ABSTRACT

Title of Thesis: DEVELOPMENTAL CELL DEATH IN THE MIDLINE GLIA CELLS OF *DROSOPHILA* EMBRYO

Vanaja Jaligam, Master of Science, 2004

Thesis directed by: Professor Eric H. Baehrecke

Department of Cell Biology and Molecular Genetics

Apoptosis is conserved in worms, flies and mammals. My goal was to identify new Drosophila midline glia cell death genes. Caspase substrate Nuclear Lamin and the midline glia specific reporter slit-lacZ were in Drosophila embryonic development cell death studies. Midline glia cell death is prevented in H99 mutants that delete hid, reaper and grim, and in p35 flies that over-express the pan-caspase inhibitor. Homozygous deficiency Df(3R)E79 flies possess a defect in midline glia cell death. Df(3R)E79 midline glia appear to be phagocytosed but do not undergo Lamin degradation suggesting that a caspase regulator resides in this deleted region of the genome. Df(3R)E79 removes

112 genes, including several interesting candidates including mus309, a protein with similarity to a RING finger protease reported to regulate apoptosis in humans. Future studies will determine the nature of the cell death regulator in Df(3R)E79 and how this gene functions in apoptosis.

DEVELOPMENTAL CELL DEATH IN THE MIDLINE GLIA CELLS OF *DROSOPHILA* EMBRYO

by

VANAJA JALIGAM

Thesis submitted to the faculty of the Graduate school of the
University of Maryland, College Park in partial fulfillment
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ADVISORY COMMITTEE:

Professor Eric H. Baehrecke, Chair
Professor Stephen M. Mount
Professor Louisa Wu
Professor Eric S. Haag

Dedicated to my Parents who are passionate about science:
for their unstinting love, tremendous support,
sacrifice and solid faith in me.

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Introduction

Types of cell death

Programmed cell death is an important component of animal development (Jacobson et al., 1997). During programmed cell death, dying cells are removed without any leakage of cellular contents avoiding an inflammatory response (Jacobson et al., 1997). By contrast, necrotic cell death that occurs due to injury results in leakage of cellular contents leading to an inflammatory response. Two major forms of programmed cell death known as apoptosis and autophagic cell death occur during development that can be distinguished based on morphology (Schweichel and Merker, 1973). Apoptosis is observed in isolated dying cells that undergo nuclear and cytoplasmic condensation leading to fragmentation and eventual phagocytosis by neighboring cells or macrophages (Kerr et al., 1972; Abrams et al., 1993; Jacobson et al., 1997; Song et al., 1999; Baehrecke, 2002). Autophagic cell death is seen in dying tissues or group of cells that sequester their cytoplasmic material in a multilayered membrane and degrade contents using their own lysosomal machinery with no support from phagocytes (Ogier et al., 2003; Baehrecke 2003).

Programmed cell death occurs in animals that are as different as worms and humans (Abrams et al., 1993; Jacobson et al., 1997; Baehrecke., 2002). Physiological cell death is seen in several developmental situations such as sculpting structures like the digits in some higher vertebrates, deletion of structures like Wolffian duct in females, eliminating unwanted or harmful cells like the lymphocytes that fail to make useful antigen receptors or make self reactive receptors in vertebrate immune system, and regulating cell numbers during development like the neurons of vertebrate or insect

nervous systems that are made in excess (Jacobson et al., 1997; Baehrecke, 2002). Excess cells made during development often fulfill the purpose of their existence by aiding in signaling important information, communicating among or within cells, and contributing to modeling of the overall form of the animal before they are eventually eliminated by apoptosis (Zhou et al., 1995; Jacobs, 2000). Another reason for elimination of excess cells might be a selective pressure for the best cells which can thrive in the limiting amounts of survival signals (Jacobson et al., 1997). Finally, tissues may be made for use in one stage of life, such as the tail of a swimming tadpole, which is lost by cell death when the terrestrial frog with legs is formed (Baehrecke, 2002).

Genetic regulation of cell death

Several stimuli activate cell death during development including steroid hormones, withdrawal of trophic factors, membrane bound death receptors, stress and DNA damaging agents (Abrams et al., 1993; Zhou et al., 1995; Baehrecke, 2002). The core cell death machinery is highly conserved from worms, flies, mice to humans as shown in Figure 1 (Budihardjo et al., 1999). *Caenorhabditis elegans* is a good model to study cell death due to the well-characterized cell lineages of the animal (Ellis and Horvitz, 1986; Baehrecke, 2002). Core cell death genes *ced-3, ced-4* and *ced-9* were identified in *C. elegans* by ethyl methane sulfonate mutagenesis and complementation screening for genes that are required for cell death (Ellis and Horvitz, 1986). The relationship between the core cell death genes *ced-3, ced-4* and *ced-9* in *C. elegans* was determined by epistasis analyses, Figure 1 (Ellis and Horvitz, 1996). *ced-3* encodes a caspase (cysteine aspartate protease) homologous to mammalian caspases which are proteolytically

activated during cell death. *ced-4,* which is homologous to human *Apaf1,* promotes caspase activation using *cytochrome c* and ATP (Ellis and Horvitz, 1986; Baehrecke, 2002). *ced-4* is blocked by the anti-apoptotic gene *ced-9*, which is homologous to human proto-oncogene *BCL-2* (Ellis and Horvitz, 1986; Baehrecke, 2002). *ced-9* is inhibited by death gene *egl-1,* which in turn is regulated by the genes that control the fate of a cell whether to live or die (Ellis and Horvitz, 1986). Animals with mutations in *ced-3* and *ced-4* genes had extra cells while *ced-9* mutations rendered the animals dead during development because of ectopic cell death (Budihardjo et al., 1999; Baehrecke, 2002).

Drosophila has the homologues of cell death genes that are present in worms and mammals (Baehrecke, 2002). Caspases, which are the cysteine proteases that play a central role in apoptosis, share similarities in amino acid sequence, structure and substrate specificity (Thornberry and Lazebnik, 1998; Budihardjo et al., 1999). Caspases are present as inactive forms in the cell and are activated by the interaction of cell surface death recptors with their specific ligands (Thornberry and Lazebnik, 1998). The fly genome encodes 3 initiator caspases DREDD, DRONC, DREAM/STRICA (Chen et al., 1998; Dorstyn et al., 1999; Doumanis et al., 2001) that cleave and activate 4 downstream executioner caspases DCP-1, DRICE, DECAY, DAMM (Song et al., 1997; Fraser and Evan, 1997; Dorstyn et al., 1997; Harvey et al, 2001). These executioner caspases then cleave their target substrates such as Lamins, Tubulins and Actin (Budihardjo et al., 1999; Green, 2000; Kumar and Doumanis, 2000; Daniel and Korsmeyer, 2004). REAPER (White et al, 1994), HID (Grether et al, 1995) and GRIM (Zhou et al., 1997) are potent activators of apoptosis through interaction with inhibitors of apoptosis (IAPs) and targeting them for degradation, thus enabling the activation of caspases (Green, 2000;

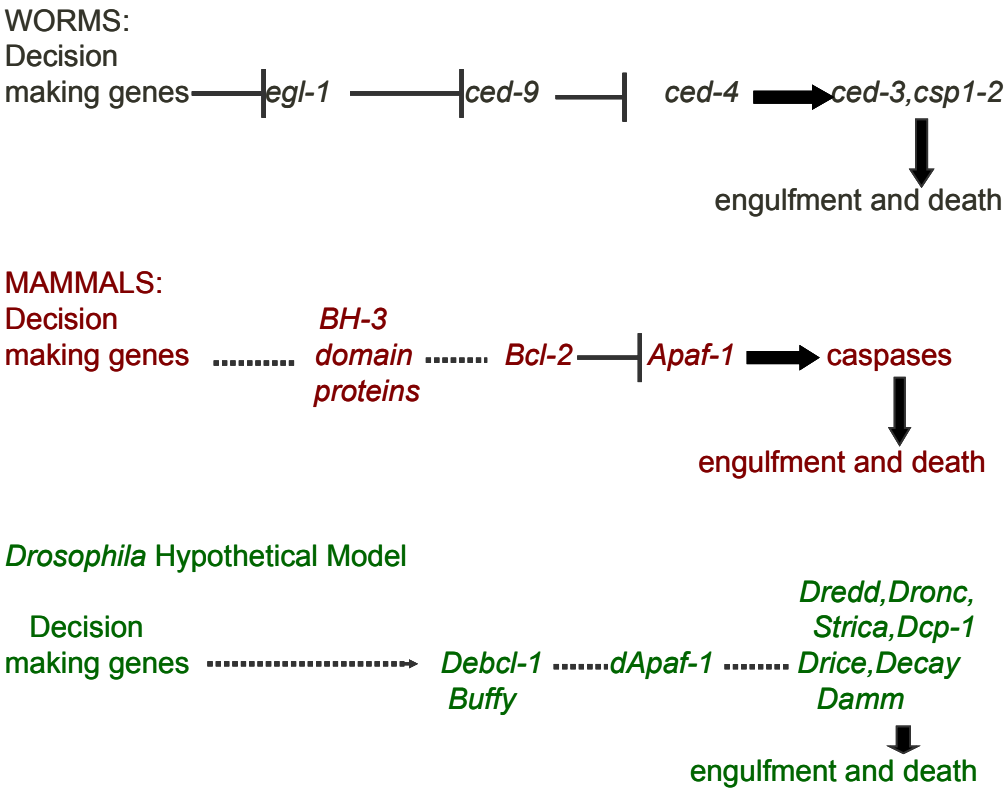

Figure 1: Core cell death machinery. Modified from Baehrecke, 2002

Daniel and Korsmeyer, 2004). IAPs physically interact with caspases and prevent their activation (Budihardjo et al., 1999; Green, 2000).

Drosophila midline glia as a model system for studies of cell death

The midline glia of *Drosophila* are derivatives of embryonic mesectoderm that are similar to the vertebrate floor plate in terms of origin, gene expression and function (Jacobs, 2000). During gastrulation, these mesectodermal cells gather at the ventral midline and form a row of 8 cells per segment (Klambt et al.,1999; Peter, 1992). During germ band elongation, these 8 cells migrate dorsally and divide into 16 cells which form the precursor cells for midline glia during germ band retraction (Klambt et al.,1999; Peter, 1992). Klambt et al. (1999) isolated 20 genes by mutagenesis that play a key role in the differentiation of midline glia which in turn influence the architecture of axon commisures (Hummel et al., 1999). Midline glia have several critical functions in the development of the commissures (Klambt et al., 1991; Hummel et al., 1999; Jacobs, 2000). They guide the commissure growth cones toward and across the midline, their migration separates anterior and posterior commissure, and help in the ensheathment of the commissural axons (Klambt et al., 1991; Hummel et al., 1999; Jacobs, 2000). *Drosophila* embryonic midline glia provide an excellent model for studies of apoptosis (Zhou et al., 1995; Sonnenfeld and Jacobs, 1995; Bergmann et al., 2002). Starting at stage 12 of embryogenesis (7hrs after egg laying at 25^{o}C) glia reduce in cell number from 10 cells per segment to 3 cells per segment by late in embryogenesis, Figure 2 (Sonnenfeld and Jacobs, 1995; Bergmann et al., 2002). The TGFα-like ligand Spitz is a critical midline glia survival factor that is secreted by neurons in limiting quantities (Stemerdink

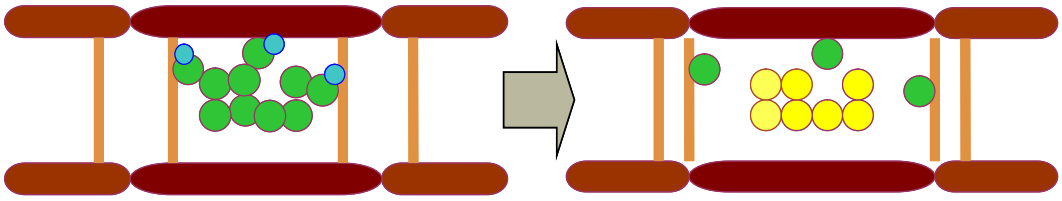

Figure 2: Cartoon of midline glia undergoing cell death.

The panel on the left shows early stage of embryogenesis (7hrs after egg laying at 25^{o}C) and the panel on the right shows late stage of embryogenesis. Surviving Midline glia are shown in green. Dying midline glia are shown in yellow. Spitz is shown in blue. Thin brown rods represent commissures and thick dark brown rods represent axons.

and Jacobs, 1997; Bergmann et al., 2002). Midline glia that lack contact with neurons and access to Spitz die by apoptosis (Sonnenfeld and Jacobs, 1995; Bergmann et al., 2002). The TGFα-like ligand Spitz, is a critical midline glia survival factor that is secreted by neurons in limiting quantities (Stemerdink and Jacobs, 1997; Bergmann et al., 2002). Midline glia that lack contact with neurons and access to Spitz die by apoptosis (Sonnenfeld and Jacobs, 1995; Bergmann et al., 2002). The apoptotic regulators *hid, reaper* and *grim* control midline glia cell number (Zhou et al, 1997; Jacobs, 2000; Bergmann et al., 2002). When spitz is present the EGFR/RAS/MAPK pathway gets turned on and the activated MAP kinase inhibits HID by phosphorylation as shown in Figure 3 (Bergmann et al., 2002; Baehrecke, 2002). Conversely, in the absence of the trophic factor Spitz, MAP kinase does not get activated and hence unphosphorylated HID induces cell death via interaction with the caspase inhibiting DIAP1, enabling caspase activation, as shown in Figure 3 (Bergmann et al., 2002; Baehrecke, 2002).

My goal was to study the function of new genes in midline glia apoptosis. Previous studies resulted in the identification of gene transcripts that were induced by radiation during embryonic apoptosis (Lee et al., 2003). I tested if deletion of these radiation-induced genes prevents midline glia apoptosis (Table 1).

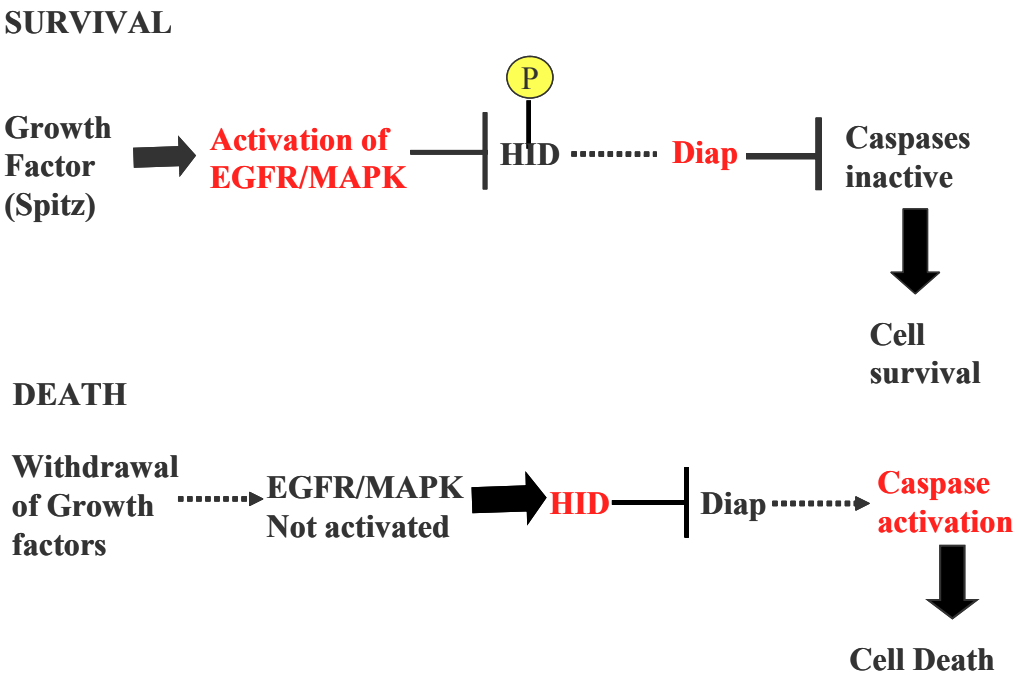

Figure 3: EGFR activation in midline glia survival.

A model for midline glia cell death.

Materials and methods

Drosophila Maintenance

Drosophila were reared on media containing corn meal, agar, molasses, yeast, proprionic acid and water.

Preparation of egg lay caps

Egg lay caps were made using bacto-agar, molasses and water. After autoclaving, the media was cooled to 60°C and acid mix A (propionic acid, phosphoric acid and water) was added. This was dispensed into 35 mm Petri dishes, allowed to solidify and stored at 4°C.

Collection and Staging of eggs

Fly embryos were collected on egg caps and staged at 25°C for specific time intervals depending on the stage of the embryo desired. In case of wild type fly embryos, early stage corresponded to ~9.20-10.20 hours after egg laying, middle stage corresponded to ~11.20-13.30 hours after egg laying and late stage corresponded to ~16.00-20.00 hours after egg laying. In case of embryos of p35, H99 and the deficiency lines with the reporter for midline glial cells, late stage corresponded to ~ 18.00- 20.00 hours after egg laying.

Antibody staining and Confocal microscopy

Embryos were staged for the desired time and harvested using a mesh basket. They were dechorinated for 2 minutes in 50% bleach with periodic agitation and transferred to PBST

Deficiency	Chromosome deleted	Gene of interest
1. Df(1)RA2 (Corp)	X	CG10965
2. Df(1)260-1	X	EG:65F1.1
3. l(2)k10127	2	CG18623
4. EP(2)2001	2	CG2064
5. Df(3R)E79	3	mus309
6. Df(3L)ru-22	3	pUbsf

Table 1: Candidate genes for midline glia cell death phenotype.

These deficiency lines deleted the gene of our interest. These genes were up regulated 5 times or greater during radiation-induced apoptosis (Lee et al., 2003). These deficiency lines were chosen after excluding deficiencies that perturb neural development to further characterize deficiencies with normal nervous system for defects in midline glia cell death.

(phosphate buffered saline with 0.1% tritonX -100) for several washes followed by PBS. The embryos were then fixed in 4% paraformaldehyde/heptane for 20 minutes at room temperature, blocked for 1 hour in 1% BSA and 0.1% triton-X (PBSBT) and incubated with primary mouse monoclonal antibody against Nuclear Lamin Dm0 (gift from Paul Fisher) at 4°C overnight. This was followed by washes in PBSBT for 2 hours and incubation with Alexafluor 546 goat anti mouse secondary antibody (from Molecular Probes) at 4°C overnight. This was followed by washes in PBSBT for 2 hours and incubation with anti-β-galactosidase rabbit antibody (from Cappel) at 4°C overnight for staining midline glia. This was followed by washes in PBSBT for 2 hours and incubation with Oregon green 488 goat anti rabbit secondary antibody (from Molecular Probes) at 4°C overnight. This was followed by washes in PBSBT for 2 hrs and the embryos were mounted in Vectashield (Vector Laboratories). The embryos were analyzed for a phenotype for midline glial cell death using a Zeiss Axiovert 100 M confocal microscope. The confocal settings were identical in all analyses.

p35 inhibition of midline glia cell death

Virgin females of *w*; *UAS- p35/UAS-p35* (p35 is a broad spectrum caspase inhibitor) were crossed to males of *P[52AGAL4],P[UAS-lac-Z]/Cyo* and their late stage eggs were collected. These embryos were stained with antibodies against nuclear Lamin and β-galactosidase as described above for confocal microscope analysis to determine the number of midline glia cells per segment.

H99 inhibition of midline glia cell death

A *lac-Z* reporter was crossed into the *H99* deletion mutant that lacks the *hid, reaper* and *grim* apoptotic genes. *Slit-lacZ* (on the second chromosome) is expressed in midline glia and enables the determination of the impact of mutations on glia cell death. Late stage embryos were collected and stained with antibodies against nuclear Lamin and β-galactosidase as described above for confocal microscopy analysis to see the inhibition of midline glial cell death in the H99 mutant. For this I genotyped the embryos as control *(slit-lacZ;H99/TM6B Ubx-lacZ)* and mutant *(slit-lacZ; H99/ H99)* embryos based on the presence or absence of *Ubx-lacZ* expression (expressed in middle segments 6-12).

Crossing midline glia reporter *lacZ* into deficiency lines

The third chromosome deficiency lines were combined with a *slit-lacZ* reporter gene such that the final progeny were *slit-lacZ*; *Df 6159/TM6B Ubx-lacZ* and *slit-lacZ; Df 4214/TM6B Ubx-lacZ.* The X-chromosome deficiency lines were combined with the midline glia reporter *AA142e-lacZ* to get the final progeny *Df 950/FM7 ACT GFP; AA142e-lacZ /AA142e-lacZ* and *Df 722/FM7 ACT GFP; AA142e-lacZ/AA142e-lacZ.*

Late stage embryos from these final progeny were collected and stained with antibodies against nuclear Lamin and β-galactosidase as described above for confocal microscopy analysis to determine the number of midline glia cells in these mutants late during embryogenesis. This required the genotyping the embryos into control with *lacZ* and mutant embryos without *lacZ.*

Results

Nuclear Lamins are Caspase substrates that can be used as a marker during apoptosis of midline glia

Several laboratories have used *slit-lacZ* as a marker of midline glia cell death (Zhou et al., 1995; Klambt et al, 1919; Bergmann et al, 2002). While *slit-lacZ* enables the identification of the number of glia per segment, it does not allow determination of Caspase activity. Nuclear Lamins are a known Caspase substrate (Rao and White 1996; Martin and Baehrecke, 2004), and we used this biochemical change in dying cells as a method to identify the function of new cell death genes. In wild-type Canton S embryos there is a reduction in the number of *slit-lacZ*-labeled midline glia cells per segment and Nuclear Lamin (red) from 10 in the early stage to 6-7 in the middle stage to 3 in the late stage of embryogenesis (Figure 4). The *H99* deficiency lacks the core cell death genes *hid, reaper* and *grim* (White et al., Zhou et al 1995) and lacks apoptosis of midline glia. As shown previously, homozygous *H99* mutants exhibit defective midline glia cell death with more than 9 cells surviving per segment even at the end of embryogenesis (Figure 5). Control embryos (*slit-lacZ; H99 / TM6B, Ubx-lacZ)* possess 3 midline glia cells per segment (Figure 5). To complement this experiment, we over-expressed the pan-caspase inhibitor p35 using *UAS-p35 / P [52AGAL4], P [UAS-lacZ]*. These embryos also had decreased midline glia apoptosis, leaving at least 8 midline glial cells surviving per segment (Figure 5). These studies indicate that the combined approach using *slit-lacZ* and nuclear Lamin degradation may serve as valuable markers of midline glia cell death.

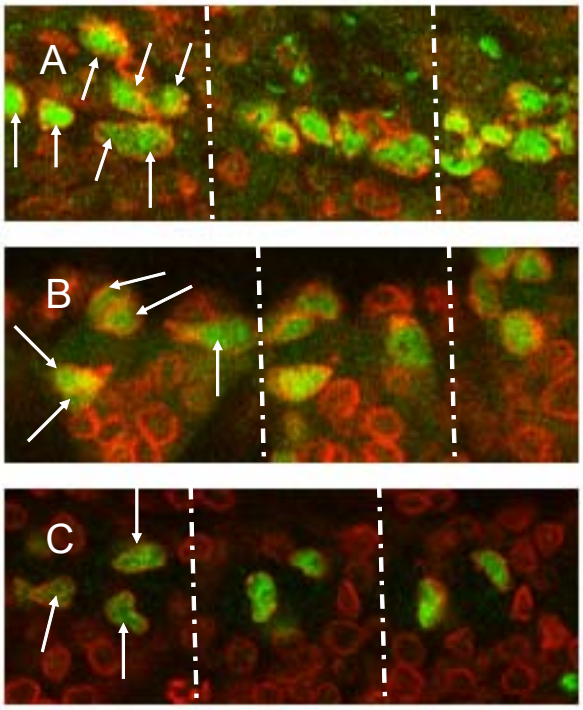

Figure 4:Analysis of Nuclear Lamin (in red) combined with β-galactosidase staining (in green) in midline glia cells in *slit-lacZ* embryos.

White arrows indicate the number of midline glia in one segment of the embryo. White dashed lines demarcate one segment.

A) There are around 7 midline glia cells surviving per segment at early stage of embryogenesis.

B) There are around 5 midline glia cells surviving per segment at middle stage of embryogenesis.

C) There are 3 midline glia surviving per segment at late stage of embryogenesis

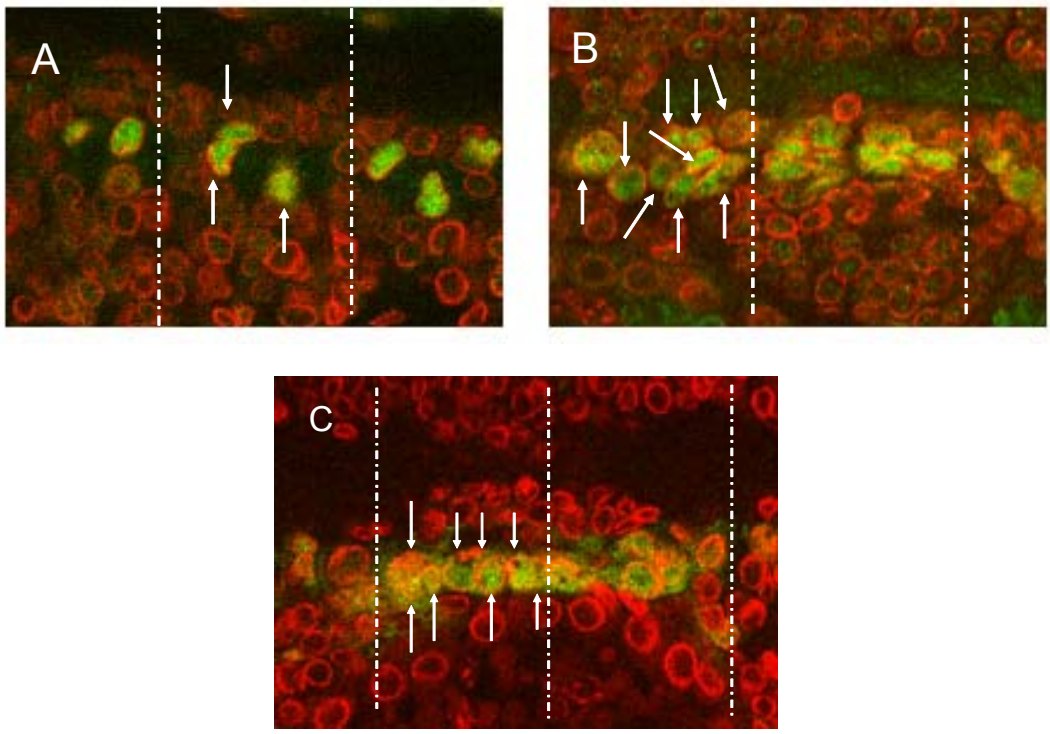

Figure 5:Analysis of Nuclear Lamin (red) midline glia cells (green) in late stage *H99* and *p35* embryos.

A) Control embryos (*H99 / TM6B, Ubx-lacZ)* possess 3 surviving midline glia cells per segment.

B) Homozygous *H99* embryos have at least 9 surviving midline glia cells per segment.

C) *p35*-expressing embryos have 8 or greater midline glia per segment.

Identification of a genomic deletion with defects in midline glia cell death

Previous studies resulted in the identification of candidate genes that are involved in radiation-induced apoptosis and steroid-induced autophagic cell death by DNA microarray analyses of nearly the entire *Drosophila* genome (Lee et al, 2003). To test if these genes are required for midline glia cell death, deficiency lines that delete candidate cell death genes were obtained. These lines were initially screened for defects in neural patterning and development to eliminate the possibility of non-specific defects in programmed cell death, and such lines were eliminated from further analysis. This was done by Jamie Cranford in the lab, by staining the embryos with axon marker BP102, to test for proper neural development (Baehrecke and Cranford, unpublished). These deficiencies were combined with either *slit-lacZ* or *AA142-lacZ* midline glia reporters and were maintained with marked balancer chromosomes so that homozygous mutant embryos could be identified and analyzed for a phenotype. The deficiency *Df (3R) E79* on the third chromosome deletes *mus309* which functions in double-stranded DNA repair, and was induced by radiation in embryos. Greater than 7 midline glia cells were present per segment in *Df (3R) E79 / Df (3R) E79* as compared to the control *Df (3R) E79 / TM6B, Ubx-lacZ* where 3 midline glia cells were present per segment during the late stage of embryogenesis (Figure 6).

These results suggest that, similar to the positive controls *H99* and p35 (Figure 4), homozygous *Df (3R) E79* embryos are defective in midline glia cell death. Homozygous *H99* deficiency and over- expression of p35 embryos possess extra midline glia cells with intact nuclear Lamin staining. By contrast, homozygous *Df (3R) E79* mutant embryo midline glia appear different; the β-galactosidase marker of midline glia has changed

localization and size such that it surrounds clusters of nuclear Lamin staining (Figure 6). These data suggest that multiple midline glia have been engulfed by phagocytes in the absence of caspase activation. The persistence of Lamin stain in spite of the observation that these cells appear to be engulfed by phagocytes argues that these results are novel. Such a result has not been previously reported and suggests that *Df (3R) E79* contains a gene that regulates caspase activation and that caspase activation is not required for phagocytosis. This result could be verified by double staining for a marker of phagocytes of midline glia cells combined with β-galactosidase. Croquemort, which is a phagocytic marker for cell death in embryos (Franc et al., 1999), is not expressed in the phagocytes of midline glia. Caspase-3 antibody staining of the whole embryo combined with β-galactosidase stain for midline glia did not provide useful results as caspase-3 stained many cells in the embryo and midline glia could not be identified (data not shown).

The deficiency lines for X chromosomes requires two more generations of crosses to complete similar analyses. The deficiency for third chromosome Df (3L) ru-22 presented many problems during crosses due to the balancer TM6B that rendered the animals lethal in combination with the deficiency on the third chromosome. The crosses involving the deficiencies on the second chromosome needed few more months for generating the final progeny that required 5 generation more of crossing.

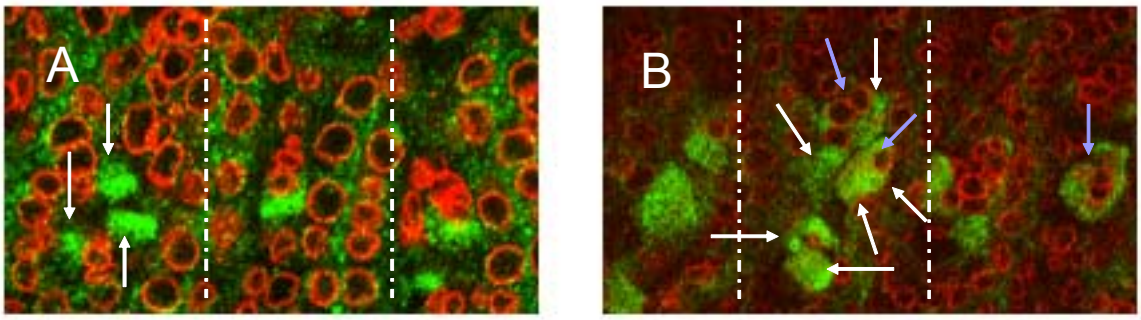

Figure 6: Analysis of Nuclear Lamin (red) and midline glia cells (green) in late stage *Df (3R) E79* embryos.

A) Control *Df (3R) E79* embryos (*Df (3R) E79 / TM6B, Ubx-lacZ)* have 3 surviving midline glia cells per segment

B) Homozygous *Df (3R) E79 / Df (3R) E79 embryos* have 6 or greater surviving midline glia cells per segment. Multiple intact nuclear Lamin rings that occur within the β-galactosidase stain for midline glia cells (Purple arrows), which appear to have been engulfed by phagocytes.

Discussion and future directions

The *Df (3R) E79* deletion line is defective in cell death of midline glial cells during development. Confirmation that multiple midline glia with intact lamin rings are being phagocytosed should be performed by immuno EM. The other possible experiment is to stain for a phagocyte marker along with the β-galactosidase marker for midline glia cells. Once we confirm the phenotype for this defect in midline glia cell death, the immediate concern is to determine which gene in this interval is responsible for this defect in cell death. *Df (3R) E79* contains 112 genes based on genome annotation (Table 2). Careful analyses of this list of the genes, gene ontology and protein domains reveal some interesting genes (Table 2). These are *mus 309, CG14722, CG6923, Ect3, CG3571,* serine proteases such as *CG1704, CG3916, CG12256, CG10041* and lastly a metallopeptidase family member *Dip-C.* Not many P-elements were present in the coding regions of interesting genes in this deletion interval. There were only 3 P-elements in regions of this interval. None of these genes except *mus309* were up regulated greater than 1.1 fold during radiation-induced cell death (Lee et al., 2003). *ECT-3* gene transcription was induced 20.5 fold in steroid-induced autophagic cell death (Lee et al., 2003).

mus309 (mutagen-sensitive 309) has a DEAD/DEAH box helicase. The DEAH/D-box proteins are a large family of ATPases that have been proposed to mediate RNA structural rearrangements in a variety of cellular processes including nuclear pre-mRNA splicing, ribosome assembly, protein synthesis, nuclear transport, and RNA degradation (Aubourg, 1999). Although one P-element enhancer trap exists in *mus309*, no strong mutations appear to be available in this gene. The *mus309* gene is most closely

related to the Homo sapiens BLM gene (involved in premature aging), suggesting functional similarity (Kusano 1999). This gene is involved in double stranded break repair and has ATP- dependent helicase activity based on direct assay and sequence similarity in humans.

CG14722 is thought to have apoptotic protease activator activity due to sequence similarity to human and mouse *EBAG9,* and protein interactions in a high throughput yeast two hybrid screen (Giot et al., 2003). *EBAG9 was* identified as an estrogen responsive gene in humans and the encoded protein is a tumor-associated antigen that is expressed at high frequency in a variety of cancers (Takahashi et al., 2003). One transposable element insertion exists in *CG14722*. *CG6923* has a RING finger domain, which is also present in IAP's. Because of this, *CG6923* may play a role in cell death, and might be a candidate gene for the defective midline glia cell death phenotype. This gene has no transposable P-element insertions available. *Ect3* is also interesting since it is induced during autophagic cell death of *Drosophila* salivary glands (Lee et al., 2003). Similar genes are present in mice, worms and humans, and no P-element insertions are available in this gene. *CG3571* might have apoptotic activity according to sequence similarity, actin binding and protein interactions in a high throughput yeast two hybrid screen (Giot et al., 2003). Hence, this might serve as a candidate gene for the midline glia cell death. This gene has one transposable element insertion available. Finally, one could investigate the genes encoding serine proteases to test if they are responsible for the midline glia cell death phenotype. Since serine proteases are required for degradation of the proteins in the cell, they might function during apoptosis.

TABLE 2			
CG #	**Gene Ontology**	**Protein Domain**	**FlyBase ID**
CG14717	Lipid metabolism	Alpha/beta-Hydrolases	FBgn0037938
l-t	Protein phosphatase inhibitor activity		FBgn0025821
CG14720			FBgn0037940
CG12594		Thrombospondin N-terminal -like domains, Concanavalin A-like lectins/glucanases	FBgn0037941
CG14721	Thiamin diphosphokinase activity		FBgn0037942
mus309	DNA repair synthesis	DEAD/DEAH box helicase	FBgn0002906
CG14722	Apoptotic protease activator activity	Trp-Asp repeat	FBgn0037943
CG6923	Development; putative transcription regulator activity	RING finger domain	FBgn0037944
Lk6	Intracellular signaling cascade	Eukaryotic protein kinase	FBgn0017581
CG6930	Transcription regulator activity	Zinc finger	FBgn0037947
l(3)neo38			FBgn0011471
CG17360	Cell communication	PH domain-like	FBgn0037949
HisCl1	Signal transduction	Gamma-aminobutyric acid A (GABAA) receptor, Neurotransmitter-gated ion-channel	FBgn0037950
Sbf	Protein metabolism	PH domain-like	FBgn0025802
CG31116	Chloride transport	Voltage gated chloride channels	FBgn0051116
CG6946	Nuclear mRNA splicing	RNA-binding domain, RBD	FBgn0037954
CoVa	Electron transport	Cytochrome c oxidase	FBgn0019624
CG6950	Amino acid biosynthesis	PLP-dependent transferases	FBgn0037955
CG6959	Cell adhesion	RNI-like,	FBgn0037956
CG6962			FBgn0037958
sad	Ecdysone biosynthesis	Cytochrome P450 enzyme	FBgn0003312
CG31368	Cell cycle	P-loop containing nucleotide triphosphate hydrolases	FBgn0051368
mthl5	Response to stress	G-protein coupled receptors family 2 (secretin-like)	FBgn0037960
CG6971	Cell motility		FBgn0037962
Cad87A	Signal transduction	Cadherin	FBgn0037963

TABLE 2			
CG #	**Gene Ontology**	**Protein Domain**	**FlyBase ID**
CG14731		Phosphatase/sulfatase	FBgn0037964
CG31211	Cytoskeletal protein binding		FBgn0051211
Hsp70Aa	Defense response; protein folding		FBgn0013275
Hsp70Ab	Defense response; protein folding		FBgn0013276
CG3281	Transcription regulator activity	Zinc finger	FBgn0037967
aur	Intracellular signaling cascade; mitosis	Eukaryotic protein kinase	FBgn0000147
CG12213	Cytoskeleton organization and biogenesis		FBgn0037968
CG18347	Lipid metabolism	Mitochondrial carrier protein	FBgn0037969
CG12201	Lipid metabolism	Mitochondrial carrier protein	FBgn0037970
CG10007			FBgn0037971
CG10005			FBgn0037972
CG18547	Oxidoreductase activity	NAD(P)-linked oxidoreductase	FBgn0037973
CG12224	Oxidoreductase activity	NAD(P)-linked oxidoreductase	FBgn0037974
CG3397	Potassium ion transport	NAD(P)-linked oxidoreductase	FBgn0037975
mfas	Cell-cell adhesion	Beta-Ig-H3/Fasciclin domain	FBgn0024211
Tk	Receptor binding		FBgn0037976
Ect3	Autophagic cell death,Salivary gland cell death	(Trans)glycosidases	FBgn0037977
CG3571	Actin binding, Apoptosis	POZ domain	FBgn0037978
CG3532	Cell cycle, Mitosis		FBgn0037979
CG3313		Trp-Asp repeat	FBgn0037980
Spt3	Transcription factor activity	Histone-fold	FBgn0037981
CG31358	Cytoskeleton organization and biogenesis		FBgn0051358
CG14735	Cytoskeleton organization and biogenesis		FBgn0037985
CG14736	Cytoskeleton organization and biogenesis		FBgn0037986
CG14739	Protein metabolism	Ubiquitin-conjugating enzymes	FBgn0037987
CG14740	Tricarboxylic acid cycle	Citrate synthase	FBgn0037988
CG14741	Lipid metabolism	H+/K+ and Na+/K+ transporting ATPase	FBgn0037989

TABLE 2			
CG #	**Gene Ontology**	**Protein Domain**	**FlyBase ID**
CG31361	Cell adhesion molecule activity	Immunoglobulin	FBgn0051361
CG4702			FBgn0037992
CG10095	Cell adhesion	Immunoglobulin	FBgn0037993
Cpn	Calcium ion binding		FBgn0010218
CG4810	Regulation of cell cycle		FBgn0037994
CG3809	Nucleic acid metabolism	Adenosine kinase	FBgn0037995
CG4830	Ligase activity	AMP-dependent synthetase and ligase	FBgn0037996
CG4848	Intracellular protein transport		FBgn0037998
CG4860	Acyl-CoA metabolism	Acyl-CoA dehydrogenase	FBgn0037999
CG10014	Cytoskeleton organization and biogenesis	Protein kinase-like	FBgn0038000
CG17404	Proteolysis and peptidolysis	Serine proteases	FBgn0038001
CG3916	Proteolysis and peptidolysis	Serine proteases	FBgn0038003
Pglym87	Glycolysis	Phosphoglycerate mutase family	FBgn0011270
CG12256	Proteolysis and peptidolysis	Serine proteases	FBgn0038002
Cyp313a3	Steroid metabolism	Cytochrome P450	FBgn0038007
CG3942		Starch-binding domain	FBgn0038008
svp	Signal transduction	Nuclear receptor ligand-binding domain	FBgn0003651
CG17738			FBgn0038009
CG4066			FBgn0038011
CG10013			FBgn0038012
CG10038		Alpha/beta-Hydrolases	FBgn0038013
MBD-R2		Zinc finger	FBgn0038016
CG10041	Proteolysis and peptidolysis	Serine proteases	FBgn0038014
CG4115		C-type lectin-like	FBgn0038017
Tim17a1	Protein translocase activity	Mito translocase subunit	FBgn0038018
GstD10	Defense response	Glutathione S-transferases	FBgn0042206
GstD9	Defense response	Glutathione S-transferases	FBgn0038020
GstD1	Defense response	Glutathione S-transferases	FBgn0001149
GstD2	Defense response	Glutathione S-transferases	FBgn0010038
GstD3	Defense response	Glutathione S-transferases	FBgn0010039
GstD4	Defense response	Glutathione S-transferases	FBgn0010040

TABLE 2			
CG #	**Gene Ontology**	**Protein Domain**	**FlyBase ID**
GstD5	glutathione transferase activity	Glutathione S-transferases	FBgn0010041
GstD6	Defense response	Glutathione S-transferases	FBgn0010042
GstD7	Defense response	Glutathione S-transferases	FBgn0010043
GstD8	Defense response	Glutathione S-transferases	FBgn0010044
CG10035			FBgn0038028
CG17639	Defense response	Glutathione S-transferases	FBgn0038029
CG17793			FBgn0038030
CG33098			FBgn0053098
CG31218	metallopeptidase activity		FBgn0051218
CG32919			FBgn0052919
CG17227	nucleic acid binding	ATP-dependent DNA ligase	FBgn0038035
CG4940			FBgn0038036
Cyp9f2	electron transporter activity	Cytochrome P450	FBgn0038037
CG5167		NAD(P)-binding Rossmann-fold domains	FBgn0038038
CG5196			FBgn0038039
Spp	transcription factor activity	PHD-finger	FBgn0038041
Dip-C	proteolysis and peptidolysis	metallopeptidase family M24	FBgn0000455
CG17202	protein binding		FBgn0038043
Pp1-87B	protein amino acid dephosphorylation; neurogenesis	Serine/threonine specific protein phosphatase	FBgn0004103
Sas	carbohydrate metabolism; protein amino acid glycosylation		FBgn0038045
CG17207			FBgn0038051
desat2	fatty acid biosynthesis	Fatty acid desaturase, type 1	FBgn0043043
CG5844	fatty acid beta-oxidation	Enoyl-CoA hydratase/isomerase	FBgn0038049
Scgβ	muscle contraction		FBgn0038042
CG5641	defense response	2'-5'-oligoadenylate synthetase	FBgn0038046
CG5245	cell proliferation	Zinc finger	FBgn0038047
Pros25	ubiquitin-dependent protein catabolism	Proteasome A-type subunit	FBgn0010405
Aos1	protein modification; protein targeting	UBA/THIF-type NAD/FAD binding fold	FBgn0029512
CG5538			FBgn0038052
CG18549			FBgn0038053
desat1	fatty acid biosynthesis		FBgn0043044

Several strategies could be taken to identify the specific gene(s) required for proper cell death in this interval including mapping using smaller deletions, EMS-induced mutagenesis and RNAi-induced silencing of candidate genes. The intact and phagocytosed Lamin rings in these midline glia cells raise the question of why midline glia cells are phagocytosed in the absence of caspase activation. If these midline glia cells are internalized, why aren't they completely degraded?

Previous studies have observed phagocytosis of dying cells in the absence of apoptosis markers (White et al, 1994; Lee and Baehrecke, 2001), suggesting that this result may point to an unknown cell death gene. The answers to these questions should be obtained by determining the gene(s) responsible for this defect.

Molecular approaches can also be taken to possibly speed the discovery of the gene responsible for the cell death defect in *Df (3R) E79*. DNA microarray analyses of gene transcript levels in *Df (3R) E79* mutant embryos compared with control embryos could help identify a gene transcript that is altered in the mutant. Given that such RNA preparations would need to be isolated from intact embryos, subtle differences in midline glia RNA levels may not be detected. Alternatively, *in situ* hybridization of genes in the *Df (3R) E79* interval may reveal differences in homozygous mutant and control embryos that will enable more rapid focus on functional studies. Once the gene(s) that are responsible for this interesting phenotype are identified, many projects can be pursued to determine the relationship to the existing cell death pathway.

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