilhelm His. This work was a collection of microscopic slides that was the first to map the development of the kidney, limbs, central nervous system, heart and eyes in vertebrate embryogenesis (National Research Council, 2000). Moreover it was during this time period that the first instance of cell death was recorded by Karl Vogt during his observations of amphibian metamorphosis (Vogt 1842).

The late 19th century was an era during which experimental biology was on the rise and many systems were used to model development. In 1888 Wilhelm Roux conducted a hallmark study using a two-cell stage frog embryo. Roux killed one of the cells in this stage and observed that half an embryo developed. He concluded that cell fate was determined at the two cell and four cell stage (Roux, 1888). Seven years later

this was refuted by the work of Hans Driesch, who repeated Roux's experiments in sea urchin embryos and showed that it was possible to remove portions from a developing embryo without affecting the resulting organism (Driesch, 1895).

By the early 1900s techniques in experimental biology had advanced such that they involved tissue removal. One of the hallmark studies in this era was the transplantation experiment conducted by Hans Spemann and Hilde Mangold, in which they published their discovery of the effect of "organizers" in the African clawed toad *Xenopus laevis* (Spemann & Mangold, 1924). They described the organizer as a cluster of cells that induces development of the central nervous system. Spemann won the 1935 Nobel Prize in Physiology or Medicine for this very discovery.

As techniques in cellular biology expanded, the next era in developmental biology focused on changes at the cellular level (Horder, 2010). Specifically, many studies sought to elucidate the role of the nucleus. Following his Nobel Prize, in 1938 Spemann published another landmark paper demonstrating that the nucleus of a cell remains totipotent after division (Spemann, 1938). By tying a piece of hair around a fertilized salamander egg, Spemann separated the nucleus from other cellular components and allowed the nuclear side of the constricted cell to divide until it reached the 16-cell stage. He then allowed the second half of the restricted cell to divide by removing the hair and allowing the nucleus to rejoin the other cellular components. The result of this experiment was the birth of a twin set of salamanders.

This observation, along with the advances in nuclear transplantation techniques by Robert Briggs and Thomas J. King (Briggs and King, 1952), catalyzed the famous work of John Gurdon in 1962 in which he transplanted the nucleus from a fully

differentiated intestinal epithelium cell of feeding tadpoles into a enucleated *Xenopus laevis* egg to demonstrate that the nucleus contained the genetic information necessary to allow for full development (Gurdon, 1962). The advances by Spemann, Briggs and King and by Gurdon illustrated that developmental research began to focus more on mechanisms that regulated development. One such mechanisms was cell death.

Cell Death

Cell death was first observed over 150 years ago by Karl Vogt (Vogt, 1842) during his observations of amphibian metamorphosis, but it wasn't until a review in 1951 by Glucksman that cell death returned as a focus of developmental research. In his review, Glucksman "rediscovered" developmental cell death in embryological tissues by observing that the core of the notochord disappears (Glucksman, 1951).

Glucksman's review catalyzed many to pursue cell death, specifically Richard Lockshin and Caroll Williams. Lockshin and Williams examined the disappearance of the intersegmental muscles (ISMs), that was originally observed in 1935 (Kuwana, 1935) in the *Pernyi* silkmoth following adult eclosion (emergence) at the end of metamorphosis. The term Programmed Cell Death (PCD) was coined by Lockshin and Williams (1965) to describe the temporally predictable death of these muscles. Since Lockshin's studies, the field of PCD has exploded: the Pubmed search engine lists more than 225,000 papers that use the term "PCD" as a descriptor.

Today cell death is recognized to be a vital component of development and homeostasis in unicellular organisms (Shemarova, 2010), vertebrates and invertebrates (Ellis et al., 1991) and plants (Sergovia et al., 2003; Reape and McCabe, 2008).

Numerous phenomena during mammalian development have been attributed to PCD. For example, by using differential labelling of nuclei of human blastocysts *in vitro* Hardy et al (1989) observed that a vast number of cells undergo cell death during development. *In vitro* data from mouse embryos was used to show that cavitation of the proamniotic cavity depends upon cell death of the inner ectodermal cells (Coucouvanis et al., 1995). One classic example of cell death during development in humans is the disappearance of the interdigital cells during the formation of the fingers (Lindsten et al., 2000). In addition, cell death mechanisms are also involved in generating the four chambers of the heart (Abdelwahid et al., 2002).

In 2002, H. Robert Horvitz received the Nobel Prize for Physiology or Medicine for elucidating the genetic pathway for the most common form of PCD, apoptosis, in the nematode *Caenorhabditis elegans*. Horvitz and colleagues identified mutations that specifically enhanced or prevented PCD and developed a pathway implicating specific genes in the process. In his work, Horvitz identified the first pro-apoptotic cell death genes, ced-3 and ced-4 (Yuan et al., 1990). Later, he identified ced-9, a homolog of Bcl-2, as a gene that protects cells from death (Hengartner et al., 1994). The conservation of this pathway is so extreme, that human Bcl-2 can substitute for ced-9 in worms and prevent cell death, despite more than 1.1 billion years of evolution (Vaux et al., 1992).

Markers of Death

Ultrastructural studies by Kerr, Wylie and Currie were the first to identify a common set of morphological changes that occurred in dying cells: nuclear and cytoplasmic condensation and fragmentation of the cell (Kerr et al., 1972), which they

termed “apoptosis”. These morphological changes warranted more mechanistic associated investigation. Consequently, today there are many molecular markers that are associated with cell death.

One of the hallmarks of apoptotic cell death is the activation of endonucleases that cleave genomic DNA at the inter-nucleosomal linker which generates the classic “ladder” pattern on agarose gels therefore creating the classic ladder pattern that is seen during electrophoresis (Williams et al., 1974; Wyllie, 1980).

In addition to nuclear changes, mitochondria undergo important changes during cell death. Cytochrome *c*, which normally resides inside the inner membrane of the mitochondria, is an important marker of death. In response to apoptotic stimuli, mitochondrial membrane permeability increases and cytochrome c is released into the cytosol where it binds Apaf-1 (Kroemer et al., 2007; Liu et al., 2004) and eventually leads to activation and proteolytic cleavage of pro-caspase 3 (Zou et al., 1997). Caspase-3 then cleaves a variety of substrates within the cell that leads to the rapid demise of the cell (Porter et al., 1999). For example, caspase-dependent cleavage of flippase allows the phosphotidylserine to flip from the inner layer of the cell membrane to the outer layer, where it acts as a signal for phagocytosis of the dying cell by macrophages and other cells (Fadok et al., 1992).

Manduca sexta

One tool for gaining new insights into the control of PCD is to exploit an invertebrate model system. In the Schwartz laboratory, we employ the tobacco

hawkmoth *Manduca sexta*, a moth that is related to the silk moths studied by Lockshin. In fact, many labs use the two species interchangeably.

Manduca require approximately 6 weeks to develop from an egg to an adult during which time they grow approximately 10,000 fold. They begin life as a spherical egg (diameter of 1.5 mm) deposited on a tobacco plant and hatch approximately 5 days after they are laid. The larval form lasts approximately 20 days during which the animal grows up to 70-80 mm in length. During the larval stage the animal spends the majority of its time and energetic resources feeding on tobacco or tomato leaves. Mature larvae wander away from the food source and burrow 10 to 15 cm into the ground where they pupate. *Manduca* pupa are large and oval shaped and measure an average of 45 to 60 mm in length. Under laboratory conditions, *Manduca* remain in their pupal state for 18 days and the adult moth emerges late on day 18. Adult moths live for approximately 10 days during which they allocate the majority of their energy and resources towards reproduction.

PCD of Manduca ISMs

The ISMs of *Manduca* are formed in the embryo and are the major abdominal muscles of the larva, pupa and pharate adult. They are divided into separate pairs of bilaterally symmetric bundles. The ISMS are composed of striated skeletal muscle fibers that are approximately 5 mm long and 1 mm diameter. During the larval stage, *Manduca* use these muscles for locomotion whereas the pupa uses them for abdominal movement during the eclosion behavior of the adult moth. The ISMs represent the major amino acid stores required by the adult which are liberated when the cells undergo PCD (Schwartz,

2008; Figure 1.1). In contrast to many experimental models of cell death that employ chemical or physical insults to initiate cell death, the loss of the ISM is a normal developmentally controlled process which offers a physiological means to the endogenous mechanisms that regulate PCD.

Three days prior to eclosion, these muscle cells undergo normal hormonally controlled atrophy during which the cells lose 40% of their muscle mass and 15-20% of their volume (Schwartz and Truman, 1983). During this phase, there is no change in resting potential or force per cross-sectional area (Schwartz and Ruff, 2002). However, there is an increase in polyubiquitin mRNA expression and ubiquitin dependent proteolysis which presumably facilitates protein turnover (Schwartz et al., 1990; Haas et al., 1995).

ISM death occurs coincident with adult eclosion and is marked by the rapid loss of mass (~ 4% per hour) and the resting potential (~ 2.5 mV per hour) (Schwartz and Ruff, 2002). By 30 hrs post-eclosion, almost all the muscle mass is lost and all that remains are whirls of connective tissue (Beaulaton and Lockshin, 1977). Muscles at day 15, day 18 and 13 hours post-eclosion are depicted in Figure 1.2.

Although these cells undergo PCD, they do not exhibit the classical signs of apoptosis, as there is no membrane blebbing, DNA fragmentation or chromatin condensation (Schwartz et al., 1993). Instead, these cells die via autophagy. They exhibit membrane wrinkling, pyknosis and the retention of high molecular weight genomic DNA/

The trigger for ISM PCD in the adult is a fall in the steroid molting hormone 20-hydroxyecdysone (20E) late on day 17 of the pupal stage (Schwartz and Truman, 1983).

The decline in 20E cause release of the peptide hormone Eclosion Hormone (EH) which acts as the proximal trigger for death. Addition of exogenous 20E during the time of eclosion blocks ISM PCD (Schwartz and Truman, 1984).

In 1969 Lockshin demonstrated that ISM PCD in silkmoths could be blocked by inhibitors of RNA or protein synthesis (Lockshin, 1969) suggesting that death requires *de novo* gene expression. The Schwartz lab demonstrated that this was true and using the *Manduca* ISM model, they were the first group to clone death associated genes from any system (Schwartz et al., 1990a). Some of these genes encoded previously characterized proteins, including: polyubiquitin (Schwartz et al., 1990b); 20S and 26S proteasome subunits (Jones et al., 1995; Sun et al., 1997), and apolipophorin III (Sun et al., 1995). Others encoded novel proteins whose role in death and development was poorly understood, such as: SCLP (Small Cytoplasmic Leucine Rich Repeat Protein) (Kuelzer et al., 1999), DALP (Death Associated LIM-Only Protein) (Hu et al., 1999) and Acheron (Valavanis et al., 2007).

Acheron

One of the novel genes that emerged from this screen encodes the protein Acheron, a 55 kDa protein that is a member of the Lupus Antigen (LA) family of RNA binding proteins (Valavanis et al., 2007). Northern blot analysis of Acheron mRNA expression in the ISMs during various stages of pupal/adult development in *Manduca* demonstrated that Acheron is expressed on day 18 of pupal development when the muscles become committed to die. qPCR data shows an approximately 980 fold induction of Acheron mRNA on day 18 of pupal development (Oppenheimer and

Schwartz, unpublished). When animals are injected with the insect molting hormone 20-hydroxyecdysone on the day preceding adult eclosion (day 17 of pupal-adult development), both ISM death and the expression of Acheron are delayed (Valavanis et al., 2007).

Immunohistochemical analysis of the ISMs demonstrated that Acheron localizes to the presumptive M band in the muscles, suggesting that it is not freely dispersed within the myoplasm. In agreement with this hypothesis, preliminary studies have shown that Acheron is not soluble in low salt extraction buffer and instead pellets with the contractile apparatus (Sheel, unpublished).

In addition to expression in the ISMs of *Manduca*, Acheron is also expressed in the fruit fly *Drosophila melanogaster.* Microarray analysis during development in the fruit fly *Drosophila melanogaster* has demonstrated that Acheron expression increases dramatically just prior to adult eclosion, consistent with its expression in *Manduca* (http://flybase.org/reports/FBgn0033936.html). Additional studies have demonstrated that Acheron is preferentially expressed in pharate adult fly heads (unpublished). We are yet to resolve if Acheron is expressed in the brain or the eclosion associated ptilinal muscles (Laing, 1935).

Acheron is well conserved across phylogeny and shares high sequence identity between insects and mammals, especially in certain domains (Valavanis et al., 2007). In addition to an RNA binding motif, it also contains functional nuclear localization (NLS) and nuclear export signals (NES) (Shao et al., 2012). Acheron binds to several proteins, including CASK (Weng et al., 2009) and the ubiquitin E3 ligase Ariadne-1 (Wang et al.,

2003). Acheron also binds to the 5' untranslated region of the collagen I mRNA and regulates both its subcellular localization and translatability (Cai et al., 2010).

In summary, several lines of evidence suggest that Acheron is a phylogenetically conserved protein that may play key roles in normal development and pathogenesis. The mechanisms by which Acheron mediates these effects have not been defined. The purpose of this thesis is to elucidate the role Acheron plays in programmed cell death in a mammalian model and to use this information to determine the role of Acheron during PCD of the ISMs.

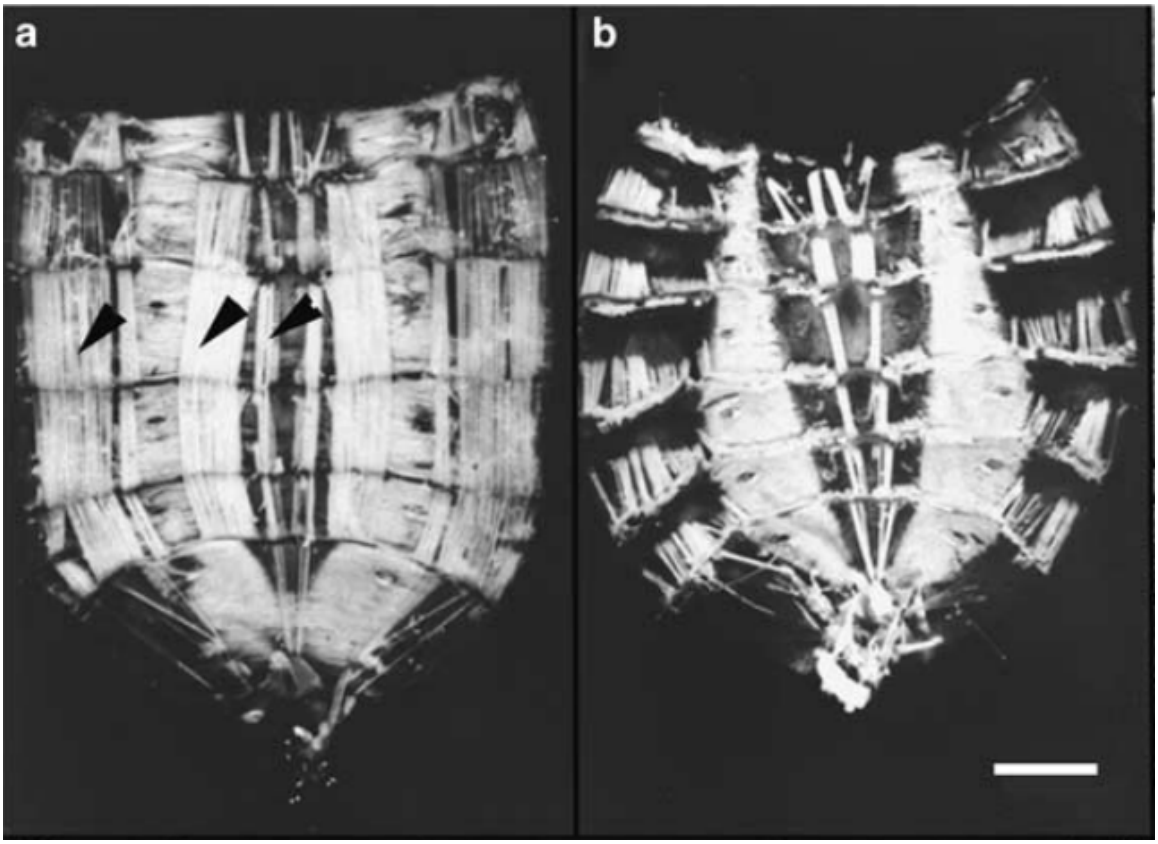

Figure 1.1: ISM morphology in adult moths.

Morphology of the ISMs of a newly eclosed adult (a) and a 30 hour old adult (b) via light microscopy. Solid headed arrows show the muscles in panel A, whereas 30 hours later these muscles have disappeared. Scale bar is approximately 5 mm. Adapted from Schwartz (2008).

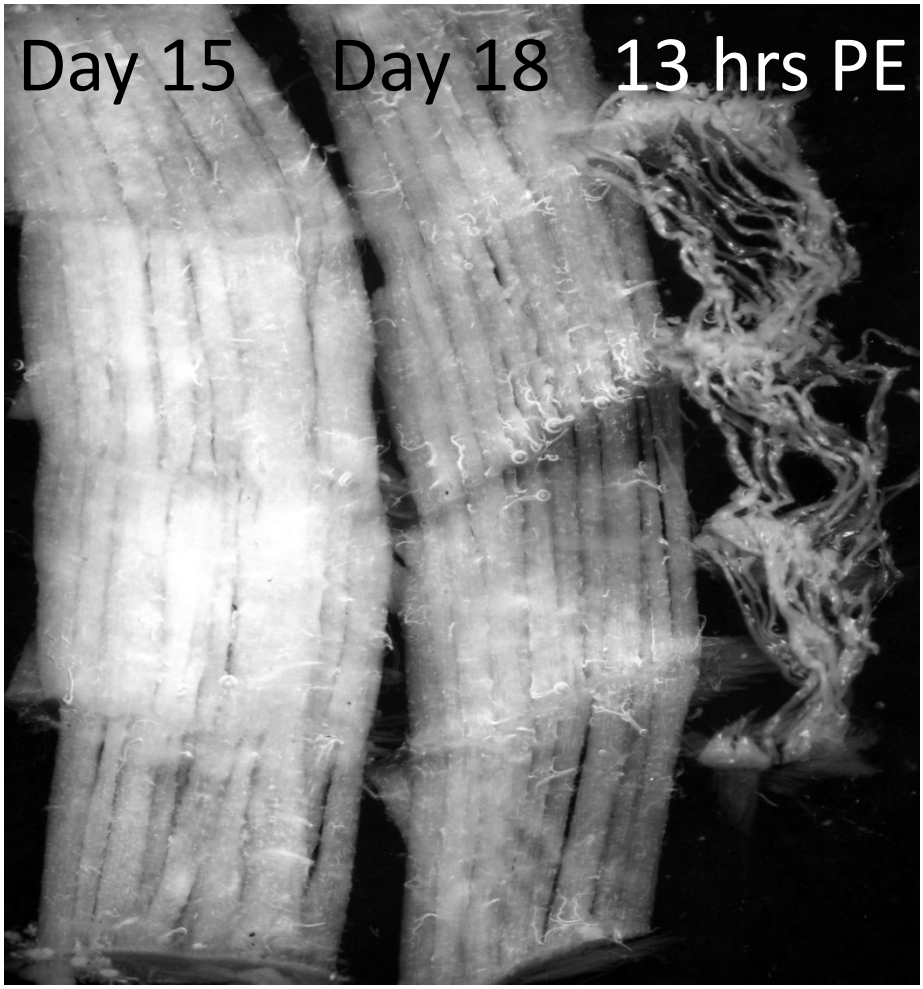

Figure 1.2 ISM morphology during pupal development

ISM morphology determined via light microscopy during pupal development on Day 15, Day 18 and 13 hours post eclosion. Prior to day 18, there is a slight decrease in muscle mass with very little change in cell number, however 13 hours after eclosion there is a dramatic decrease in cell muscle mass.

CHAPTER II

THE ROLE OF ACHERON IN DETERMINING CELL SURVIVAL

Introduction

The sudden and dramatic appearance of Acheron during day 18 of *Manduca* pupal-adult development is intriguing. Specifically, why is the gene expressed when the ISMs become committed to die at this stage? Perhaps Acheron is sensitizing the ISMSs for death.

Several studies have attempted to elucidate the role of Acheron in other invertebrate and vertebrate systems. One of the first studies investigating the function of this protein demonstrated that Acheron played an essential role during differentiation in myoblasts (Wang et al., 2009). Ectopic expression of Acheron in the mouse muscle satellite cell line C_2C_{12} resulted forced cells to either differentiate or die. It also drove the expression of the myogenic regulatory factor, MyoD. Moreover upon differentiation from myoblasts to multinucleated myotubes, Acheron expressing C_2C_{12} cells formed larger myotubes than did vector control cells. Taken together, the data suggested that Acheron plays a role in driving muscle differentiation. In the same study, this hypothesis was tested *in vivo* using zebrafish embryos. A reduction in Acheron expression, via antisense morpholinos, led to a significant reduction in muscle fiber formation during embryogenesis, while ectopic Acheron expression enhanced muscle fiber formation (Wang et al., 2009).

During myogenesis, differentiation cues are provided by interactions between neighboring cells and the extracellular matrix. This interaction is mediated by transmembrane proteins known as integrins (Boettiger et al., 1995). Studies in C_2C_{12} cells have explored the role of Acheron during the integrin-extracellular matrix interactions required during myogenesis (Glenn et al., 2010). Control and Acheron-expressing myoblasts each expressed the fibronectin receptor, composed of integrin subunits α5 and β1, and the laminin receptor upon differentiation, composed of integrin subunits α7 and β1. Inhibiting Acheron expression using antisense RNA or a dominant negative form of the Acheron protein blocked expression of the laminin receptor. However, there was no change in expression of the fibronectin receptor. Taken together the data suggested that during differentiation Acheron may play a role in regulating cell adhesion and dynamics (Glenn et al., 2010).

In a more recent study, the Schwartz laboratory demonstrated that Acheron is expressed in several human tissues, including certain neurons, cardiac muscle, kidney, skeletal muscle and mammary glands, specifically the myoepithilial cells. Relative to normal mammary tissue in women, Acheron expression is elevated in some basal- like breast cancers and suppressed in some non basal-like breast cancers (Shao et al., 2012). MDA- MB-231 human breast cancer cells that were engineered to express ectopic Acheron became more motile and expressed higher levels of VEGF (vascular endothelial growth factor) and MMP-9 (Matrix metallopeptidase 9), two proteins associated with metastasis. Consistent with these *in vitro* data, transplantation of Acheron-engineered MDA-MB-231 human breast cancer cells into athymic mouse xenographs resulted in

enhanced angiogenesis and tumors that were five times larger than those obtained with vector control cells (Shao et al., 2012).

Previous studies of the Acheron in mammalian models indicate that Acheron plays a role in differentiation and cell survival. The study by Shao et al. (2012), suggested that Acheron enhances tumor growth. To test the hypothesis that Acheron enhances tumor growth by functioning as a survival factor that protects cells from apoptosis, I treated the Acheron expressing MDA-MD-231 cells, used by Shao et al., (2012), with various toxic insults (Table 2.1). To mimic nutrient deprivation, I deprived the cells of serum supplemented media. To induce proteosome inhibition, I treated the cells with MG132. To induce oxidative stress I treated the cells with H_2O_2. To mimic heat stress, I cultured the cells at 44^{o}C for 20 minutes. To disrupt mitochondrial function, I treated cells with Rotenone. To induce DNA damage, I treated the cells with the anti-cancer drug camptothecin. In addition, I also disrupted cellular respiration by inhibiting oxidative phosphorylation via carbonyl cyanide-3-chlorophenyl hydrazine (CCCP), a drug that inactivates cellular respiration by interfering with ATP synthesis via uncoupling the proton gradient that exists in the inner mitochondrial membrane. To determine if Acheron was indeed protecting the cells from the above mentioned insults, survival was determined using the XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt) cell viability assay.

Materials and Methods

Cell Culture

MDA-MB-231 cells expressing ectopic Acheron have been described previously (Shao et al., 2012). Briefly, the human Acheron coding sequence was subcloned into the pCMV-neo retroviral vector and MDA-MB-231 cells were transfected using Fugene 6 (Roche, Indianapolis, IN). In order to generate a stable cell line, cells were selected with 800 µg/mL G418 48 hours after transfection for a total of 2 weeks. Cells were cultured at 5% CO_2 and 37°C in Dulbecco's modified Eagle medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and penicillin/streptomycin.

Western Blot

Wildtype (WT), pCMV-Neo and pCMV-neo-Acheron cells were lysed in Lamelli buffer and centrifuged at 10,000xg at 4^{o}C for 8 minutes. The resulting supernatant were boiled for 5 minutes and centrifuged for 1 min at 14,000xg at room temperature. 10 µg of protein was loaded on a 12% SDS-PAGE (Bio-Rad) and was electrophoresed for 1 hour at 150 volts at room temperature. Proteins were transferred to a PVDF membrane (Millipore). Membrane was blocked in 5% nonfat dry milk in PBST and incubated with a rabbit anti-*Human* Acheron antibody (1:100, Schwartz Laboratory). HRP labeled goat anti-rabbit antisera (Bio-Rad, 1:3000) and an ECL kit (VWR) were used for detection.

Cytotoxicity Assays

Cells were washed twice with warm phosphate buffered saline (PBS) and $1x10^5$ cells were plated in a volume of 100µL per well in a Costar 96 well flat bottom plate (Corning Life Sciences). Cells were allowed to adhere overnight and were then challenged with various noxious treatments that would normally induce cell death. To induce oxidative stress using hydrogen peroxide (H_2O_2), cells were grown in DMEM lacking sodium pyruvate. For serum withdrawal, cells were incubated with DMEM lacking FBS for the times indicated.

Cells were incubated with noxious treatments for 24 hours except in the case of serum withdrawal, where cells were deprived of serum supplemented media for 6, 24 and 48 hours. MG132 (Sigma) was used at 0.25 µM, 0.5 µM, 1 µM and 2 µM. H_2O_2 (30%, Fisher) was used at 2 µM, 4 µM, 6 µM and 8 µM. For heat induced death, cells were cultured at 44°C for 20 minutes followed by 24 hour recovery. Rotenone (Sigma) was used at 0.25 µM, 1 µM, 2.5 µM and 5 µM. Camptothecin (Sigma) was used at 100 nM, 200 nM, 300 nM and 400 nM. Carbonyl cyanide-3-chlorophenyl hydrazone (Sigma) was used at 100 µM, 250 µM, 500 µM and 1000 µM.

Cell viability in the presence of noxious treatment was determined using XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt) (Sigma) assay. Briefly, cells were cultured in the presence of the noxious agent for the indicated times and then centrifuged for 3 minutes at 800rpm at 37°C. After centrifugation, the media was removed and cells were resuspended in fresh growth media containing XTT at a final concentration of 0.5mg/mL. To allow for the reduction of the tetrazolium ring of XTT, cells were incubated at 5% CO_2 and 37°C for 2 hours.

Absorbance values were determined at 490nM via the Flurostar Optima Plate reader. Cell survival was determined by dividing the absorbance of the treated sample by the absorbance of the sample treated with the appropriate vehicle control multiplied by 100 for a percentage value. Each plate had 8 replicates and each experiment was repeated at least two times.

Statistics

Student's two-tailed T tests were conducted using Microsoft Excel. Statistical significance is denoted by asterisks (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.005$).

Results

Western blot analysis of lysate from WT, pCMV-neo empty vector and pCMV-neo-Acheron transfected MDA-MB-231 cells shows a strong band at 55kDa present in only in the Acheron construct (Figure 2.1).

To test the hypothesis that ectopic expression of Acheron protected cells from toxicity induced by nutrient starvation, I cultured cells in serum free media (Figure 2.2). Acheron expressing cells deprived of serum for 6, 24 and 48 hours had an average survival of 89.16% ± 6.59%, 88.96% ± 4.54% and 58.91% ± 2.54% respectively compared to cells not deprived of serum. Vector control cells deprived of serum for 6, 24 and 48 hours had an average survival of 91.03% ± 5.19%, 52.99% ± 2.99%, and 45.82% ± 3.55% respectively. Acheron conferred protection to cells that had been deprived of nutrients for 24 hours ($p<0.005$) and 48 hours ($p<0.005$).

Next, Acheron expressing and vector control cells were incubated for 24 hours in the presence of the proteosome inhibitor, MG132 at concentrations of 0.25 µM, 0.5 µM, 1.0 µM, and 2.0 µM (Figure 2.3). Ectopic expression of Acheron protected cells from toxicity induced by proteosome inhibition. Acheron expressing cells had average survivals of 97.45% ± 3.69%, 89.75% ± 7.39%, 76.82% ± 6.81%, and 63.07% ± 7.31% when exposed to 0.25 µM, 0.5 µM, 1.0 µM, and 2.0 µM MG132 respectively. Vector control cells treated with 0.25 µM, 0.5 µM, 1.0 µM, and 2.0 µM MG132 had average survivals of 66.37% ± 4.74%, 50.07% ± 3.92%, 39.53% ± 3.87% and 26.04% ± 3.28% respectively. Acheron conferred protection to cells treated with 0.25 µM MG132 (($p<0.005$), 0.5 µM MG132 ($p<0.005$), 1.0 µM MG132 ($p<0.005$), and 2.0 µM MG132 ($p<0.005$).

Next, oxidative stress was induced by treatment with H_2O_2 (Figure 2.4). Acheron expressing cells treated with 2 mM, 4 mM, 6 mM and 8 mM H_2O_2 had average survivals of 64.05% ± 3.26%, 39.69% ± 3.24%, 35.42% ± 2.54% and 33.57% ± 2.96% respectively whereas vector control cells had average survivals of 94.46% ± 7.41%, 65.69% ± 7.77%, 63.94% ± 6.17%, 49.24% ± 4.82%. Ectopic expression of Acheron did not protect cells from toxicity induced by oxidative stress.

Heat stress was tested next (Figure 2.5). Cells, normally cultured at 37^{o}C, were placed at 44^{o}C for 20 minutes and then allowed to recover for 24 hours. Cells that ectopically expressed Acheron had an average survival of 70.99% ± 3.44% compared to unstressed Acheron expressing cells, whereas vector control cells had an average survival of only 58.11% ± 3.28% compared to unstressed vector control cells. Overall, Acheron conferred protection against heat stress ($p<0.005$).

To determine if Acheron protected against mitochondrial toxins, cells were treated with rotenone (Figure 2.6). Acheron expressing cells treated with 0.25 µM, 1.0 µM, 2.5 µM and 5 µM Rotenone had average survivals of 80.58% ± 6.18%, 71.04% ± 5.49%, 23.31% ± 5.82% and 6.78% ± 2.14% respectively compared to untreated Acheron expressing cells, whereas vector control cells had average survivals of 68.93% ± 6.07%, 47.87% ± 6.67%, 10.08% ± 2.52% and 2.66% ± 0.74% respectively compared to untreated vector control cells. Acheron conferred protection for cells exposed to rotenone concentrations of 1.0 µM ($p<0.05$) and 2.5 µM ($p<0.005$).

Next, cells were treated with the topoisomerase I inhibitor camptothecin, which is used clinically as an anti-cancer therapeutic (Figure 2.7). Acheron expressing cells treated with 100 nM, 200 nM, 300 nM and 400 nM camptothecin had average survivals

of 100.01% ± 1.95%, 97.51% ± 3.59%, 79.01% ± 4.24% and 48.49% ± 5.05% respectively compared to untreated Acheron expressing cells, whereas vector control cells had average survivals of 80.33% ± 3.22%, 57.79% ± 7.91%, 53.75% ± 7.15% and 35.59% ± 8.52% respectively compared to untreated vector control cells. Acheron conferred protection when exposed to camptothecin concentrations of 100 nM ($p<0.005$) and 200 nM ($p<0.005$).

Finally, cells were treated with carbonyl cyanide-3-chlorophenyl hydrazone in order to inhibit cellular respiration by blocking oxidative phosphorylation (Figure 2.8). Acheron expressing cells treated with 100 µM, 250 µM, 500 µM and 1000 µM carbonyl cyanide-3-chlorophenyl hydrazone had average survivals of 91.19% ± 3.85%, 72.33% ± 8.14%, 66.87% ± 6.31% and 23.33% ± 1.64% respectively compared to untreated Acheron expressing cells, whereas vector control cells had average survivals of 69.74% ± 1.51%, 49.47% ± 5.64%, 36.98% ± 3.55% and 21.6% ± 2.71% respectively compared to untreated vector control cells. Acheron conferred protection when exposed to carbonyl cyanide-3-chlorophenyl hydrazone concentrations of 100 µM ($p<0.005$), 250 µM ($p<0.05$), and 500 µM ($p<0.005$).

Discussion

The purpose of this study was to test the hypothesis that Acheron functions, in part, as a survival factor that can protect cells from a variety of insults. I utilized the human breast cancer cell line MDA-MB-231 that had been engineered to ectopically express Acheron. Both vector control and Acheron engineered cells were then challenged with stressors that act on multiple pathways with cells and determine is the cells could survive these insults.

The first insult I employed was nutrient withdrawal via serum starvation. In MDA-MB-231 cells, the absence of nutrients due to serum starvation triggers cell cycle arrest and death through a p53 independent mechanism (Jang et al., 2000). Ectopic expression of Acheron protected cells from death after they were serum deprived for 24 hours and 48 hours (Figure 2.2).

The next class of toxins induced involved the buildup of misfolded and unfolded proteins. MG132 is toxic to cells because it binds to the N-terminal threonine residue of the β1 subunit of the 26S proteosome and blocks proteolysis (Myung et al., 2001). This results in a buildup of toxic levels of unfolded and misfolded proteins in the cell (Sherman and Goldberg, 2011). Of all the toxic stimuli tested in this study, ectopic expression of Acheron conferred the greatest degree of protection against MG132 toxicity (Figure 2.3). In line with toxicity due to unfolded and misfolded proteins, the cells were also subjected to heat stress since elevating the temperature increases in the amount of misfolded and unfolded proteins in cancer cells (Jolly and Morimoto, 2000). Similar to MG132, ectopic expression of Acheron protected cells against toxicity due to heat stress (Figure 2.5). Taken together, the data from Figure 2.3 and Figure 2.5 suggests

that Acheron protects cells that are challenged with stress associated with misfolded and unfolded proteins.

Many cells are subjected to oxidative stress due to transient increases in levels of reactive oxygen species (ROS). Oxidative stress can be caused by a variety of endogenous and exogenous factors such as cellular senescence (Hagen et al., 1997), changes in levels of certain growth factors (Yu et al., 2006), and environmental factors such as UV radiation (Zhang et al., 1997). In order to determine if Acheron protects cells from ROS damage, I treated cells with H_2O_2. Acheron did not confer any protection against H_2O_2 treatment- in fact Acheron expression sensitized the cells to death as survival in control cells was significantly greater (Figure 2.4).

The fact that Acheron sensitized cells to hydrogen peroxide induced toxicity warranted further investigation. Hydrogen peroxide can induce cell death via two different mechanisms: apoptosis (Cerella et al., 2009) and necrosis (Miyoshi et al., 2006). The process of death induced during these two pathways are drastically different. Structurally, cells undergoing necrosis lose cell membrane integrity, swell and ultimately rupture thereby releasing their cellular contents whereas cells undergoing apoptosis maintain membrane integrity, shrink and generally do not rupture. Moreover at the nuclear level, cells undergoing necrosis have random degradation of DNA whereas cells undergoing apoptosis undergo chromatin condensation and non-random fragmentation of DNA.

Studies in human T-lymphoma Jurkat cells have shown that the mechanism of hydrogen peroxide induced death (apoptosis or necrosis) is dependent on concentration and time of exposure (Saito et al., 2006). Furthermore, studies treat MDA-MB-231 with

3mM hydrogen peroxide as a positive control for necrosis (Galluzzi et al., 2012). Given the concentration of hydrogen peroxide used in this study, the data from Figure 2.4 indicated that Acheron does not protect cells from necrosis triggered cell death. Perhaps Acheron mediates its necrosis effect through increased integrin expression, as necrosis is often observed with increased expression of α and β integrins (Bachmann et al., 2008). However, the question if Acheron protected against ROS induced apoptosis remained.

Given that cell death was not induced in these cells unless the concentration of hydrogen peroxide was greater than 2mM for 24 hours, the data from Figure 2.4 suggest that ROS-induced apoptosis in MDA-MB-231 cells could not be achieved via hydrogen peroxide. Therefore toxicity due to rotenone was tested. Rotenone inhibits transfer of electrons from the iron-sulfur centers in the mitochondrial respiratory chain complex I to ubiquinone, which leads to elevated ROS production and cell death via apoptosis (Li et al., 2003). Ectopic Acheron expression did protect cells from rotenone toxicity (Figure 2.6).

In order to determine if Acheron mediates its effect with toxicities associated with mitochondria, cells were exposed to carbonyl cyanide-3-chlorophenyl hydrazine (CCCH). CCCH is an inhibitor of cellular metabolism as it interferes with oxidative phosphorylation during the electron transport chain by inhibiting cytochrome *c* oxidase (Tsyrlov et al., 1976). Acheron conferred protection against CCCH (Figure 2.8) indicating that it protected against mitochondrial associated toxicities.

Finally, given that Acheron has a functional NLS and NES and the DNA binding winged helix-turn-helix domain (Brennan et al., 1989), I tested the hypothesis that Acheron protected against DNA damage by treating cells with camptothecin.

Camptothecin induces DNA damage by blocking the re-ligation step of the cleavage reaction of topoisomerase I during S-phase of the cell cycle (Liu et al., 2000). Indeed Acheron protected against toxicity due to camptothecin (Figure 2.7). One interpretation of this data is that Acheron may translocate into the nucleus and possibly utilize its winged helix-turn-helix domain in order to bind DNA thereby shielding it from the fragmentation that is typically observed during. However, this may not be the case since ISM death is not associated with fragmentation of DNA (Schwartz et al., 1993).

Basal-like breast cancers are more aggressive and are typically associated with poor prognosis compared to non basal-like breast cancers (Rakha et al., 2008). Shao et al., (2012) demonstrated that basal-like breast cancers have greater levels of Acheron mRNA, and that xenographed human tumors that ectopically expressed Acheron had higher rates of proliferation and angiogenesis suggesting that Acheron enhanced tumor growth.

The data in this study suggests that Acheron may be enhancing tumor growth, in part, by protecting cells from death via apoptosis. One interpretation of these data is that tumors that express Acheron, such as basal-like breast cancers, may be less sensitive to chemotherapeutic intervention. Studies have shown that invasive breast cancer tumors also have high expression of Laminin-332 (Kim et al., 2012), which plays an important role in conferring cell migration and survival by binding to integrins composed of α3β1 subunits (Zahir et al., 2003). Given that inhibiting Acheron expression results in no expression of the Laminin receptor, composed of α7β1 subunits, another interpretation of the data is that Acheron promotes tumor growth through regulation of the Laminin receptor-perhaps Acheron mediates its effect in breast cancers through the β1 subunit.

Future studies need to be conducted in order to further investigate the mechanism of Acheron action. Figure 2.1 shows that MDA-MB-231 cells do not natively express Acheron, therefore a more appropriate study would be to repeat the cytotoxicity experiments from this chapter using a cell line in which endogenous Acheron expression has been silenced. Furthermore, this proposed study will have more mechanistic relevance if these studies were done in the *Manduca* GV1 cell line as these arise from *Manduca* embryos and natively express Acheron (Sheel, unpublished).

Furthermore the Acheron gene used in this study was fused to a HA epitope tag at the N-terminus of Acheron via PCR (Shao et al., 2012). Using an anti-HA antibody, co-immunoprecipitation (co-IP) of Acheron in the MDA-MB-231 cells can be used to precipitate Acheron while in a complex with its binding partners. The Acheron complexes can then be separated via SDS-PAGE and the resulting proteins can then be identified via Mass Spectroscopy. Utilizing this approach, we can identify binding partners and possibly elucidate the biochemical pathways that mediate Acheron action.

The role of Acheron in the ISMs can be further elucidated using the data from this chapter. Given that Acheron protects MDA-MB-231 cells from death inducing toxicities, the initial predication that Acheron sensitizes the ISMs for death is likely incorrect. In the ISMs, one possible role for Acheron is that it serves to protect the cells from endogenous death-inducing factors until the actual initiation of death, and then it becomes modified, sequestered and/or lost just prior to the actual initiation of PCD.

Table 2.1: Stressors used in MDA-MB-231 cytotoxicity assays

Class	Reagent	Range Used
Nutrient Withdrawal	Serum Starvation	0 to 48 hours
Proteosome Inhibition	MG132	0.25 µM to 2 µM
Oxidative Stress	H_2O_2	2 µM to 8 µM
Heat Shock	Increased Temperature	44^{o}C
Mitochondrial toxin	Rotenone	0.25 µM to 5 µM
DNA Damage	Camptothecin	0.25 µM to 5 µM
Inhibition of cellular respiration	Carbonyl Cyanide-3-Chlorophenyl Hydrazone	100 µM to 1000 µM

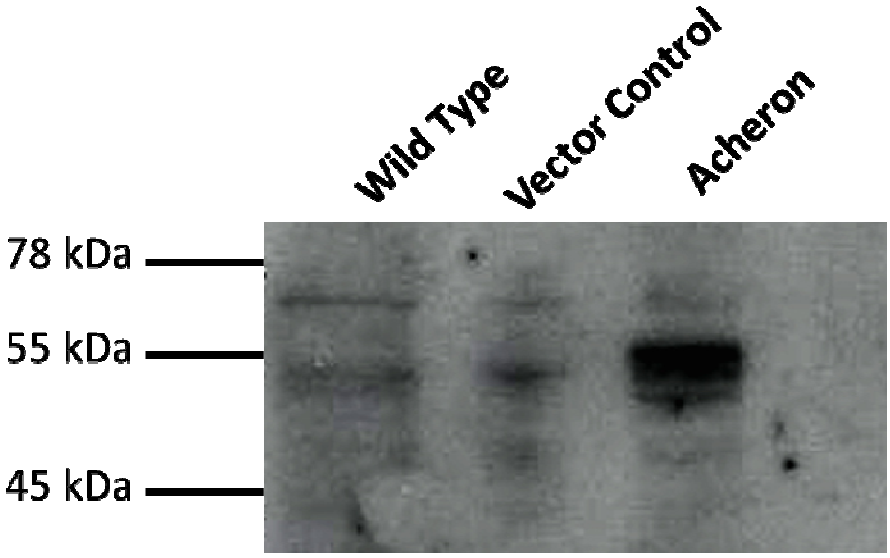

Figure 2.1: Acheron expression in MDA-MB-231 cells used in cytotoxicity assays

MDA-MB-231 cells were transfected with pCMV-Neo empty vector or pCMV-Neo-Acheron and antibiotic selected. Cells were lysed and expression of Acheron was determined in wild type cells, pCMV-Neo transfected cells and pCMV-Neo-Acheron transfected cells.

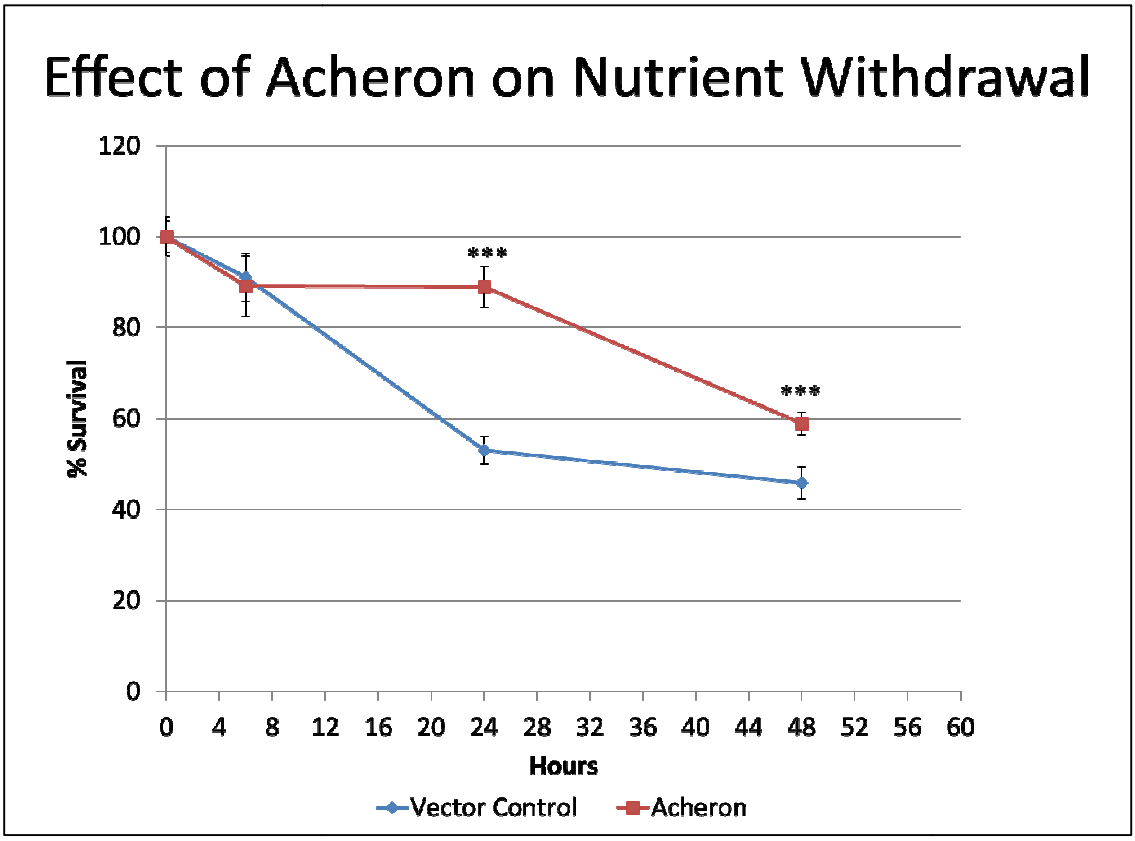

Figure 2.2: Effect of Acheron on Nutrient Withdrawal Induced Toxicity

Percent viabilities for Acheron expressing or Vector Control MDA-MB-231 cells following serum starvation for 6, 24 and 48 hours. Data was pooled from 3 independent experiment of which each had at least 8 replicates. Error bars represent ± S.E.M.

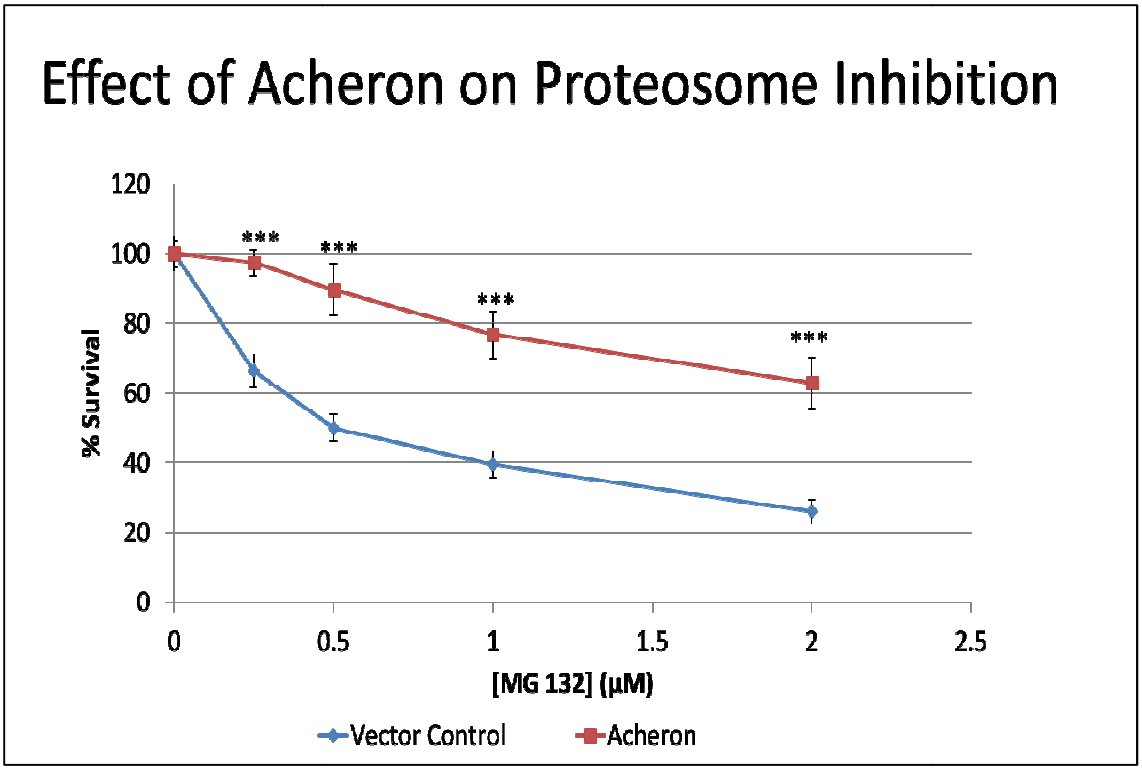

Figure 2.3: Effect of Acheron on Proteosome Inhibition Induced Toxicity.

Percent viabilities for Acheron expressing or Vector Control MDA-MB-231 cells following treatment with MG132 at 0.25 µM, 0.5 µM, 1 µM and 2 µM. Data was pooled from 2 independent experiment of which each had at least 8 replicates. Error bars represent ± S.E.M.

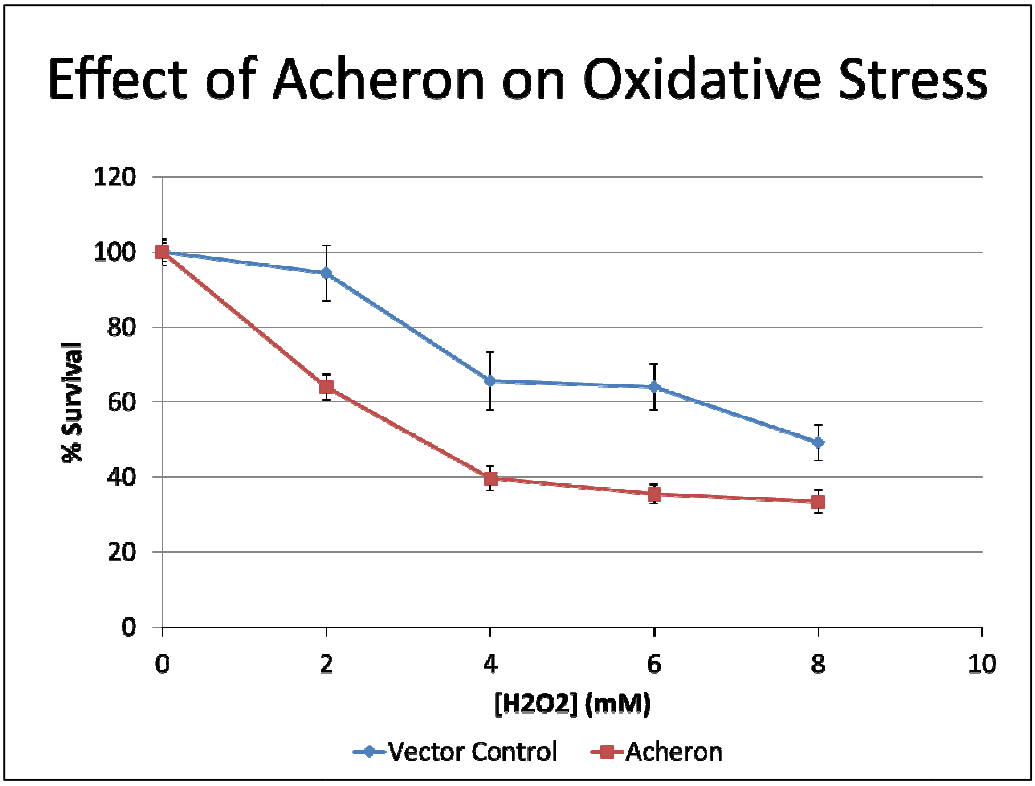

Figure 2.4: Effect of Acheron on Oxidative Stress Induced Toxicity.

Percent viabilities for Acheron expressing or vector control MDA-MB-231 cells following treatment with H_2O_2 at 2 mM, 4 mM, 6 mM and 8 mM. Data was pooled from 3 independent experiment of which each had at least 8 replicates. Error bars represent ± S.E.M.

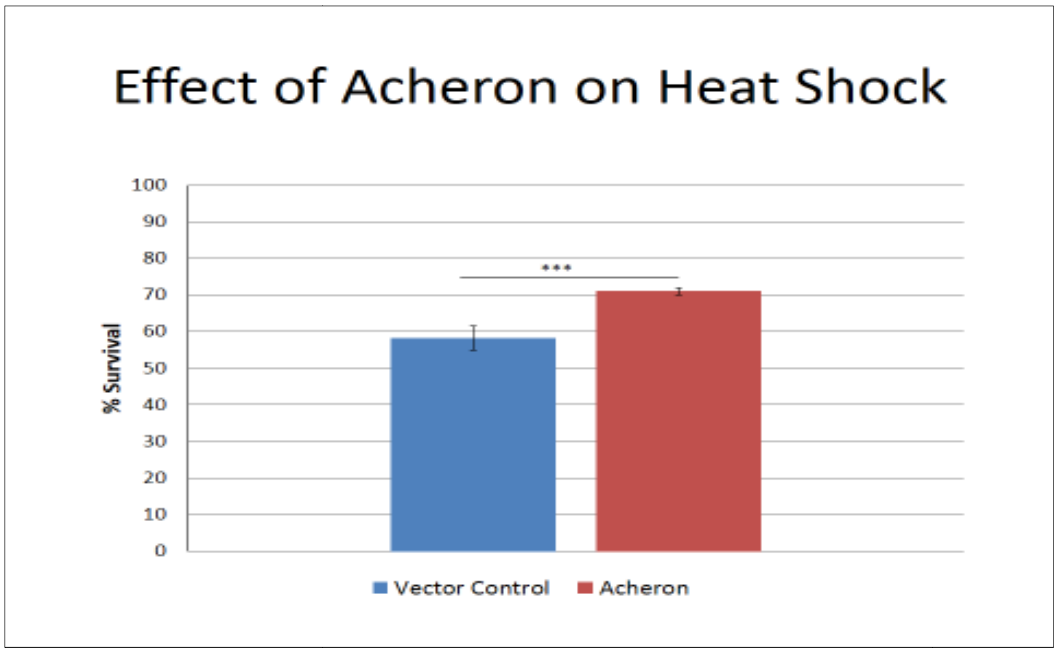

Figure 2.5: Effect of Acheron on Heat Stress Induced Toxicity.

Percent viabilities for Acheron expressing or vector control MDA-MB-231 cells following treatment at 44^{o}C for 20 minutes. Data was pooled from 2 independent experiment of which each had at least 20 replicates. Error bars represent ± S.E.M.

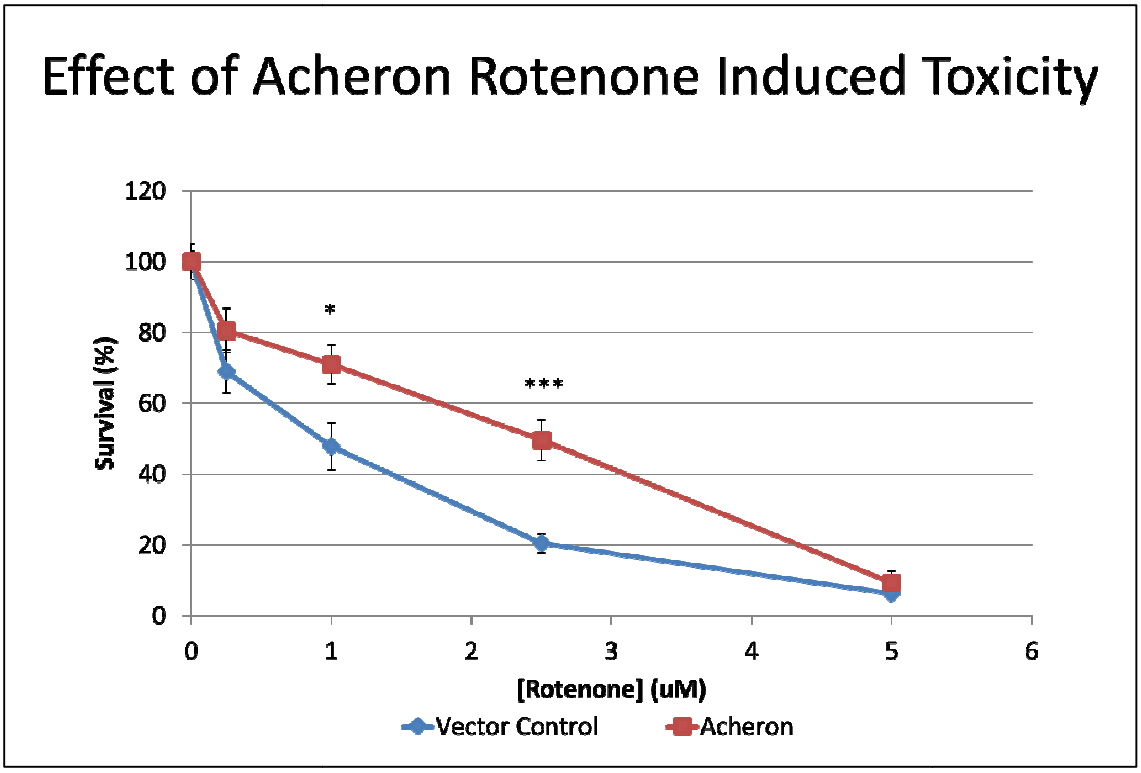

Figure 2.6: Effect of Acheron on Rotenone Induced Toxicity.

Percent viabilities for Acheron expressing or vector control MDA-MB-231 cells following treatment with Rotenone at 0.5 µM, 1.0 µM, 2.5 µM and 5.0 µM. Data was pooled from 2 independent experiment of which each had at least 8 replicates. Error bars represent ± S.E.M.

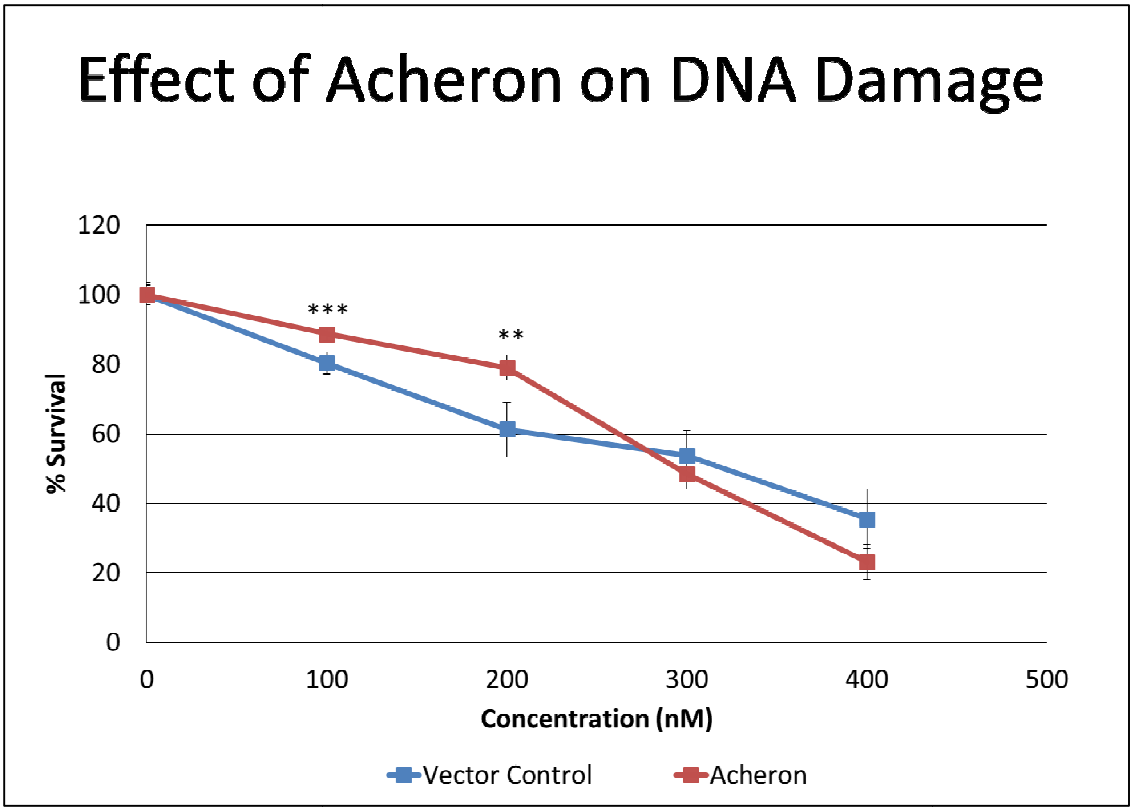

Figure 2.7: Effect of Acheron on DNA Damage Induced Toxicity.

Percent viabilities for Acheron expressing or vector control MDA-MB-231 cells following treatment with Camptothecin at 100 nM, 200 nM, 300nM, and 400 nM. Data was pooled from 2 independent experiment of which each had at least 8 replicates. Error bars represent ± S.E.M.

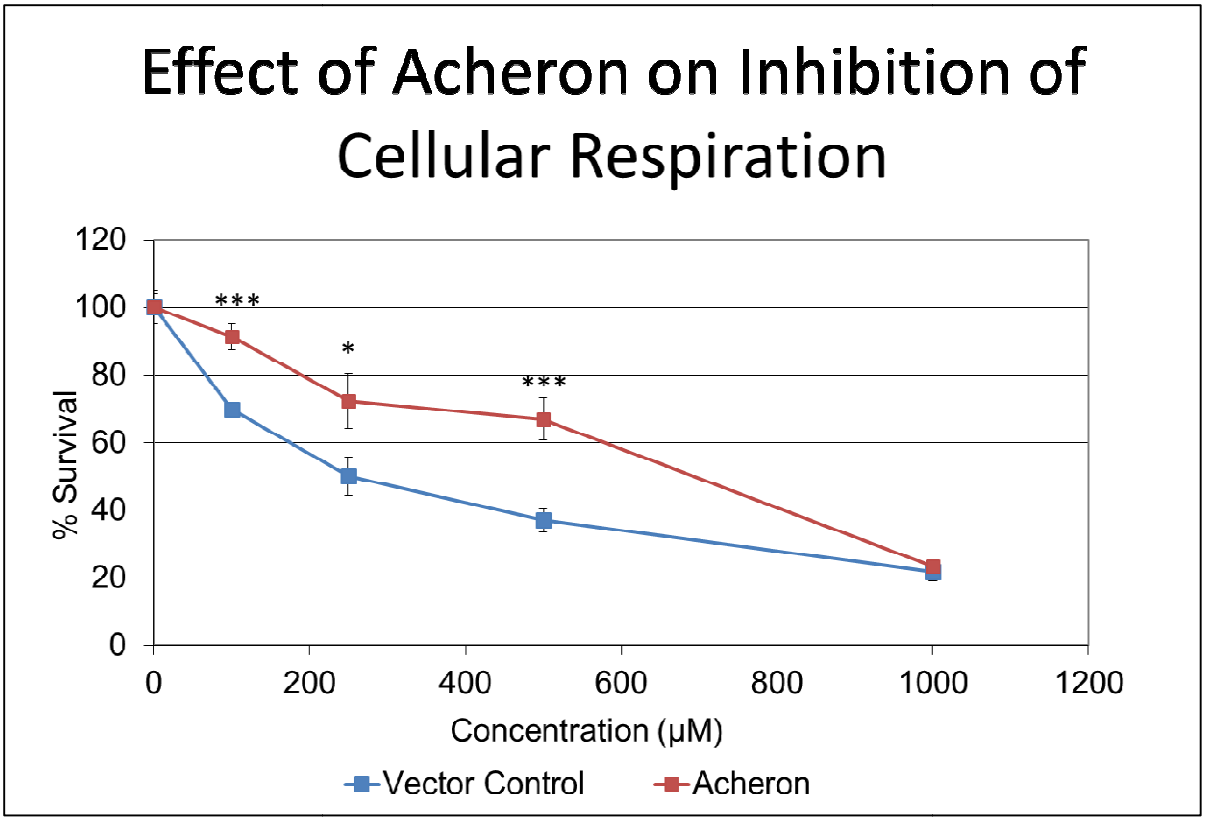

Figure 2.8: Effect of Acheron on Inhibition of Cellular Respiration Induced Toxicity.

Percent viabilities for Acheron expressing or Vector Control MDA-MB-231 cells following treatment with carbonyl cyanide-3-chlorophenyl hydrazone at 100 μM, 250 μM, 500 μM and 1000 μM. Data was pooled from 2 independent experiment of which each had at least 8 replicates. Error bars represent ± S.E.M.

CHAPTER III

DEGREDATION OF ACHERON VIA A cGMP MECHANISM

Introduction

In 1971 Earl Sutherland proposed the Second Messenger hypothesis in which he set forth four criteria that need to be fulfilled in order to identify a cyclic nucleotide as a second messenger that mediates the action of a given hormone (Sutherland and Robison, 1966). First, the cell must synthesize the cyclic nucleotide in response to the hormone. Second, exogenous addition of the hormone must trigger synthesize of the cyclic nucleotide in a dose and time dependent manner. Third, drugs that inhibit phosphodiesterase should mimic the action of the hormone. Finally, the fourth criteria of this hypothesis was that addition of exogenous cyclic nucleotide should mimic the action of the hormone.

Studies in the silkmoth *Antheraea polyphemus* have demonstrated that EH induces the expression cyclic GMP as a second messenger, and this in turn activates the death machinery within the cell (Schwartz et al., 1984). However, the downstream steps that couple hormone action to the endogenous "death program" have yet to be identified. The study by Schwartz et al., (1984) was novel in that it was one of the first publication to fulfill all four of Sutherland's criteria in order to establish that cGMP is a second messenger in any system.

Western blot analysis has shown that Acheron expression is induced late on day 17 of pupal-adult development when the ISMs become committed to die late on day 18. ("Committed" in this context means that the addition of exogenous 20E will no longer

delay the timing of death). The Acheron protein is present in the ISMs throughout all of day 18 and then is dramatically and specifically lost from the cells during the few minutes before adult eclosion and the activation of the death machinery (Brown and Schwartz, unpublished). One interpretation of this expression data is that Acheron actually serves as a survival protein that protects the ISMs from the action of the death machinery. When Acheron is then lost, the death program becomes de-repressed and serves to kill the cell, making Acheron the proximal trigger for the initiation of death.

Since EH triggers cGMP, which works via protein kinases that phosphorylate specific protein substrates, the Schwartz lab began testing the hypothesis that Acheron may be phosphorylated in the ISMs. Western blotting with anti-phospho serine/threonine antibody has demonstrated that Acheron is labeled in condemned ISMs (Smith, Brown and Schwartz, unpublished), suggesting that it may be subject to regulation in a cGMP manner.

In this chapter I use *in vitro* cell-free ISMs extracts and a novel non-radioactive kinase assay to provide evidence that Acheron is phosphorylated in a cGMP dependent manner and that this phosphorylation triggers Acheron degradation.

Materials and Methods

DNA Constructs

ISMs from day 18 animals were dissected at various times relative to adult eclosion, snap frozen on dry ice and stored in liquid nitrogen until used. RNA was isolated via RNeasy Mini Kit (Qiagen) and cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen) per manufacturer's instructions. The following primers were used to amplify the Acheron ORF from the synthesized cDNA:

For: 5' TATTGCGTGAATGCGCGCGC 3'

Rev: 5' TCGAACACCGCCACAGCGT 3'

The PCR product was cloned into the pCR2.1-TOPO vector via the TOPO TA Cloning kit (Invitrogen) to create pCR2.1-TOPO-M44a. pCR2.1.TOPO-M44a was digested with EcoRI and the Acheron ORF was subcloned into the EcoRI site of the pMT/V5-HisB vector such that Acheron expression was under control of the metallothionine promoter (Bunch et al., 1988). The pMT/V5-HisB vector was a generous gift from Dr. Tom Maresca.

Cell Culture

Drosophila S2 cells were cultured at 28^{o}C in S2 insect media containing 10% FBS, penicillin (50 units/mL) and streptomycin (50µg/mL). S2 cells were transfected with the pMT/V5-HisB-M44a construct in a 6-well plate using Effectene (Qiagen) according to manufacturer's instructions. 24 hours post transfection, cells were antibiotic bulk selected with 25µg/mL Blasticidin (Life Technologies) for 2 weeks. Stable

expression of ectopic protein was determined by Western blotting using rabbit an anti-Acheron antiserum (1:200, Schwartz laboratory).

Preparation of Intersegmental Muscle Extracts

ISMs were isolated from animals at various time on day 18, including 1 hr post-eclosion (PE). The tissues were snap frozen on dry ice and stored in liquid nitrogen until used for assays. Strips of ISM tissue from individual animals (two strips of four segments each) were pulverized in liquid nitrogen via mortar and pestle, and then homogenized using a Type B Dounce Homogenizer at 4^{o}C in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) supplemented with cOmplete protease inhibitor (Roche) and PhosStop phosphatase inhibitor (Roche) in order to create a broken cell prep (BCP).

Kinase Assay

5 µL D18 ISM BCP or S2 extract was combined with 5 µL 1 hour PE extract or 5 µL D18 ISM BCP and 5 µL of Reaction Mixture (30 mM MgCl2, 1.5 mM DTT, 75 mM β-glycerophosphate, pH 7.3, 0.15 mM sodium orthovanadate, 3.75 mM EGTA and 0.3 mM ATP). 8-Bromo-cGMP, sodium salt (8-bromoguanosine cyclic 3',5'-monophosphate sodium salt) (Tocris Bioscience) was added to a final concentration of 10mM. IBMX (3,7-Dihydro-1-methyl-3-(2-methylpropyl)-1H-purine-2,6-dione) (Tocris Bioscience) was added to final concentration of 10µM. The resulting mixture was incubated at room temperature for 20 minutes and then the reaction was stopped by adding an equal volume of Sample Buffer (Bio-Rad).

Western Blot Analysis

Samples were boiled for 5 minutes and centrifuged for 1 min at 14,000xg at room temperature. Samples were loaded on a 12% SDS-PAGE (Bio-Rad) and were electrophoresed for 1 hour at 150 volts at room temperature. Proteins were transferred to a PVDF membrane (Millipore). Membrane was blocked in 5% nonfat dry milk in PBST and incubated with a rabbit anti-*Manduca* Acheron antibody (1:200, Schwartz Laboratory). HRP labeled goat anti-rabbit antisera (Bio-Rad, 1:3000) and an ECL kit (VWR) were used for detection.

Results

ISMs from the day 18 pupal stage of *Manduca* shows that Acheron, visualized by a strong band at 55kDa, is solubilized in the presence of Laemmli buffer as well as in the PBS BCP (Figure 3.1). However, in the absence of the phosphodiesterase inhibitor IBMX, neither the addition of 8-bromo-cGMP nor addition of ATP triggers a shift in molecular weight of Acheron (Figure 3.1).

Addition of IBMX resulted in an upward shift of the Acheron band on the gel, marked by a black arrow. This sample is noted as D18-P. As can be seen in the figure, this shift corresponds to an increase in molecular weight compared to both endogenous Acheron in the D18 BCP and Acheron ectopically expressed by *Drosophila* S2 cells (Figure 3.2). Figure 3.2 also shows that the higher molecular weight Acheron band disappears upon addition of extract from ISMs 1hr PE. However, the modified Acheron band remains when the 1hr PE extract is added in the presence of Laemmli buffer (Figure 3.2).

Degradation of Acheron was only observed after cGMP and IBMX had been added to the day 18 BCP (Figure 3.3). As observed before, addition of the 1 hr PE extract resulted in disappearance of the Acheron band seen in the D18-P Acheron sample, whereas the Acheron band was still present when the D18-P sample was added to the 1 hr PE extract in the presence of Laemmli buffer to arrest any biochemical reactions. However addition of D18 extract, without cGMP and IBMX, to the 1 Hr PE extract did not result in disappearance of the Acheron band (Figure 3.3).

I next engineered *Drosophila* S2 cells to express *Manduca* Acheron and used that as a source of Acheron that would lack the machinery for its eclosion associated

degradation. The stability of the exogenous Acheron appeared to be regulated similarly to endogenous ISM Acheron. A band at 55 kDa corresponding to Acheron from S2 extract could be visualized by Western blotting. Upon addition of the D18 extract along with cGMP and IBMX, a thicker band with a slightly higher molecular weight than native Acheron was observed (Figure 3.4). However when extract from the 1 hr PE sample was added, there was a dramatic reduction in the intensity of this band (Figure 3.4).

Our hypothesis is that cGMP dependent kinase phosphorylates Acheron in a sequence-specific manner. As a first step to test this hypothesis, I aligned the Acheron protein sequences from *Manduca sexta*, *Drosophila melanogaster*, *Mus musculus*, *Danio rerio*, and *Homo sapiens* to look for phylogenetically conserved serine residues and threonine residue (Figure 3.5). I observed 7 conserved serine residues at residues 84, 107, 112, 113, 135, 163 and 284 and one conserved threonine at residue 121 in the *Manduca sexta* Acheron coding sequence. Further motif analysis revealed that the Ser 163 residue was a potential PKG binding site (Figure 3.5).

Discussion

Given that death of the ISMs is dependent on cGMP as a second messenger, the purpose of this study was to test the hypothesis that Acheron degradation is mediated by a cGMP-dependent mechanism. In order to test this hypothesis, ISMs were isolated from animals at two developmental stages: pre-eclosion day 18and 1 hr PE. The pre-eclosion ISMs contain endogenous Acheron which is missing an hour later. ISMs were homogenized in PBS buffer to create a broken bell preparation (BCP) that should contain both Acheron and the enzymatic machinery that modifies the protein.

As seen in Figure 3.1, there is no shift in molecular weight of Acheron upon the addition of either cGMP or ATP in the absence of the phosphodiesterase inhibitor IBMX. However upon addition of both IBMX and cGMP, there is an increase in molecular weight of Acheron (Figure 3.2). Based on the pattern of the shift, and separate studies that demonstrate the presence of phosphor-ser/thr (Smith, Brown and Schwartz, unpublished) this shift in molecular weight is presumed to represent a phosphorylation event. The fact that the presumptive phosphorylation was dependent on the addition of the phosphodiesterase provides further support for the fact that this is likely a cGMP mediated process.

Furthermore, the presumptive phosphorylated Acheron is degraded upon addition of the 1 hr PE extract (Figure 3.2). This indicates that the enzymes and proteases required to degrade Acheron are present and active in the 1 hr PE extract but not in the D18 extract. These data are consistent with previous Western data demonstrating that Acheron is present on day 18 but the degraded just prior to adult eclosion and absent post-eclosion.

Phosphorylation of Acheron appears to be required for its degradation (Figure 3.3). Figure 3.3 shows that when D18 extract, which contains native Acheron, is mixed with 1 hr PE ISM extract, Acheron protein is retained. However, if the same experiment is performed in the presence of the phosphodiesterase inhibitor IBMX plus cGMP, then Acheron is degraded. These data support the hypothesis that Acheron phosphorylation is required for degradation by enzymes present in the 1 hr PE extract. Finally, exogenously synthesized Acheron can also be phosphorylated using the kinases and enzymes present in the D18 extract and then degraded after phosphorylation by the 1 hr PE extract (Figure 3.4).

One interesting point to emphasize, is that these assays were conducted in the presence of Roche's cOmplete protease inhibitor. Therefore, the degradation of Acheron was facilitated by an enzyme or protease that was insensitive to this broad series of inhibitors. In theory, this means that the eclosion associated degradation of Acheron is not mediated by the serine proteases trypsin and chymotrypsin, the cysteine protease papain, and the metallo-protease thermolysin (Roche). Therefore the "Acheronase" pathway utilizes different enzymatic machinery or the protease inhibitors present in this cocktail are not very active against the endogenous *Manduca* proteases used to degrade Acheron.

Given that phosphorylation occurs on serine, threonine and tyrosine residues, in order to further validate that Acheron can be phosphorylated, I aligned the Acheron sequences from *Manduca sexta*, *Drosophila melanogaster*, *Mus musculus*, *Danio rerio*, and *Homo sapiens*. The alignment revealed that there are seven evolutionary conserved serine residues and one evolutionary conserved threonine residue in the full length

Manduca sequence (Figure 3.6). Given that cGMP mediates its effects through Protein Kinase G (PKG), analysis of kinase binding motifs of these conserved phosphorylation residues, revealed Ser 163 as a possible PKG binding site (Figure 3.6). Taken together, the data in this chapter suggests that the dramatic disappearance of Acheron on Day 18 of development is due to phosphorylation via a cGMP mediated mechanism, possibly on Ser 163.

To further emphasize that this shift corresponds to phosphorylation and not acetylation, methylation or another post translational modification, these kinase assays need to be repeated using purified Acheron, purified PKG and radioactive ^{32}P. Further studies can also be conducted using the above mentioned kinase assays on Acheron versions in which the presumed serine residue is mutated to a phospho-null variant, such as alanine (Pearlman et al., 2011), or to a phospho-mimic residue such as glutamate or aspartate (Pearlman et al., 2011) in order to determine if Acheron can be degraded.

In Chapter II, I provided evidence that Acheron could possibly function by protecting cells. Given the dramatic disappearance of Acheron immediately preceding ISM death one possible explanation is that Acheron is protecting the ISMs from death. Taken together with the fact that ISM death in *Manduca* occurs via a cGMP-dependent mechanism, the evidence from this chapter suggests that cGMP is responsible for triggering the phosphorylation and subsequent degradation of Acheron by Acheron specific degradation enzymes present 1 Hr post eclosion.

Moreover, the data presented here are consistent with the hypothesis that loss of Acheron triggers ISM death. A proposed model for the eclosion hormone dependent degredation of Acheron and subsequent death of the ISMs is depicted in Figure 3.6. This

hypothesis is being tested directly using Acheron RNAi in *Drosophila* (Johnson, Sheel, Markstein and Schwartz, unpublished). Additional studies are focused on performing co-immunoprecipitation of Acheron from day 18 ISMs to identify presumptive Acheron binding partners. This might help delineate the mechanism by which Acheron may inhibit PCD.

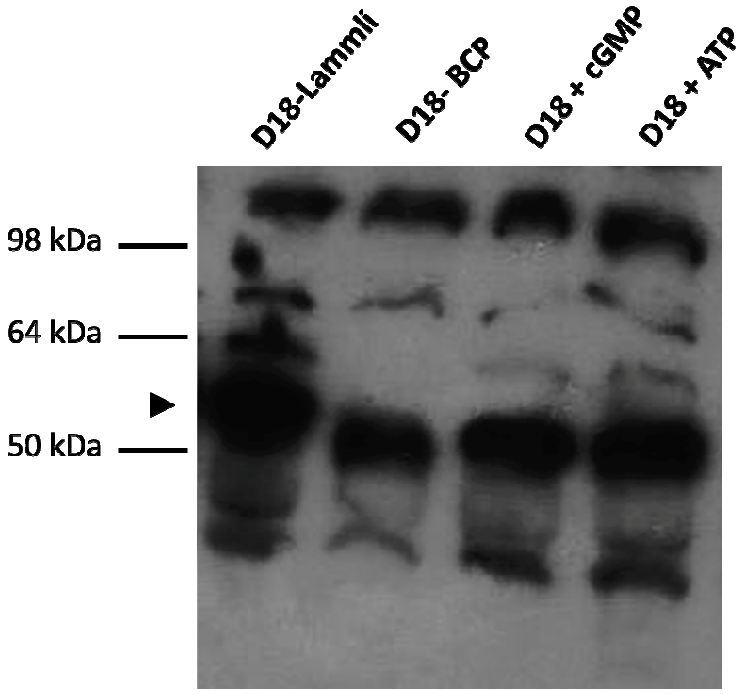

Figure 3.1: Acheron modification in the absence of the phosphodiesterase inhibitor IBMX

Analysis of Acheron in expression in D18 ISMs with anti-*Manduca* Acheron via Western blotting. The first lane (D18-Laemmli) contains lysate from D18 extracted in Laemmli buffer. The second lane (D18-BCP) contains the D18 PBS broken cell prep. The third lane (D18+cGMP) contains the products of the kinase reaction with D18 PBS broken cell prep and exogenous cGMP in the absence of IBMX. The fourth lane (D18+ATP) contains the products of the kinase reaction with D18 PBS broken cell prep, exogenous cGMP and ATP in the absence of IBMX. The bands corresponding to Acheron is denoted by a black arrow.

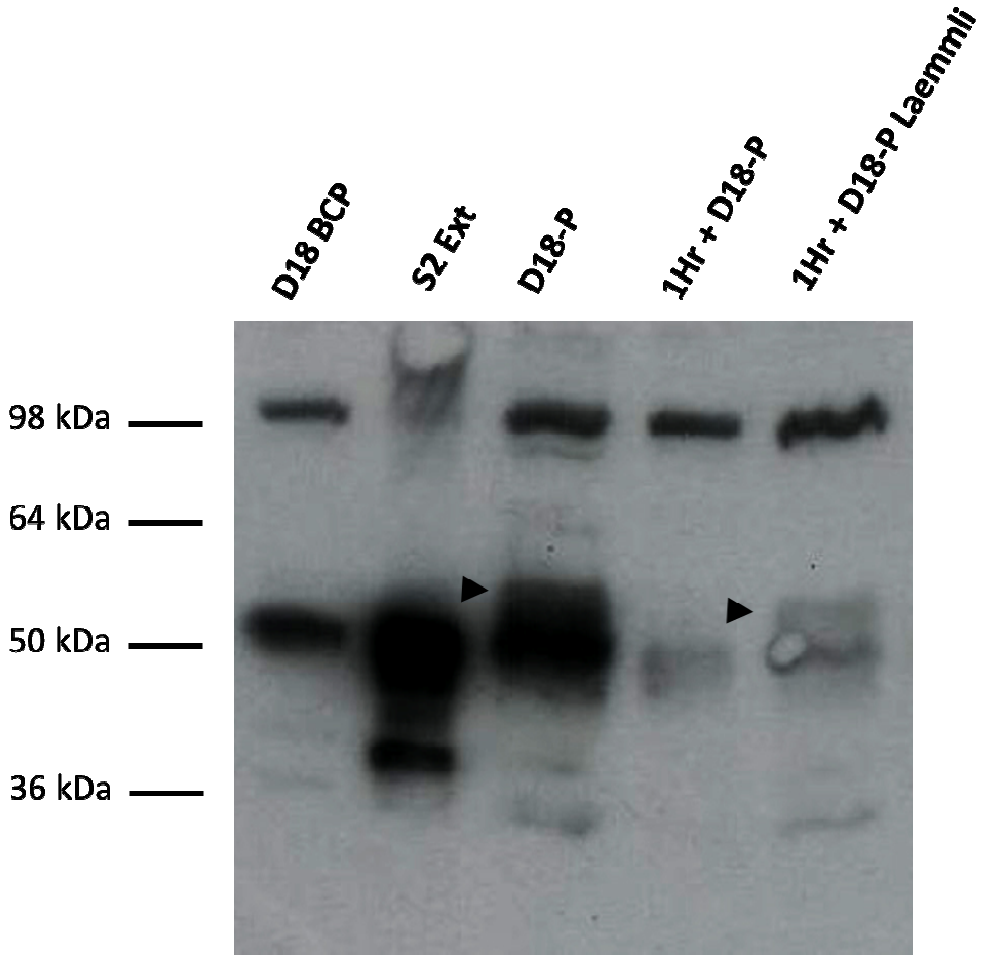

Figure 3.2: Endogenous Acheron Modification and Degradation

Analysis of Acheron after the kinase assay using D18 ISMs detected with anti-*Manduca* Acheron via Western blotting. The first lane (D18 BCP) contains the D18 PBS broken cell prep. The second lane (S2 Ext) contains the extract from *Drosophila* S2 cells engineered to express ectopic *Manduca* Acheron. The third lane (D18-P) contains the products of the kinase assay using the D18 PBS broken cell prep with exogenous cGMP and IBMX. The fourth lane (1Hr + D18-P) contains the products of the reaction when BCP from ISMs staged at 1hr PE is added to the D18-P sample. The fifth lane (1Hr + D18-P in Laemmli) contains the products of the reaction when BCP from ISMs staged at 1hr PE is added to the D18-P sample in the presence of Laemmli buffer. The presence of the modified Acheron is denoted by a black arrow.

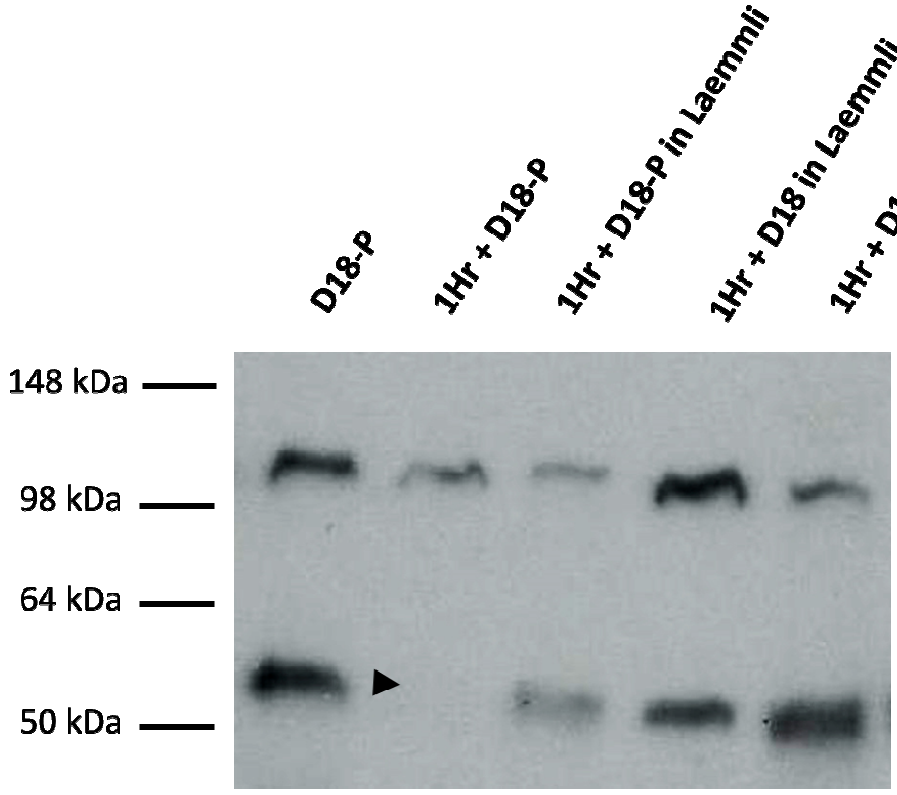

Figure 3.3: Presumptive Phosphorylation of native Acheron is required for degradation

Analysis of Acheron after the kinase assay using D18 ISMs detected with anti-*Manduca* Acheron via Western blotting. The first lane (D18-P) contains the products of the kinase assay using the D18 PBS broken cell prep with exogenous cGMP and IBMX. The second lane (1Hr + D18-P) contains the products of the reaction when BCP from ISMs staged at 1hr PE is added to the D18-P sample. The absence of Acheron protein is marked by an arrow. The third lane (1Hr + D18-P in Laemmli) contains the products of the reaction when BCP from ISMs staged at 1hr PE is added to the D18-P sample in the presence of Laemmli buffer. The fourth lane (1hr + D18 in Laemmli) contains the products of the reaction when BCP from ISMs staged at 1hr PE is added to the BCP from D18 in the presence of Laemmli buffer. The fifth lane (1hr + D18) contains the products of the reaction when BCP from ISMs staged at 1hr PE is added to the BCP from D18.

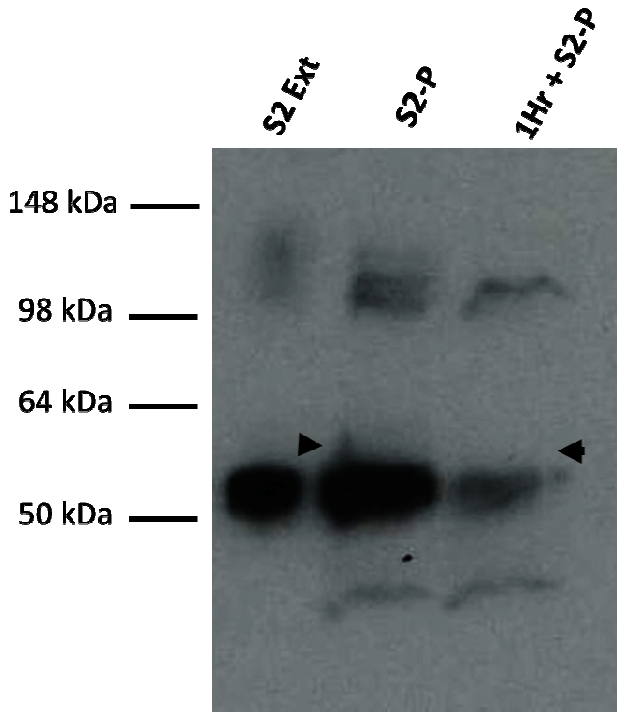

Figure 3.4: Ectopic Acheron can be modified and degraded.

Analysis of Acheron synthesized ectopically in S2 cells detected with anti-*Manduca* Acheron via Western blotting. The first lane (S2 Ext) contains the extract from the S2 cells. The second lane (S2-P) contains the products of the reaction when Day 18 ISM was added to the S2 Ext sample along with cGMP and IBMX. The presence of the modified Acheron is denoted by a black arrow. The third lane (1Hr+S2-P) contains the products of the reaction when extract from ISMs staged at 1 hr PE is added to the S2-P sample. The absence of the modified Acheron is denoted by a black arrow.

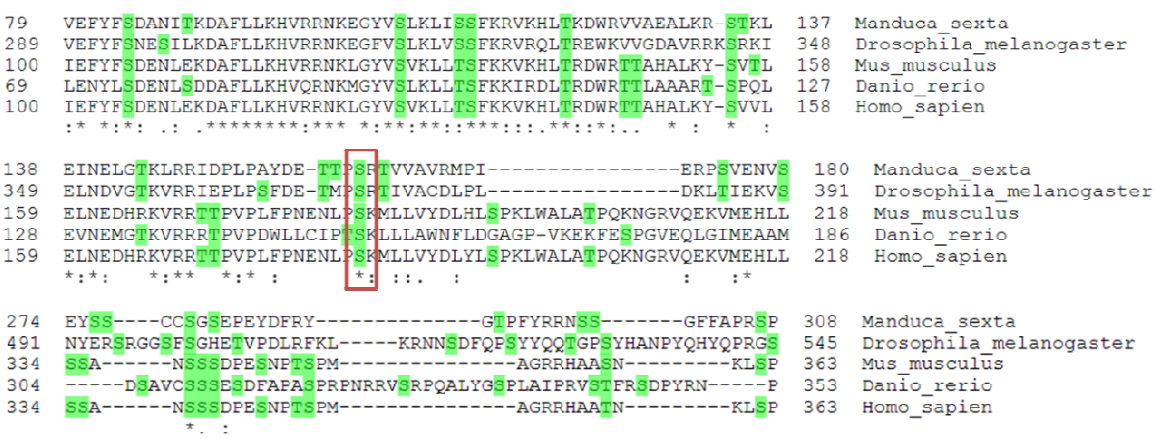

Figure 3.5: Acheron protein sequence alignment

Alignment of the Acheron protein sequence for *Manduca sexta, Drosophila melanogaster*, *Mus musculus, Danio rerio* and *Homo sapien* with conserved Serine and Threonine residues highlighted in green and the potential PKG phosphorylation site boxed in red.

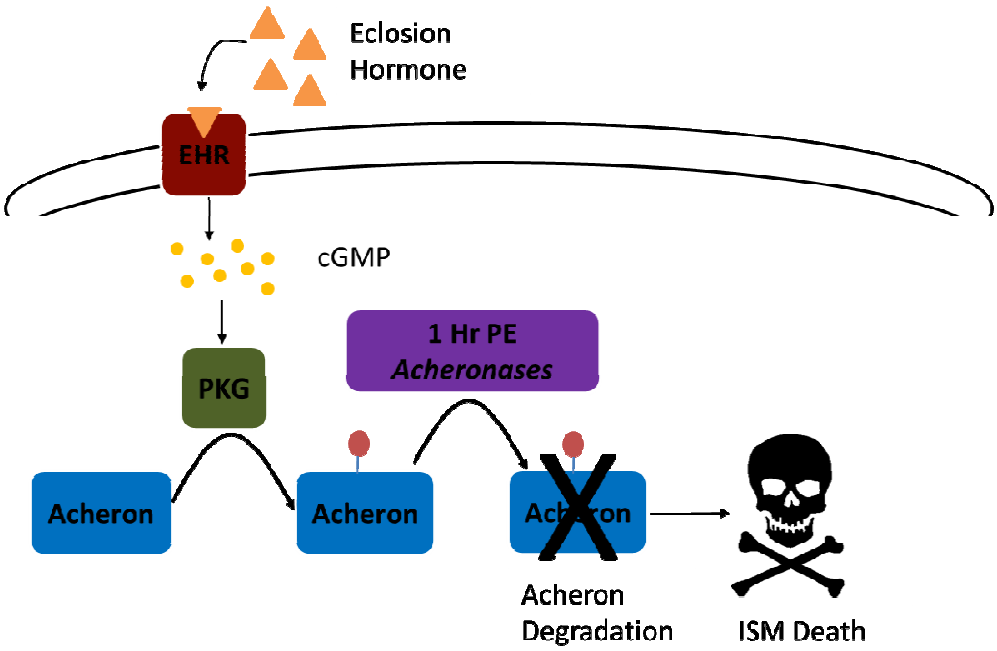

Figure 3.6: Proposed Model of Acheron's Role in ISM Cell Death.

Proposed model of ISM death. The decline in 20E triggers the release of Eclosion Hormone. Eclosion hormone binds to its receptors and causes a release in cGMP. Activation of cGMP presumably activates PKG which phosphorylates Acheron. Presumptive phosphorylation of Acheron targets it for degradation via Acheron specific machinery present 1 Hr P.E. Finally, loss of Acheron triggers cell death.

BIBLIOGRAPHY

Abdelwahid, E., Pelliniemi, L. J., & Jokinen, E. (2002). Cell death and differentiation in the development of the endocardial cushion of the embryonic heart. Microscopy research and technique, 58(5), 395-403.

Beaulaton, J., & Lockshin, R. A. (1977). Ultrastructural study of the normal degeneration of the intersegmental muscles of Antheraea polyphemus and Manduca sexta (Insecta, Lepidoptera) with particular reference to cellular autophagy. Journal of morphology, 154(1), 39-57.

Boettiger, D., Enomoto-Iwamoto, M., Yoon, H. Y., Hofer, U., Menko, A. S., & Chiquet-Ehrismann, R. (1995). Regulation of integrin α5β1 affinity during myogenic differentiation. Developmental biology, 169(1), 261-272.

Brennan, R. G., & Matthews, B. W. (1989). The helix-turn-helix DNA binding motif. J. biol. Chem, 264(4), 1903-1906.

Briggs, R., & King, T. J. (1952). Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. Proceedings of the National Academy of Sciences, 38(5), 455-463.

Cerella, C., Coppola, S., Maresca, V., De Nicola, M., Radogna, F., & Ghibelli, L. (2009). Multiple Mechanisms for Hydrogen Peroxide–Induced Apoptosis. Annals of the New York Academy of Sciences, 1171(1), 559-563.

Coucouvanis, E., & Martin, G. R. (1995). Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo. Cell, 83(2), 279-287.

Driesch, H. (1895). Zur Analysis der Potenzen embryonaler Organzellen. Archiv für Entwicklungsmechanik der Organismen, 2(2), 169-203.

Ellis, H. M., & Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode C. elegans. Cell, 44(6), 817-829.

Ellis, R. E., Yuan, J., & Horvitz, H. R. (1991). Mechanisms and functions of cell death. Annual review of cell biology, 7(1), 663-698.

Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., & Henson, P. M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. The Journal of Immunology, 148(7), 2207-2216.

Galluzzi, L., De Santi, M., Crinelli, R., De Marco, C., Zaffaroni, N., Duranti, A., & Magnani, M. (2012). Induction of endoplasmic reticulum stress response by the indole-3-

carbinol cyclic tetrameric derivative CTet in human breast cancer cell lines. PloS one, 7(8), e43249.

Glenn, H. L., Wang, Z., & Schwartz, L. M. (2010). Acheron, a Lupus antigen family member, regulates integrin expression, adhesion, and motility in differentiating myoblasts. American Journal of Physiology-Cell Physiology,298(1), C46.

Glücksmann, A. (1951). Cell deaths in normal vertebrate ontogeny. Biological Reviews, 26(1), 59-86.

Gurdon, J. B. (1962). The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. Journal of embryology and experimental morphology, 10(4), 622-640.

Haas, A. L., Baboshina, O., Williams, B., & Schwartz, L. M. (1995). Coordinated induction of the ubiquitin conjugation pathway accompanies the developmentally programmed death of insect skeletal muscle. Journal of Biological Chemistry, 270(16), 9407-9412.

Hagen, T. M., Yowe, D. L., Bartholomew, J. C., Wehr, C. M., Do, K. L., Park, J. Y., & Ames, B. N. (1997). Mitochondrial decay in hepatocytes from old rats: membrane potential declines, heterogeneity and oxidants increase. Proceedings of the National Academy of Sciences, 94(7), 3064-3069.

Hengartner, M. O., & Horvitz, H. R. (1994). C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. Cell, 76(4), 665-676.

Horder, T. 2010. History of Developmental Biology. eLS.

Hardy, K. A. T. E., Handyside, A. H., & Winston, R. M. (1989). The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. Development, 107(3), 597-604.

Jacobson, M. D., Weil, M., & Raff, M. C. (1997). Programmed cell death in animal development. Cell, 88(3), 347-354.

Jang, B. C., Sanchez, T., Schaefers, H. J., Trifan, O. C., Liu, C. H., Creminon, C., & Hla, T. (2000). Serum withdrawal-induced post-transcriptional stabilization of cyclooxygenase-2 mRNA in MDA-MB-231 mammary carcinoma cells requires the activity of the p38 stress-activated protein kinase. Journal of Biological Chemistry, 275(50), 39507-39515.

Jolly, C., & Morimoto, R. I. (2000). Role of the heat shock response and molecular chaperones in oncogenesis and cell death. Journal of the National Cancer Institute, 92(19), 1564-1572.

Jones, M.E.E., Haire, M.F., Kloetzel, P.-M., Mykles, D., and Schwartz, L.M. (1995) Changes in structure and function multicatalytic proteinase (proteasome) activity during the programmed death of intersegmental muscles of the Hawkmoth Manduca sexta. Developmental Biology, 169:436-447.

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British journal of cancer, 26(4), 239.

Kim, B.G., Gao M.Q., Choi Y.P., Kang S., Park H.R., Kang K.S., & Cho N.H. (2012). Invasive breast cancer induces laminin-332 upregulation and integrin β4 neoexpression in myofibroblasts to confer an anoikis-resistant phenotype during tissue remodeling. Breast Cancer Res, 14 (3), R88.

Kroemer, G., Galluzzi, L., & Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. Physiological reviews, 87(1), 99-163.

Kuwana. (1936). Degeneration of muscles in the silkworm moth. Zool. Mag. Tokyo, 48, 881-884.

Lindsten, T., Ross, A. J., King, A., Zong, W. X., Rathmell, J. C., Shiels, H. A., & Thompson, C. B. (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Molecular cell, 6(6), 1389-1399.

Li, N., Ragheb, K., Lawler, G., Sturgis, J., Rajwa, B., Melendez, J. A., & Robinson, J. P. (2003). Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. Journal of Biological Chemistry, 278(10), 8516-8525.

Liu, L. F., Desai, S. D., LI, T. K., Mao, Y., Sun, M., & SIM, S. P. (2000). Mechanism of action of camptothecin. Annals of the New York Academy of Sciences, 922(1), 1-10.

Liu, X., Kim, C. N., Yang, J., Jemmerson, R., & Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell, 86(1), 147-157.

Lockshin, R. A. (1969). Programmed cell death. Activation of lysis by a mechanism involving the synthesis of protein. Journal of insect physiology,15(9), 1505-1516.

Lockshin RA, and Williams CM. (1965) Programmed Cell Death--I. Cytology Of Degeneration In The Intersegmental Muscles Of The Pernyi Silkmoth. J Insect Physiol. 1965 Feb;11:123-33.

Metzstein, M. M., Stanfield, G. M., & Horvitz, H. R. (1998). Genetics of programmed cell death in C. elegans: past, present and future. Trends in Genetics, 14(10), 410-416.

Miyoshi, N., Oubrahim, H., Chock, P. B., & Stadtman, E. R. (2006). Age-dependent cell death and the role of ATP in hydrogen peroxide-induced apoptosis and necrosis. Proceedings of the National Academy of Sciences of the United States of America, 103(6), 1727-1731.

Myung, J., Kim, K. B., & Crews, C. M. (2001). The ubiquitin-proteasome pathway and proteasome inhibitors. Medicinal research reviews, 21(4), 245-273.

National Research Council. (2000). Scientific frontiers in developmental toxicology and risk assessment.

Pearlman, S. M., Serber, Z., & Ferrell Jr, J. E. (2011). A mechanism for the evolution of phosphorylation sites. Cell, 147(4), 934-946.

Porter, A. G., & Jänicke, R. U. (1999). Emerging roles of caspase-3 in apoptosis. Cell death & differentiation, 6(2).

Rakha, E. A., Reis-Filho, J. S., & Ellis, I. O. (2008). Basal-like breast cancer: a critical review. Journal of Clinical Oncology, 26(15), 2568-2581.

Reape, T. J., & McCabe, P. F. (2008). Apoptotic-like programmed cell death in plants. New Phytologist, 180(1), 13-26.

Reynolds, S. E., Löw, P., & Sass, M. (2001). Proteolytic activity of 26S proteasomes isolated from muscles of the tobacco hornworm, Manduca sexta: differences between surviving muscles and those undergoing developmentally programmed cell death. Acta Biologica Hungarica, 52(4), 435-442.

Roux, W. (1888). Beiträge zur Entwickelungsmechanik des Embryo. Archiv für pathologische Anatomie und Physiologie und für klinische Medicin, 114(2), 246-291.

Saito, Y., Nishio, K., Ogawa, Y., Kimata, J., Kinumi, T., Yoshida, Y., & Niki, E. (2006). Turning point in apoptosis/necrosis induced by hydrogen peroxide. Free radical research, 40(6), 619-630.

Schwartz, L. M. (2008). Atrophy and programmed cell death of skeletal muscle.Cell Death & Differentiation, 15(7), 1163-1169.

Schwartz, L. M., Myer, A., Kosz, L., Engelstein, M., & Maier, C. (1990). Activation of polyubiquitin gene expression during developmentally programmed cell death. Neuron, 5(4), 411-419.

Schwartz, L.M., Kosz, L., and Kay, B.K. (1990) Gene Activation is Required for Developmentally Programmed Cell Death. Proceedings of the National Academy of Science 87:6594-6598.

Schwartz, L. M., & Ruff, R. L. (2002). Changes in contractile properties of skeletal muscle during developmentally programmed atrophy and death. American Journal of Physiology-Cell Physiology, 282(6), C1270-C1277.

Schwartz, L. M., Smith, S. W., Jones, M. E., & Osborne, B. A. (1993). Do all programmed cell deaths occur via apoptosis?. Proceedings of the National Academy of Sciences, 90(3), 980-984.

Schwartz, L.M. and Truman, J.W. (1983) Hormonal control of rates of metamorphic development in the tobacco hornworm Manduca sexta. Developmental Biology 99:103-114

Segovia, M., Haramaty, L., Berges, J. A., & Falkowski, P. G. (2003). Cell death in the unicellular chlorophyte Dunaliella tertiolecta. A hypothesis on the evolution of apoptosis in higher plants and metazoans. Plant Physiology, 132(1), 99-105.

Shao, R., Scully, S. J., Yan, W., Bentley, B., Mueller, J., Brown, C., Bigelow, C. and Schwartz, L. M. (2012). The novel lupus antigen related protein acheron enhances the development of human breast cancer. Int. J. Cancer, 130: 544–554.

Shemarova, I. V. (2010). Signaling mechanisms of apoptosis-like programmed cell death in unicellular eukaryotes. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 155(4), 341-353.

Sherman, M. Y., & Goldberg, A. L. (2001). Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. Neuron, 29(1), 15-32.

Spemann, H.. (1938). Embryonic Development and Induction. Silliman Lectures. Yale University Press, New Haven.

Spemann, H. & Mangold, H. (1924). Über die Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. W. Roux' Arch. f. Entw. d. Organis. u. mikrosk. Anat. 100: 599-638.

Sun, D., Sathyanarayana, U.G., Johnston, S.A. and Schwartz, L.M. (1996) A member of the phylogenetically conserved CAD family of transcriptional regulators is dramatically upregulated during the programmed cell death of skeletal muscle in the tobacco hawkmoth Manduca sexta. Developmental Biology, 173:499-509.

Sun, D., Ziegler, R., Milligan, C.E., Fahrbach, S. and Schwartz, L.M. (1995) Apolipophorin III is Dramatically Up-Regulated During the Programmed Death of Insect Skeletal Muscle and Neurons. Journal of Neurobiology 26:119-129.

Sutherland, E. W., & Robison, G. A. (1966). A. THE ROLE OF CYCLIC-3', 5'-AMP IN RESPONSES TO CATECHOLAMINES AND OTHER HORMONES. Pharmacological reviews, 18(1), 145-161.

Tsyrlov, I. B., Gromova, O. A., & Lyakhovich, V. V. (1976). Mechanism of inhibition by carbonyl cyanide m-chlorophenylhydrazone and sodium deoxycholate of cytochrome P-450-catalysed hepatic microsomal drug metabolism. Biochem. J, 160, 75-83.

Valavanis, C., Wang, Z., Sun, D. Vaine, M., and Schwartz, L.M. (2007) Acheron, a novel member of the Lupus Antigen family, is induced during the programmed cell death of skeletal muscles in the moth Manduca sexta. Gene 393:101–109.

Vogt, C. (1842). Untersuchungen u¨ ber die Entwicklungsgeschichte der Geburtshelferkroete (Alytes obstertricians). (Solothurn, Switzermatogenesis: Jent und Gassman).

Wang Z, Glenn H, Brown C, Valavanis C, Liu JX, Seth A, Thomas JE, Karlstrom RO, Schwartz LM. "Regulation of muscle differentiation and survival by acheron." Mech Dev 2009; 126: 700–9.

Weng, H., Kim, C., Valavanis, C., Wang, Z., and Schwartz, L.M. (2009) Acheron, a Novel La Antigen Family Member, Binds To CASK And Forms A Complex With Id Transcription Factors. Cell. Mol. Biol. Letters, 14:273-287.

Weil, M., Jacobson, M. D., & Raff, M. C. (1997). Is programmed cell death required for neural tube closure? Current Biology, 7(4), 281-284.

Williams JR, Little JB, Shipley WU. (1974). Association of mammalian cell death with a specific endonucleolytic degradation of DNA. Nature, 252: 754-755.

Yu, T., Robotham, J. L., & Yoon, Y. (2006). Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. Proceedings of the National Academy of Sciences of the United States of America, 103(8), 2653-2658.

Yuan, J., & Horvitz, H. R. (1990). Genetics mosaic analysis of ced-3 and ced-4, two genes that control programmed cell death in the nematode C. elegans. Dev. Biol,, 138: 33-41.

Zahir, N., Lakins, J. N., Russell, A., Ming, W., Chatterjee, C., Rozenberg, G. I., & Weaver, V. M. (2003). Autocrine laminin-5 ligates α6β4 integrin and activates RAC and NFκB to mediate anchorage-independent survival of mammary tumors. The Journal of cell biology, 163(6), 1397-1407.

Zhang, X., Rosenstein, B. S., Wang, Y., Lebwohl, M., & Wei, H. (1997). Identification of possible reactive oxygen species involved in ultraviolet radiation-induced oxidative DNA damage. Free Radical Biology and Medicine, 23(7), 980-985.

Zhaohui Wang, "Acheron, a novel regulator of myoblast differentiation" (January 1, 2003). Electronic Doctoral Dissertations for UMass Amherst. Paper AAI3078726.

Zou, H., Henzel, W. J., Liu, X., Lutschg, A., & Wang, X. (1997). Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome *c*–dependent activation of caspase-3. Cell, 90(3), 405-413.