Pursuit - The Journal of Undergraduate Research at The University of Tennessee

Volume 3
Issue 1 *Fall 2011*

Article 8

December 2011

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Faith V. Creekmore
fcreekm1@utk.edu

Pursuit: The Journal of Undergraduate Research at the University of Tennessee

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Neurogenetic Studies on the Role of DIAP1 in Neuronal Programmed Cell Death in *Drosophila*

FAITH V. CREEKMORE
Advisor: Jae H. Park

Department of Biochemistry and Cellular and Molecular Biology
University of Tennessee, Knoxville

The ventral Corazonin (vCrz) neurons provide a model system to study neuronal programmed cell death (PCD) in Drosophila*, as they undergo caspase-dependent PCD during early metamorphosis between 1 and 7 hours after puparium formation (APF). DIAP1 (*Drosophila *inhibitor of apoptosis) is a key survival factor, preventing accidental cell death at the wrong stage. In response to death signals, caspase activation resulting from DIAP1 degradation is a primary cause of the PCD in many tissues. However, transgenic over-expression of the wild type* diap1 *gene in vCrz neurons failed to block PCD completely. This previous result suggested its role as a survival factor in the vCrz neurons. To gain definitive evidence for* diap1*'s roles as a survival factor, two P-elements, UAS-diap1$^{(6\text{-}3s)}$ and UAS-diap1$^{\Delta RF}$, were constructed and injected into* Drosophila *embryos. Transgenic lines were established and crossed to a Crz-gal4 line for targeted over-expression. The result showed that this type of transgenic manipulations blocked vCrz neuronal PCD significantly, supporting the critical role of* diap1 *in the prevention of premature vCrz death.*

Introduction

Programmed cell death (PCD) is an integral part of normal animal development. It is a highly conserved process necessary for the removal of damaged and unwanted cells (Abrams et al., 1993; Baehrecke, 2000). Eliminating unneeded cells is vital for an organism's maintenance of homeostasis (Lee and Baehrecke, 2000).

Apoptosis is a type of PCD, and the apoptotic degeneration of unwanted or obsolete neurons in the larval central nervous system (CNS) during metamorphosis plays an important role in sculpting the adult CNS. Recent studies discovered that a specific group of peptidergic neurons, termed vCrz neurons, undergo caspase-dependent apoptotic death during early metamorphosis and therefore serve as a model system to understand the mechanisms of developmentally-regulated neuronal PCD (Choi et al., 2006; Lee et al., 2011).

Caspases play an important role in PCD, functioning as parts of a cascade resulting in cell death. Activation of initiator caspases in response to death signals begin the process by cleaving and activating executioner caspases, which are subsequently involved in proteolytic degradation of cellular proteins (Wang et al., 1999). Therefore, precise regulation of the caspase activity is extremely important for the timely death of target cells (Perez-Garijo et al., 2004).

Drosophila Inhibitor of Apoptosis Protein (DIAP1) is a 438 amino acid protein necessary to prevent inappropriate caspase activation (Steller, 2008). IAPs contain at least one Baculovirus IAP Repeat (BIR) motif, which is crucial for their anti-apoptotic function (Cashio et al., 2005). BIRs are sequences of 65 amino acids vital for both protein-protein interactions and cell death inhibition (Clem and Duckett, 1997). BIRs have the ability to bind and inhibit caspases (Steller, 2008). BIRs also bind and inactivate proapoptotic proteins, such as *Reaper, Hid, and Grim* (Wang et al., 1999).

IAP levels are controlled by ubiquitin-mediated degradation. Many IAPs, including DIAP1, contain an approximately 100 amino acid long zinc-binding RING (Really Interesting New Gene) finger domain (for short, RF). This domain is a cysteine and histidine rich zinc finger motif located on the C-terminus (Clem and Duckett, 1997). In its absence, DIAP1 is stabilized and protein levels increased (Schile et al., 2008). The RING domain acts as an E3-ubiquitin ligase responsible for recruiting E2 ubiquitin-conjugating enzyme to promote DIAP1 degradation in the presence of death signals (Vaux and Silke, 2005). When a death signal is present, up-regulation of proapoptotic genes elevates the level of proapoptotic proteins that bind to DIAP1. This molecular interaction causes self-ubiquitination of DIAP1 that leads to self-degradation (Wang et al., 1999; Goyal et al., 2000). From these results, it is conceivable that DIAP1 without the RF does not undergo self-degradation even in the presence of the proapoptotic factors.

Despite extensive studies on DIAP1's anti-apoptotic role, it is unclear whether DIAP1 is also essential for the survival of the vCrz neurons during larval stage, because over-expression of wild type *diap1* has been shown to rescue vCrz death incompletely (Choi et al., 2006). One possibility of this surprising result is because overproduced DIAP1 still undergoes self-degradation, thus failing to prevent PCD of vCrz neurons. To address this issue, this study investigated the anti-apoptotic activity of the two potentially hyperactive DIAP1s, DIAP1 lacking RF and DIAP1$^{(6\text{-}3s)}$. The former, as explained earlier, is expected to be a stable molecule to inhibit caspases. The latter was originally identified as a gain-of-function allele and carries a point mutation (from glycine to serine at the 88th position in BIR1 domain) (Goyal et al., 2000). This study found that DIAP1$^{(6\text{-}3s)}$ proteins interact with proapoptotic proteins, REAPER and HID, at much lower degree than does wild type DIAP1, thus immune to apoptosis-induced degradation. With these molecular characteristics as a basis, we hypothesize that expression of either hyperactive form of DIAP1 would more effectively block PCD of vCrz neurons than does that of wild type protein.

Materials and Methods

Purification of Genomic DNA

Genomic DNA isolation was completed using DNAzol reagent (Invitrogen). To begin, six *diap1*$^{(6\text{-}3s)}$ mutant flies were immersed in a tube containing 0.5 mL of DNAzol and ground with a pestle. The ground flies were centrifuged for 10 min at 10000x*g*. The supernatant was transferred to a new 1.5 mL tube. Next, 250 μL of 100% ethanol were added to precipitate the DNA. The contents were mixed by inversion several times and kept at room temperature for 2 min. It was again spun for 1 min at 4000x*g*. The supernatant was removed and 900 μL of 75% ethanol was added and the tube was inverted 5 times. After centrifugation for 1 min at 4000x*g*, the supernatant was removed and the DNA precipitate was washed with the 75% ethanol and spun. After removing the supernatant, the DNA pellet was air-dried for 5 min and 75 μL of elution buffer was added to dissolve the DNA for 5 min at 65° C.

Polymerase Chain Reaction (PCR) and Cloning

The diap1$^{(6\text{-}3s)}$ PCR reaction contained 2 μL of the diap1$^{(6\text{-}3s)}$ mutant fly genomic DNA, 2.5 μL each of diap1 forward and diap1 reverse primer (see Fig. 1 for primer sequences), 5μL of $MgCl_2$, 13 μL of water, and 25 μL of PCR master mix containing Taq polymerase and dNTPs (Promega). This reaction proceeded using the following cycle settings: 94ºC for 2 min, 35 cycles of 94ºC for 1 min, 55ºC for 45 sec, and 72ºC for 2 min, followed by a final extension at 72ºC for 3 min.

The diap1$^{\Delta RF}$ PCR reaction removed the RING domain. It contained 1 μL of the pre-existing UAS-diap1 plasmid diluted 1:40 in water, 4 μL each of the UAS-forward primer and the diap1$^{\Delta RF}$ reverse primer, 20 μL of PCR master mix containing Taq polymerase and dNTPs (Promega), and 11 μL of water. The reaction underwent the following cycle conditions: initial denaturation at 94ºC for 2 min, 35 cycles of 94ºC for 45 sec, 55ºC for 45 sec, and 72ºC for 2 min, and then a final extension at 72ºC for 10 min.

Each PCR product was purified using the QIAprep Spin Miniprep Kit (Qiagen). The purified DNA was then analyzed using gel electrophoresis. Next, the purified PCR products were ligated with the pGEMT-easy vector in a 10 μL ligation reaction using 10X ligation buffer and T4 ligase (Promega). After ligation was complete, the products, diap1$^{\Delta RF}$-pGEMT and diap1$^{(6\text{-}3s)}$-pGEMT, were transformed. To transform the diap1$^{(6\text{-}3s)}$-pGEMT,

PRIMER	SEQUENCE
diap1 forward	5′ CCAGAGAAGAATTCAAGAGTCAGGCA 3′
diap1 reverse	5′ TCTAGATTTGAGGACTTGGGTGCGCA 3′
UAS forward	5′ GAATACAAGAAGAGAACTCTG 3′
diap1$^{\Delta RF}$ reverse	5′ CCGATGATCTAGATTACTACTGCTCTTCC 3′

Figure 1. PCR Primer Sequences. The diap1 forward and diap1 reverse primers were used to amplify the desired gene sequence for the *diap1*$^{(6\text{-}3s)}$ mutant. The UAS forward and diap1$^{\Delta RF}$ reverse primer were used to amplify the desired gene sequence for the *diap1*$^{\Delta RF}$ mutant.

2 μL of the ligation reaction product was added to 35 μL of DH5α cells and kept on ice for 30 min. The mixture was then transferred to a 37ºC water bath for 30 sec before being returned to the ice for 2 additional min. Next, 3 mL of LB media was added and the reaction was placed in a shaker for 1 hour. Approximately 200 μL was plated onto an LB agar plate overlaid with X-gal and Isopropyl-β-D-thio-galactoside (IPTG) and kept at 37ºC overnight.

Transformation of the diap1$^{\Delta RF}$-pGEMT was done using JM110 cells because one of the restriction enzymes used, Xba I, was blocked by overlapping DNA Adenine Methylase (DAM) methylation. The enzyme's recognition site (TCTAGA) is preceded by GA causing a GATC sequence to overlap the Xba I site. The adenine in the GATC sequence is methylated by DAM and is not cut by Xba I. JM110 competent cells lack DAM activity; therefore, plasmid DNA with GATC sequence produced in the JM110 cells is not methylated and thereby sensitive to Xba I digestion. For transformation using these cells, 0.85 μL of β-mercaptoethanol was added to the 50μL JM110 competent cells. The mixture was kept on ice for 10 min during which the cells were removed every 2 min to mix. Next, 1 μL of the DNA was added to the tube. The mixture was returned to the ice for 30 min. The cells were then heat shocked for 45 sec in a 42ºC water bath before being returned to ice for 2 additional min. Next, 450 μL of LB media were added and placed in the shaker for 1 hour of incubation at 37ºC and 230 rpm. Approximately 200 μL were plated onto an LB agar plate overlaid with X-gal and IPTG and kept at 37ºC overnight.

After transformation, cells from multiple white colonies were grown separately in 3 mL LB media containing 0.1 mg/mL ampicillin overnight at 37ºC. White colonies were selected because in the presence of X-gal, white bacterial colonies are expected to contain the recombinant DNA. The plasmid DNA was purified using the QIAprep Spin Miniprep Kit according to manufacturer's manual. The presence of the insert was confirmed with a digestion using EcoR I and Xba I restriction enzymes and further verified by sequencing with SP6 and T7 primers. The positive plasmids were used to subclone the *diap1*$^{(6\text{-}3s)}$ and *diap1*$^{\Delta RF}$ fragment into the pUAST vector.

The 15 μL digestion reaction used for both constructs consisted of restriction enzymes EcoR I and Xba I. Another ligation was performed as previously described and transformed using DH5α cells for the *diap1*$^{(6\text{-}3s)}$ and JM110 cells for the *diap1*$^{\Delta RF}$. Multiple colonies were selected; the plasmid DNA was purified and digested with EcoR I and Xba I as previously described. After performing gel electrophoresis to confirm the sizes of the DNA and the vector, the potential positive clones were sequenced.

Generation of Transgenic Flies

The final plasmids were injected into *yellow white* (*y w*) embryos for germ-line transformation. As a result, five transgenic lines (one on the X, one on the second, and three on the third chromosome, one of which is homozygous lethal) were established for UAS-diap1$^{(6\text{-}3s)}$; three homozygous lines (one on the second and two on the third chromosome) were generated for UAS-diap1$^{\Delta RF}$.

Histochemical Analysis of vCrz Neuronal PCD

The presence or absence of vCrz neurons was determined by Crz-gal4 mediated expression of green fluorescence protein (GFP). For this, *UAS-mCD8-gfp;;Crz-gal4*^{T2a} virgins were crossed to *y w* (control) or various *UAS-diap1* lines (*y w; Bl/CyO, y*$^{+}$*; UAS-diap1*^{wt} positive control, *y w;;UAS-diap1*$^{(6\text{-}3s)}$*12B/TM6C*, or *y w;;UAS-diap1*$^{\Delta RF}$*17)*. The F1 progeny from each cross were then dissected at the third instar larva stage and at the 7-h APF stage.

CNSs were dissected in PBS/0.1% Triton X-100/25% ethanol (PTE) and transferred to a plate containing phosphate-buffered saline (PBS) kept on ice until the dissections were complete. The tissues were fixed in 4% paraformaldehyde (PFA) for 30 min and then washed 3 times in 500 μL PBS for 5 min each time. Next, the tissues were rinsed serially in 30%, 60%, and 90% glycerol in PBS for 10 min each. After the final rinse, they were mounted for observation; images of the vCrz neurons expressing the GFP reporter gene were acquired by fluorescence microscopy.

Results and Discussion

Cloning *diap1*$^{(6\text{-}3s)}$ and *diap1*$^{\Delta RF}$ into the pUAST vector

The UAS-Gal4 system is a targeted over-expression tool used in *Drosophila* to study gene expression and function (Brand and Perrimon, 1993). Gal4 is a transcription activator of 881 amino acids that binds to an Upstream Activation Sequence (UAS). When Gal4 binds to the UAS-responder gene, the gene becomes activated. This system allowed an investigation of the effect of *diap1* over-expression on the vCrz neuronal death in *Drosophila*.

To further address the issue of previous data not favoring DIAP1's expected anti-apoptotic function in the vCrz neurons, experiments were designed using potentially hyperactive DIAP1. The wild type DIAP1 contains two BIR domains and the C-terminal RING finger domain vital for ubiquitination-mediated degradation (Fig. 2A), while the two mutants differ in the BIR1 domain and the lack of the RING finger domain (Fig. 2B and Fig. 2C).

Fly genomic DNA was used as the template to amplify the *diap1* fragments using PCR. The *diap1* wild type and *diap1*$^{(6\text{-}3s)}$ fragments are the same size, approximately 1300 base pairs (bps), because only one nucleotide is substituted in this mutation (Fig. 3A). However, there is an apparent size difference between the *diap1* wild type and the *diap1*$^{\Delta RF}$ fragments, as expected, because the latter does not contain the approximately 300 bps that encode the RING finger (Fig. 3B).

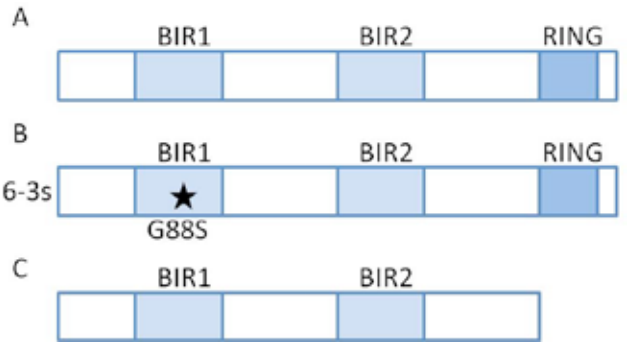

Figure 2. Protein structure of DIAP1.
A: DIAP1^{wt}.
B: DIAP1$^{(6\text{-}3s)}$ (Goyal et al., 2000).
C: DIAP1$^{\Delta RF}$.

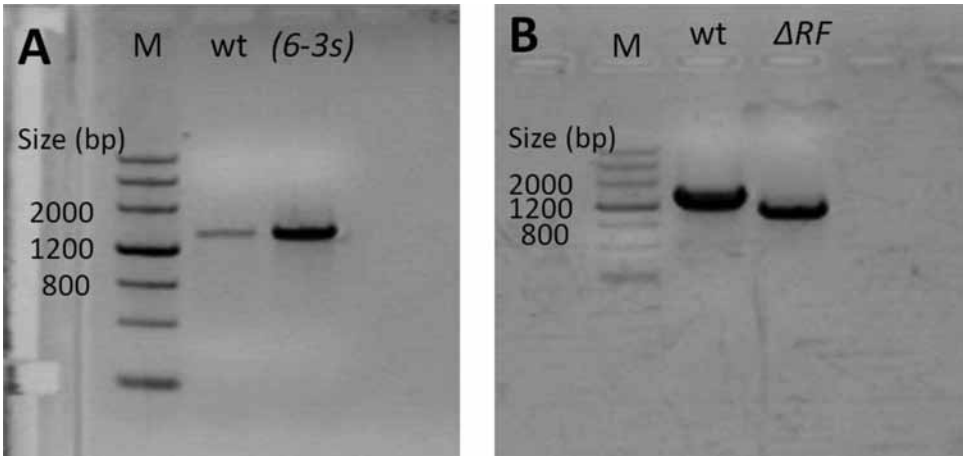

Figure 3. PCR amplification of *diap1* fragments using fly genomic DNA template (M, molecular size marker).
A: PCR of *diap1* wild type and *diap1*$^{(6\text{-}3s)}$ fragments.
B: PCR of diap1 wild type and *diap1*$^{\Delta RF}$ fragments.

EcoR I and Xba I restriction enzymes were used to digest the recombinant plasmids to confirm the presence of the insert DNA. According to the gel electrophoresis result, samples 4, 5, and 6 of *diap1*$^{(6\text{-}3s)}$-pGEMT contained the desired insert because the *diap1*$^{(6\text{-}3s)}$ fragment is approximately 1300 bps while the pGEMT vector has a length of approximately 3000 bps (Fig. 4A). All plasmid clones of *diap1*$^{\Delta RF}$-pGEMT were confirmed to have the *diap1*$^{\Delta RF}$ fragment measuring just over 1000 bps (Fig. 4B).

After the ligation of *diap1*$^{(6\text{-}3s)}$-pUAST and *diap1*$^{\Delta RF}$-pUAST, EcoR I and Xba I restriction enzymes were again used for digestion. The digestion of samples 3, 5, and 6 of *diap1*$^{(6\text{-}3s)}$-pUAST were confirmed with the *diap1*$^{(6\text{-}3s)}$ insert shown at its proper size (approximately 1300 bps) and the pUAST vector at approximately 9000 bps (Fig. 5A). All the *diap1*$^{\Delta RF}$-pUAST samples were successfully digested as the *diap1*$^{\Delta RF}$ fragment again measures approximately 1000 bps (Fig. 5B).

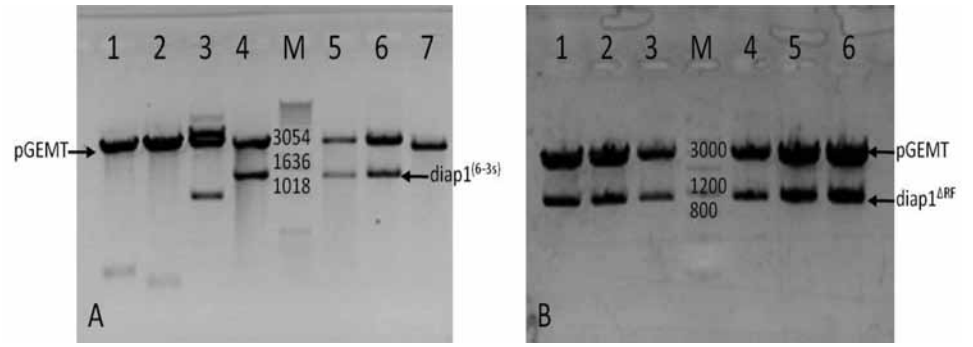

Figure 4. Digestion using EcoR I and Xba I restriction enzymes (M, molecular size marker).
A: Digestion of *diap1*$^{(6\text{-}3s)}$-pGEMT.
B: Digestion of *diap1*$^{\Delta RF}$-pGEMT.

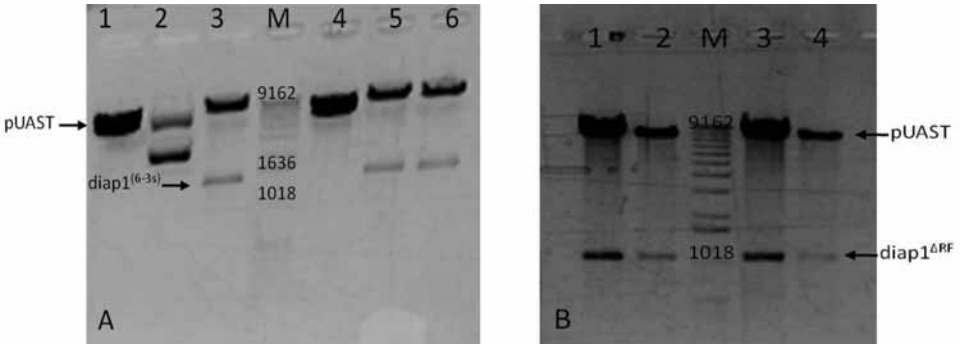

Figure 5. Digestion using EcoR I and Xba I restriction enzymes (M, molecular size marker).
A: Digestion of *diap1*$^{(6\text{-}3s)}$-pUAST.
B: Digestion of *diap1*$^{\Delta RF}$-pUAST.

Examination of vCrz neurons

After the final plasmids were confirmed by sequencing, they were injected into fly embryos for germ-line transformation. The resulting UAS-diap1$^{(6\text{-}3s)}$, UAS-diap1$^{\Delta RF}$ and pre-existing UAS-diap1^{wt} flies were crossed with Crz-gal4 flies to assess the existence of the vCrz neurons using the GFP reporter gene expression as a vital marker. When the larval CNS was examined, the 16 vCrz neurons were always present, regardless of the *diap1* transgene expressed, indicating that there is no detrimental effect by overproduction of DIAP1 (Fig. 6A). In wild type, these vCrz neurons disappear normally through PCD within 7 hours APF. However, over-expression of various diap1 constructs prevented vCrz PCD to certain degrees.

Over-expression of wild type *diap1* rescued an average of 6 neurons, demonstrating that the over-expression of the wild type gene only marginally inhibited PCD (Fig. 6B and 7). The hyperactive forms of DIAP1, however, had a stronger effect on the inhibition of PCD, both rescuing significantly more neurons than the wild type gene. Four samples of the *diap1*$^{(6\text{-}3s)}$ mutation rescued an average of 12 neurons (Fig. 6C and 7). By comparison,

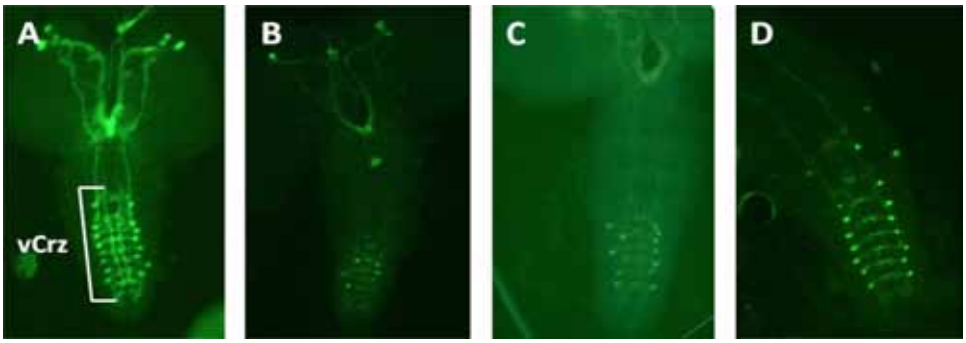

Figure 6. Detection of surviving vCrz neurons.
A: vCrz neurons at the third instar larva stage.
B: vCrz neurons of *diap1*^{wt} at 7-h APF.
C: vCrz neurons of *diap1*$^{(6\text{-}3s)}$ at 7-h APF.
D: vCrz neurons of *diap1*$^{\Delta RF}$ at 7-h APF. (See also Fig. 7)

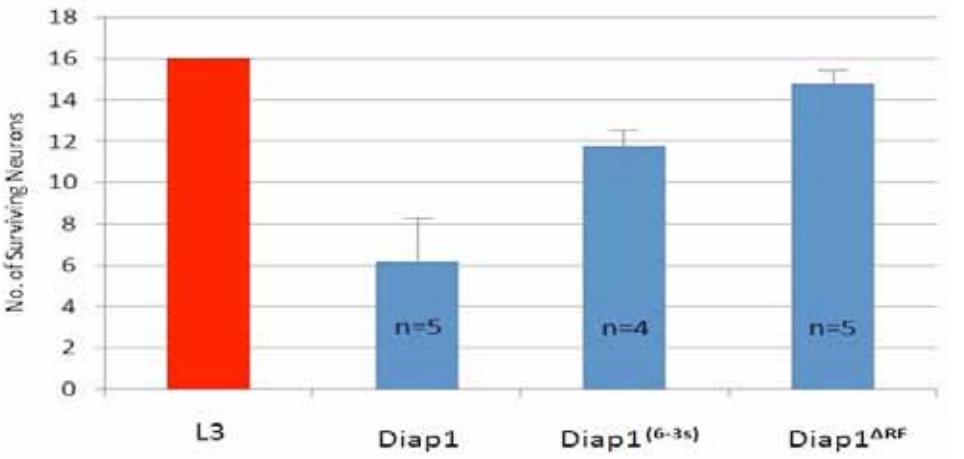

Figure 7. The average number of surviving neurons in response to the expression of *diap1* genes. The number of samples dissected and observed is represented by n. At the L3 stage, all 16 vCrz neurons were always present. At the 7-h APF stage, however, *diap1*^{wt} rescued 6.2 ± 2.2 neurons, whereas *diap1*$^{(6\text{-}3s)}$ rescued 11.8 ± 1 neurons, and *diap1*$^{\Delta RF}$ did 14.8 ± 0.8 neurons.

expression of *diap1*$^{\Delta RF}$ resulted in enhanced protection against cell death, as an average of 15 neurons were rescued, indicating that the *diap1*$^{\Delta RF}$ is an even more efficient PCD inhibitor (Fig. 6D and 7).

DIAP1$^{\Delta RF}$ protein is expected to be resistant to Reaper/Hid/Grim-mediated self-degradation because the RING finger domain regulating ubiquitination was removed. Tight interaction between DIAP1$^{\Delta RF}$ and caspases, even in the presence of death stimuli, inhibits caspase activity, disallowing PCD to take place. Consistent with this expected molecular event, DIAP1$^{\Delta RF}$ blocked PCD of vCrz neurons nearly completely. It is not clearly understood why DIAP1$^{(6\text{-}3s)}$ has a lesser effect. One possibility is that DIAP1$^{(6\text{-}3s)}$ interacts with proapoptotic proteins subnormally.

Conclusion

According to previous observations, it was not possible to draw a clear conclusion as to whether DIAP1 is the survival factor that prevents vCrz neurons from dying during larval stage. If DIAP1 is a true survival factor, then it would be expected that over-expression of DIAP1 would prevent vCrz cell death completely. However, only marginal rescue was observed, most likely because DIAP1 is autodegraded during the early pupal stage. Experiments with the hyperactive and undegradable mutants prevented cell death more efficiently, thus supporting the conclusion that DIAP1 does play a critical role in the prevention of premature death of vCrz neurons.

Acknowledgements

We would like to thank all members of the Park Lab for their assistance with this project. We especially thank Dr. Geni Lee for the injection of DNA constructs. This research was supported by a National Science Foundation grant (IOS-919797).

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About the Author

Faith V. Creekmore received her B. S. in Honors Biochemistry and Cellular and Molecular Biology with a minor in Spanish from the University of Tennessee in May 2011. She currently works as a Research Technician in the Vascular Research Laboratory at the UT Medical Center and as a Medical Assistant for Pediatric Consultants, Inc. She plans to continue her education in medical school.

About the Advisor

Dr. Jae H. Park is an associate professor in the Department of Biochemistry and Cellular and Molecular Biology at the University of Tennessee. His research focuses on the biological functions of various neuropeptide-encoding genes and their neurons in the central nervous system of the fruit fly *Drosophila melanogaster*. This past summer, Dr. Park set up a workshop for six science teachers in Knox County schools that focused on molecular biology methods and analysis.