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Shannon Smith

University of Tennessee - Knoxville, ssmit170@utk.edu

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Characterization of the apoptotic functions of the HID homolog isolated from *Megaselia scalaris*

Shannon Smith  
Department of Biochemistry and Cellular and Molecular Biology  
The University of Tennessee, Knoxville  
Faculty Advisor – Dr. Jae Park
ABSTRACT

Apoptotic cell death is crucial for the normal development of animals. Apoptosis is a form of programmed cell death in which cell death is precisely regulated by a genetic program. *Drosophila melanogaster* has been extensively used as a model system to study the molecular basis of apoptosis. *Reaper (rpr), head involution defective (hid), grim, and sickle* are the death promoters best characterized in *D. melanogaster*. The products of these death genes antagonize caspase inhibitor, thereby activating the caspases, which are the ultimate executioners of programmed cell death (Lee et al., 2012). To investigate whether apoptotic mechanisms are conserved in remotely related species, we cloned a gene from the scuttle fly, *Megaselia scalaris*, homologous to *Drosophila hid (dHID)*. The isolated scHID cDNA encodes a protein of 197 amino acids (aa), which was much shorter than that of dHID-410 aa. We then tested the killing activity of this gene in *Drosophila* neurons using a UAS-gal4 transgenic expression system. The results showed that the scHID killed most of the neurons that we tested in the *Drosophila* central nervous system. This indicates that despite the significant structural divergence between the two fly species, the death domains of both have been similarly conserved over time and are sufficient for executing cell death.
INTRODUCTION

The fruit fly *D. melanogaster* has been a model organism for nearly 100 years in the study of genetics, development, learning, behavior, and the synaptic physiology of neuromuscular junctions. However, other fly species, such as *Megaselia scalaris*, show characteristics not seen in *Drosophila*. These characteristics include novel behaviors and development that may offer insight into mechanisms and pathways such as programmed cell death (PCD) which is required for normal development and function.

PCD is a process that occurs in all animals and apoptosis is a form of PCD that is genetically controlled and characterized by distinct morphological changes (Hay et al., 2004). In a cell undergoing apoptosis, the cytoplasm and nucleus contract while the structure of the organelles remains essentially the same. Small, apoptotic bodies then bud from the dying cell and are consumed by phagocytes (Abrams et al., 1993).

Apoptosis occurs not only throughout development, but also into adulthood, serving many important functions. Apoptosis sculpts structures into their appropriate forms and removes excess cells and tissues that are no longer useful. During development, apoptosis is important for cellular proofreading; it can eliminate gametes that contain damaged DNA or an incorrect number of chromosomes. In adults, apoptosis is necessary for tissue homeostasis and works by balancing cell proliferation with death. Additionally, apoptosis can protect organisms from hazardous cells such as those infected with a virus or those which are undergoing uncontrolled proliferation, which is a hallmark of cancerous cells (Hay et al., 2004).

The study of PCD began in *C. elegans*, in which key apoptotic regulators that are evolutionarily conserved across different species were identified. These conserved key factors are caspases; the end apoptotic executioners (Lee et al., 2013). These caspases are inactivated by *Drosophila* inhibitor of apoptotic protein 1 (DIAP1) through the formation of a complex between DIAP1 and the caspase. When the cell receives a death signal, the death activators RHG, which are encoded by *rpr*, *hid*, and *grim*, either degrade DIAP1 or bind to DIAP1, breaking the DIAP1-caspase complex, which then allows apoptosis to occur (Choi et al., 2006) (See Figure 1).

Flies are a useful system to study apoptosis because of the prevalence of PCD throughout the fly life cycle (Hay et al., 2004). Additionally, there are many genetic tools available to study fly species (specifically *Drosophila*), such as gene targeting and the capacity to drive gene expression in specific tissues (Hay et al., 2004). Past studies have shown that apoptosis in
Drosophila as well as in mammals utilizes similar tools and mechanisms. However, the specific molecular mechanisms of PCD are still largely unknown (See Figure 2).

Previous studies located the hid gene in the H99 interval of the third Drosophila chromosome (Grether et al., 1995). Hid is thought to work cooperatively with the other death genes, rpr and grim, to prevent unintentional cell death due to the over activation of a single death gene. Despite the cooperative nature of RHG, each death activator is hypothesized to have distinct functions in the apoptotic pathway due to their distinct expression patterns, both temporally and spatially (Lee et al., 2013).

This project aims at investigating whether apoptotic mechanisms, specifically the apoptotic functions of hid, have been conserved evolutionarily over time by utilizing the distinct genetic makeup of the Megaselia line, which diverged from the Drosophila lineage over 100 million years ago. We hope to identify, through future research, novel functions of the hid gene by identifying regulatory mechanisms of scHID and tissues that require it for their normal development.

METHODS
Circadian rhythm analysis

Locomotor assays were used to compare the behavioral rhythms of M. scalaris to that of D. melanogaster. One or two days old virgin female flies (n=20) were placed in individual 7-mm diameter locomotor tubes containing standard cornmeal-agar medium without yeast flakes. The tubes were placed in locomotor monitors containing dual-detectors with an infrared beam passing through each of the tubes. The monitors then recorded each time the flies crossed the beam. This information was collected by a Trikinetics system interfaced with a PC computer every 30 min. The flies were entrained for four cycles of 12-hr light: 12-hr dark (LD) conditions and then assessed for free-running locomotion for the next seven days in constant darkness (DD) at 25°C. Data analysis was completed with a ClockLab program.

Corazonin-Immunohistochemistry

Immunohistochemistry was used to determine if the general neuroanatomical features of dipteran insects have been evolutionarily conserved. The central nervous systems (CNSs) of third instar larvae were dissected in phosphate-buffered saline (PBS) and fixed in 4%
paraformaldehyde and 7.5% picric acid in 0.1M sodium phosphate buffer (SPB) overnight at 4°C. The samples were rinsed three times in SPB followed by three times in TNT buffer (0.1M Tris, pH7.4, 0.3M NaCl, 0.5% Triton X-100). The samples were incubated in blocking buffer (4% normal donkey serum and 0.02% NaN3 in TNT) for 2 h at room temperature followed by overnight incubation in anti-Crz (rabbit, diluted 1:300) primary antibody. After rinsing samples in TNT, secondary antibody, conjugated with tetramethyl rhodamine (TRITC) diluted 1:200, was applied. The samples were washed three times in TNT followed by three washes in SPB. Signals were viewed with an Olympus BX61 microscope equipped with a CC12 digital camera. Images were processed with analySIS 3.0 software.

**Cloning of scHID and transgenic flies**

The *M. scalaris* homolog of *hid* was isolated in an earlier project within the lab by Haylie Lam. The full length cDNA sequence was obtained using degenerate primers followed by Rapid Amplification of cDNA Ends (RACE) methods. The product was then purified with the QIAprep Spin Miniprep Kit by Qiagen and ligated into the pGEMT-easy vector. Following ligation, scHID-pGEMT was transformed and sequenced using SP6 and T7 primers. Subsequently the *scHID* cDNA was cloned into the pUAST vector to generate the *Drosophila-UAS-scHID* transgenic lines.

**Fly crossings**

To test the pro-apoptotic function of *scHID* in different neuropeptide-producing neurons, UAS-scHID was crossed using various neuropeptide-specific gal4 lines. Each of these gal4 transgenes was combined with *UAS-mCD8-GFP* or *UAS-lacZ* to conveniently asses cell death using either GFP or lacZ markers as follows: (1) *UAS-mCD8GFP; bursicon-gal4*, (2) *UAS-mCD8GFP, DvPdf-gal4*, (3) *UAS-mCD8GFP; CCAP-gal4*, (4) *UAS-mCD8GFP; Crz-gal4*, and (5) *UAS-LacZ; npf-gal4*. Virgin females of the gal4 lines were crossed with either control flies (*w^1118*) or with *UAS-scHID* flies. The F1 progeny at the third instar larva stage were obtained from these crosses and were subjected to immunohistochemistry.

**X-gal histochemistry**
The central nervous systems (circled in Figure 3) of larva in the third instar stage from the f1 generation of the UAS/Gal4 crosses were dissected in PBS on ice. The tissues were then fixed with 4\% paraformaldehyde, PFA, for 40 min and then washed three times with 500 ul of PBS for five min each. The tissues were then washed in 30\% glycerol and 60\% glycerol for ten min each. They were then mounted on glass slides and the different GFP expressing neurons were visualized using fluorescence microscopy.

RESULTS AND DISCUSSION

Circadian locomotor activity rhythms of scuttle flies

To determine general neurological and neuroanatomical features of the scuttle flies, we measured circadian locomotor activity rhythms and examined expression patterns of the Crz neuropeptide in the CNS. About 61\% of the *Megaselia* flies (8/13 flies) were rhythmic, while the *Drosophila* flies were 100\% (20/20) rhythmic. *Megaselia* had a period of 24.55 ± 2.06 and a power of 42.07 ± 36.25. *Drosophila* had a period of 24.43 ± 0.34 and a power of 83.90 ± 33.54. Normal *Drosophila* behavior follows a crepuscular model; they are active in the morning and in the evening with the peaks at ZT 0 (light on) and ZT 12 (light off), respectively (Fig. 4). These peaks continue into the DD days, which reflect the endogenous rhythm of the flies.

Scuttle flies, while still showing some rhythmicity, do not show a rhythmic pattern similar to *Drosophila*. During the entrainment period, there are sharp peaks at ZT 0 and ZT 12 that are only present for a short amount of time. These sharp peaks dissolve into single broad peaks around ZT 12 during DD. Scuttle flies walk in rapid bursts of movement with short pauses in between, from which their name is derived (Harrison, 2003). This, along with underlying novel neuronal developmental mechanisms and functions, could be the cause of the abnormal behavioral rhythmicity.

Corazonin-Immunohistochemistry

In order to confirm that the general neuroanatomical features have been evolutionarily conserved between *Drosophila* and *Megaselia*, immunohistochemistry was used to visualize Crz neurons in third instar larvae. The two groups of neurons located in the brain and the 16 neurons in the ventral nerve cord are present in both *Drosophila* (Fig. 5A, B) and *Megaselia* (Fig. 5C, D). Although the CNS of *Megaselia* are smaller and slightly folded, as can be seen from the
necessary detachment of the brain from the ventral nerve cord in Fig. 5C, the neurons themselves are in the comparable locations and arrangement as those in *Drosophila*. This indicates that the general neuroanatomical structures are similar between the two species and allows for further neurogenetic investigations using established methods of neuronal imaging.

**Characteristics of scHID**

The specific mechanism through which *hid* works and interacts with the other apoptotic inhibitors is still largely unknown. To investigate whether the apoptotic functions of *hid* has been conserved evolutionarily, a gene encoding a *hid* homolog was isolated from *M. scalaris*.

Overall, the *scHID* cDNA encodes a protein of 197 amino acids, while *dHID* cDNA encodes a protein of 410 amino acids. Sequence alignment showed shared conserved regions in their N- and C-terminals as illustrated in Fig. 6. Thus, we wondered if such limited structural conservation is sufficient for the cell killing activity. To test this, we ectopically expressed *scHID* in the specific peptidergic neurons of *D. melanogaster* using a gal4/UAS transgenic expression system, as described in the Methods.

In Figures 8A, 8B, 8C, 8D, and 8E the wild type cross represents the normal patterns of peptidergic neurons producing bursicon, Crz, Pdf, CCAP, and npf neuropeptides, respectively. Figures 8F, 8H, and 8J show complete death for all neurons, illustrating the intact killing function of *scHID*. However, in Figures 8I and 8G there are a small number of neurons remaining in the *scHID* sample. There are a few reasons why partial killing could have occurred. First, as mentioned above, *hid* is thought to work cooperatively with *rpr* and *grim* to promote programmed cell death (Lee et al., 2013). Therefore, in these samples, there may not have been sufficient interaction between *scHID* and the other pro-apoptotic genes to result in complete neuronal death. Similarly, it was observed, in a previous study, that cell death increased when both *Anastrepha*-hid (As-hid) and As-rpr were expressed together in cells, which strengthens the hypothesis that *hid* and *rpr* work in a cooperative manner (Schetelig et al., 2011). Secondly, the strengths of the gal4 drivers and their effect on target genes can vary. *CCAP-gal4* and *Crz-gal4* may have been weaker than the other lines. Given more time, the CCAP and Crz lines may have completely killed the remaining neurons.

Based on these results, the much attenuated *scHID* sequence is hypothesized to contain critical death domains that have been conserved over time between species. Similar to findings
from studies in *A. suspensa*, this study also suggests that pro-apoptotic genes may be more evolutionarily conserved among insect species than previously thought (Schetelig et al., 2011). Future projects will aim to reveal the regulatory mechanisms of scHID in the development of *M. scalaris* in addition to identifying tissues that require scHID for normal apoptotic functions.

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**LITERATURE CITED:**


Figure 1: ARK promotes the activation of the caspase DRONC and, therefore apoptosis, in cells that should normally live. This activation is thought to be regulated by DEBCL/Buffy. DIAP1, an apoptosis inhibitor, inhibits DRONC and the effector caspases activated by DRONC, such as DRICE DIAP1 binding proteins RPR, HID, and GRIM (RHG), promote cell death by disrupting the inhibitory action of DIAP1. (Hay et al., 2004)

Figure 2: Phylogenic tree showing the divergence of Megaselia scalaris, family Phoridae, from the Drosophila lineage. (http://cedarcreek.umn.edu/insects/orderpages/diptera1.html)
Figure 3: Diagram illustrating the formation and use of the UAS/GAL4 system. (adopted from St. Johnston D. 2002. Nat Rev Genet 3: 176-188)

Figure 4: Locomotor activity rhythms. Seven Megaselia died and their data was not included in the analysis. Megaselia scalaris (left) rhythmicity = 61.5% (8/13 flies) with a period of 24.55 ± 2.06 and a power of 42.07 ± 36.24. Drosophila melanogaster (right) rhythmicity = 100% (20/20 flies) with a period of 24.43 ± 0.34 and a power of 83.90 ± 33.54. The crepuscular rhythm of Drosophila, present during both the entrainment period and DD, are represented by the peaks at ZT 0 and ZT 12, which indicate light on and light off respectively. The crepuscular rhythm of Megaselia was much less apparent during the entrainment period and absent during DD.
Figure 5: Circadian locomotor activity rhythms in *Megaselia scalaris* and *Drosophila melanogaster*. The activities were measured for four periods of 12:12 LD cycles represented by the light and dark grey shaded areas. A period of total darkness then followed for seven days.
**Figure 6:** Neurons detected with Crz-immunohistochemistry. Larval Crz neurons in *Drosophila* (A, B) and in *Megaselia* (C, D). Two groups of neurons are located in the brain while another group of 16 neurons (two rows of eights) are located in the central nerve cord. A and C are taken at 10X, while B and D are taken at 20X.

**Figure 7:** 5’ region of scHID, about 300 bp. Underlined regions at beginning and end represent the EcoR I restriction enzyme site. The portions of the sequence in red are the forward (5’) and reverse (r3) primer sequences.
Figure 8: Images of the scHID expression in different tissues using green fluorescence protein. (A) UAS-mCD8GFP; bursicon-gal4 x w^1118 (B) UAS-mCD8GFP;; Crz-gal4 x w^1118 (C) UAS-mCD8GFP; CCAP-gal4 x w^1118. (D) UAS-mCD8GFP, DvPdf-gal4 x w^1118. (E) UAS-LacZ; npf-gal x w^1118. (F) UAS-mCD8GFP; bursicon-gal4 x UAS-scHID. (G) UAS-mCD8GFP; CCAP-gal4 x UAS-scHID. (H) UAS-mCD8GFP, DvPdf-gal4 x UAS-scHID. (I) UAS-mCD8GFP;; Crz-gal4 x UAS-scHID. (J) UAS-LacZ; npf-gal4 x UAS-scHID. E and J utilized the reporter gene LacZ rather than GFP.