

MIDLINE LINEAGES IN EMBRYOS DEFICIENT FOR APOPTOSIS IN DROSOPHILA

**THE FATE OF MIDLINE LINEAGES IN THE EMBRYOS DEFICIENT
FOR APOPTOSIS IN *DROSOPHILA MELANOGASTER***

By

RONG DONG, M.D.

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree
Master of Science

McMaster University
(c) Copyright by Rong Dong, January, 1997

MASTER OF SCIENCE (1997)
(Biology)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: The Fate of Midline Lineages in the Embryos Deficient
for Apoptosis in *Drosophila melanogaster*

AUTHOR: Rong Dong, M.D.
(Shandong Medical University)

SUPERVISOR: Professor J. Roger Jacobs

NUMBER OF PAGES: ix, 92.

ABSTRACT

One function of programmed cell death in the developing CNS is the removal of excess cells that provide transient function. Previous studies in *Drosophila* observed an overpopulation of midline glia cells in embryos deficient for apoptosis. Where do these extra glia cells come from? Using different enhancer traps and antibodies as cell identity markers, the cell number of different CNS midline lineages was assessed in both wild type and *Df(3L)H99* embryos which are deficient for apoptosis. The results show that at stage 16 there are approximately 3 cells labeled by midline glia specific markers (AA142 enhancer trap & *P[slit1.0/lacZ]* reporter construct) in wild type while there are up to 12 cells in embryos deficient for apoptosis. Comparing the number of midline neurons of mutants with that of wild type embryos, there are no detectable changes labeled by the enhancer trap P223, antibody 22C10, or an antibody to Engrailed. Exceptionally, there is one more neuron labeled by enhancer trap X55 in *Df(3L)H99* embryos. Therefore, apoptosis is restricted to the midline glia lineage. Using *P[slit1.0/lacZ]* as MG marker, I observed that the extra midline glia in *Df(3L)H99* initially appear at late stage 12 or early stage 13. The expression of *reaper* mRNA precedes programmed cell death. In wild type embryos, the initial expression of *reaper* mRNA of midline cells is at late stage 11 as revealed by *in situ* hybridization. These indicate that the first programmed cell death in the midline occurs approximately at stage 12.

The supernumerary cells labeled by midline glia specific markers in *Df(3L)H99* embryos share features of the midline glia. These extra midline glia may be divided into two groups according to their differentiation. The cells of the first group strongly express the AA142 enhancer trap and ensheath the commissures. These cells are functional midline glia corresponding to the surviving midline glia in wild type embryos. The cells of the second group weakly express the AA142 and associate with but do not ensheath the commissures. These are likely the cells which normally undergo apoptosis in wild type. The results of this study indicate that the supernumerary midline glia come from neither midline glia proliferation nor other lineages. They may come from a midline glia progenitor pool in which midline glia marker expression begins at different stages. In wild type embryos, these potential midline glia die by apoptosis before activating midline glia specific genes. In *Df(3L)H99* embryos, these midline glia survive and express midline glia markers.

All the midline glia die in embryos mutant for spitz group genes. In embryos double mutant for spitz group genes and *Df(3L)H99*, supernumerary midline glia cells survive. These cells cannot totally rescue the axon tract phenotype of spitz group gene mutants indicating that spitz group genes are necessary for producing 'mature' midline glia. In *Df(3L)H99* embryos, approximately 12 midline cells labeled with the midline glia specific marker *P[slit1.0/lacZ]*. However, there is not a significant increase in the number of midline glia expressing *pnt* or *argos* compared with wild type. Therefore, the survival of supernumerary midline glia in embryos deficient for apoptosis does not require DER signaling. However, the DER pathway seems to specify which and how many midline glia progenitors avoid apoptosis.

ACKNOWLEDGEMENTS

I would like to express my appreciation to all people who contributed to the completion of this thesis. First of all, I would like to thank my supervisor Dr. J. Roger Jacobs for his invaluable encouragement, advise and criticisms during the course of my studies. My thanks also give to him for generating figure 6. I am grateful to Dr. Ana Campos for serving in my supervisory committee and sharing her time with me especially on confocal microscopy. I also thank Dr. D. Morton for serving on my defense committee. I appreciate the friendliness and aid of the other members of the laboratory, past and present, including Christian Smith, Pam Mulligan, Cathy Bucci, Sharon Arova, Ronjon Raha, Brad Lanoue, Robin Battye, Maria Guarnieri, and especially Christopher Stemerding made working in the laboratory more enjoyable. Further thanks to Brad, Robin, Maria, and Christopher for giving critical comments and corrected the English on this manuscript. My thanks to the graduate students and staffs in the Department for their kindness and helpfulness.

I would like to thank my husband, Huijun Zhong, for his patience and support. His optimism has helped me to see things in perspective. I would also like to thank my parents and family for all their love and support through my life. Finally, my son Yi has made everything worthwhile and I express my love to him.

TABLE OF CONTENTS

	Page
Title Page	i
Descriptive Note	ii
Abstract	iii
Acknowledgement	v
Table of Contents	vi
List of Figures	ix
INTRODUCTION	1
Programmed cell death	1
Genes required for programmed cell death in <i>Drosophila</i>	3
Methods for detecting apoptosis in <i>Drosophila</i>	9
Development of Mesectoderm cells in <i>Drosophila</i>	10
Mesectodern lineages	11
Interactions between midline cells and the axon commissures	13
PCD in the ventral midline of <i>Drosophila</i>	16
MATERIALS AND METHODS	19
1. <i>Drosophila</i> strains	19
1.1 Mutant flies	19

1.2 Enhancer traps and gene fusion reporter elements	20
2. Antibodies	21
3. Embryo collection and staging	22
4. Immunocytochemistry in whole mount <i>Drosophila</i> embryos	23
4.1 HRP reaction	23
4.2 Anti- β -galactosidase and anti- <i>elav</i> fluorescent double labeling	25
4.3 Anti- β -galactosidase HRP and anti-BrdU FITC double labeling	25
5. Anti- β -galactosidase and <i>in situ</i> hybridization double labeling	26
5.1 Embryo fixation	27
5.2 Antibody staining prior to RNA <i>in situ</i> hybridization	27
5.3 <i>in situ</i> hybridization	28
5.4 Solutions	30
6. Electron microscopy protocol	33
7. Cell counting	34
RESULTS	35
Lineage analysis of the mesectoderm in embryos deficient for apoptosis	35
Time course of the effects of <i>Df(3L)H99</i> on the number of midline cells	38
Expression of <i>reaper</i> in the mesectoderm	45
Supernumerary midline cells in <i>Df(3L)H99</i> embryos are MG	48

Absence of extra cell divisions in the mesectoderm of <i>Df(3L)H99</i> embryos	51
Expression of DER, pnt and argos in <i>Df(3L)H99</i> embryos	56
<i>Df(3L)H99</i> can rescue some aspects of the spitz group mutants	
midline phenotypes	60
DISCUSSION	67
PCD in the mesectoderm of <i>Drosophila</i>	67
Source of supernumerary MG in <i>Df(3L)H99</i> embryos	69
Midline glia progenitor pool	71
Function of supernumerary MG in mutant for <i>Df(3L)H99</i>	
and double mutants for spitz group genes and <i>Df(3L)H99</i>	77
DER pathway	79
REFERENCE	82
APPENDIX: Table: Cell number of different midline lineages in wild type	
and <i>Df(3L)H99</i> embryos	92

List of Figures

1. Schematic drawing of cell death pathway in <i>C. elegans</i> , <i>Drosophila</i> and mammals.	4
2. The fate of different MEC lineages in <i>Df(3L)H99</i> embryos	36
3. Time course of P[<i>sim/lacZ</i>] and P[<i>slit1.0/lacZ</i>] expression in the mesectoderm during embryogenesis	40
4. Early appearance of supernumerary MG in <i>Df(3L)H99</i> embryos	42
5. <i>reaper</i> mRNA expression in the mesectoderm	46
6. Supernumerary midline cells in <i>Df(3L)H99</i> contact commissural tracts	49
7. Expression of <i>elav</i> and AA142 in the CNS midline of wildtype and <i>Df(3L)H99</i> embryos	52
8. Immunofluorescence labeling of BrdU in wild type embryo mesectoderm	54
9. Expression of genes of the DER pathway in <i>Df(3L)H99</i> embryos	58
10. The fate of MG in embryos mutant for the spitz group genes or double mutants for the spitz group genes and <i>Df(3L)H99</i>	61
11. Axon tract morphology in embryos mutant for the spitz group genes or double mutants for the spitz group genes and <i>Df(3L)H99</i>	65
12. Schematic models of mesectoderm lineages	75

INTRODUCTION

Programmed cell death

During the development of multicellular organisms, an excess of cells is generated that is subsequently removed by a naturally occurring process termed programmed cell death (PCD; Wyllie et al., 1980; Oppenheim, 1991; Hengarten and Horvitz, 1994a). In vertebrates, cell death occurs in almost all tissues and has been extensively studied in the developing nervous system (Cowan et al., 1984; Oppenheim, 1991). In invertebrates, cell death also affects many different tissues during development (Ellis and Horvitz, 1986; Truman, 1984; Bate et al., 1981; Wolff and Ready, 1991; Fujisawa and David, 1984; Shankland, 1984). PCD recognized as an important component of normal development or homeostasis provides an efficient way for removing unwanted cells (Raff, 1993; Ellis et al., 1991). For example, PCD can significantly affect morphogenesis of the developing chicken foot; extensive cell death occurs in zones between the digits that results in formation of separated digits (Hinchcliffe and Thorogood, 1974).

PCD has a characteristic process, termed apoptosis. During apoptosis, the cytoplasm and the nucleus of a cell condense, and the dying cell often fragments into membrane-bound bodies which are rapidly removed by either phagocytes or neighboring cells. The morphology of cellular organelles remains well preserved for a certain period (Wyllie et al., 1980; Abrams et al., 1993). Apoptosis is distinct from necrotic cell death caused by an external insult. During necrosis, the necrotic cells swell and lyse, thereby releasing cytoplasmic material which can cause an inflammatory response (Wyllie et al., 1980; Arends and Wyllie, 1991). The

induction of PCD requires RNA and protein synthesis, suggesting that this process represents an active, gene-directed program (Tata, 1966; Martin et al., 1988; Oppenheim et al., 1990). The activation of apoptosis must be precisely regulated, otherwise a variety of pathologies may be elicited. Thus, studies of PCD would be beneficial to our understanding of both normal development and pathogenesis.

Genetic studies in the nematode *C. elegans* provide strong evidence for an intrinsic death program in animal cells. Two genes, *ced-3* and *ced-4* (*ced*, cell death abnormal), are required for the initiation of programmed cell death; mutations in either of these genes cause the survival of almost all cells that normally die in *C. elegans* (Ellis and Horvitz, 1986). A third gene, *ced-9*, encodes a protein that inhibits cell death program. If the *ced-9* gene is inactivated due to a mutation, most of the cells, which would normally live, will die and the animals die at early development. However, the lethality caused by the *ced-9* mutant occurs only if both *ced-3* and *ced-4* are functionally intact (Hengartner et al., 1992). This suggests that *ced-9* is a negative regulator of *ced-3* and *ced-4* and acts upstream of these genes to keep the *ced-3/ced-4* dependent cell death suppressed (Fig. 1A).

The molecular characterizations of the above genes reveal that *ced-3* and *ced-9* in *C. elegans* are homologous to vertebrate cell death genes. The *ced-3* gene shows significant similarity to a family of cysteine proteases such as interleukin-1 β converting enzyme (ICE) and CPP32 (Yuan et al., 1993), which are involved in mediating apoptosis in mammals (Nicholson et al., 1995; Lazebnik et al., 1994). The protein encoded by *ced-9* gene is homologous in both structure and function to the mammalian protein encoded by the proto-

oncogene *bcl-2* (Hengartner and Horvitz, 1994; Vaux et al., 1992). *bcl-2* expression can inhibit PCD in many mammalian cells and can even partially inhibit PCD in *C. elegans* (Vaux et al., 1992). These findings suggest that at least some components of apoptosis have been conserved through evolution (Fig. 1B). Other experiments have further supported this hypothesis. For instance, the expression of the baculovirus P35 gene inhibits PCD in a wide range of heterologous species such as mammals, *Drosophila* and nematodes (Rabizadeh et al., 1993; Hay et al., 1994; Sugimoto et al., 1994). P35 protein is an inhibitor of ICE-like proteases. Expression of P35 can block autoactivation of ICE and may effectively prevent ICE-like proteases from initiating apoptosis (Bump et al., 1995).

Genes required for programmed cell death in *Drosophila*

In *Drosophila*, cell death displays many of the morphological and biochemical hallmarks of mammalian apoptosis (Wyllie et al., 1980; Abrams et al., 1993; White et al., 1994). Studies of PCD in *Drosophila*, which provides an experimentally very accessible invertebrate model system, may be directly relevant for understanding the mechanism of apoptosis in vertebrates.

In 1994, Steller and colleagues used a genetic approach to screen for deletions that affected PCD in *Drosophila* (White et al., 1994). They found a single genomic region on the third chromosome (positioned at 75C1, 2) required for all cell death in *Drosophila* embryos. Overlapping deletions (*Df(3L)WR4*; *Df(3L)WR10*; *Df(3L)CatDH104* and *Df(3L)H99*) have been isolated in this region. Among them, *Df(3L)H99* (abbreviated as *H99*) is the smallest

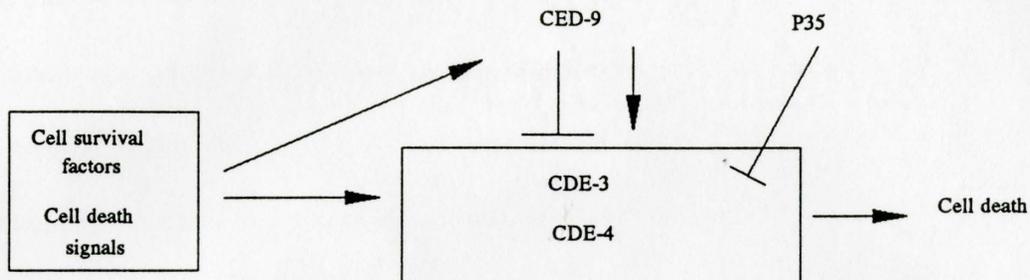
Figure 1. Schematic drawing of cell death pathway in *C. elegans*, *Drosophila*, and mammals.

A. In *C. elegans*, CED-3 and CED-4 are proteins promoting the apoptotic fate of cells that should die. Since their order of function is not known, these two proteins were put in one box. CED-9 prevents cell death from CED-3 and CED-4 indicated by horizontal bar. Previous data suggest that CED-9 also promote the death of cells that should die (indicated by arrow).

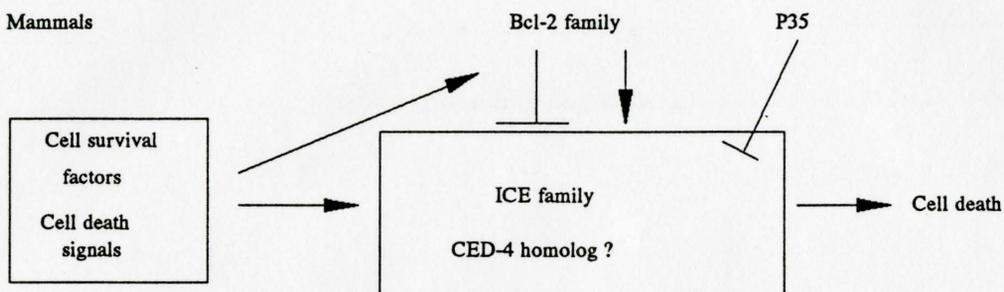
B. The cell death pathway in mammals is similar to that in *C. elegans*. Interleukin-1 β -converting enzyme (ICE) family members promote the cell death and their activities are modulated by the Bcl-2 family members.

C. In *Drosophila*, Reaper promotes cell death through a similar pathway function as in mammals and *C. elegans*. Reaper acts upstream of the apoptotic core program which is inferred from experiment data (White, et al., 1994). Hid and Grim promote death via a similar pathway as Reaper. In all the species, P35 prevents programmed cell death by inhibiting the activity of the CED-3, CED-4 or ICE family. Arrows indicate promotion and bars indicate inhibition. Dashed lines mean no direct experiment data (From Hengartner, 1996).

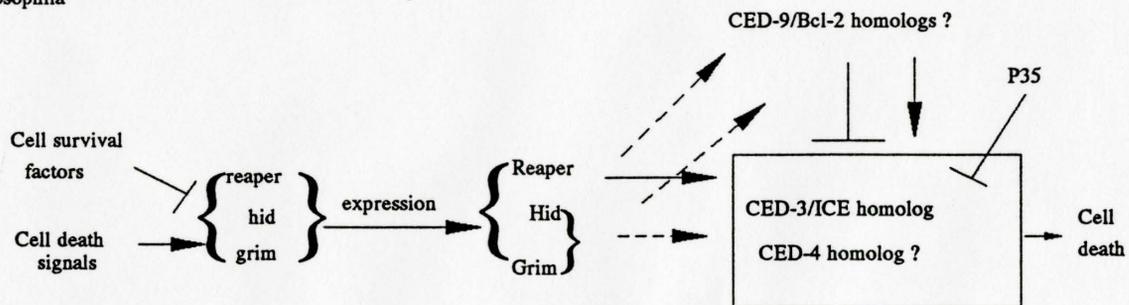
A. *C. elegans*



B. Mammals



C. *Drosophila*



cell death defective deletion, which can abolish all apoptotic cell death in *Drosophila* embryos. *H99* deficient embryos contain many extra cells and fail to hatch, but many other aspects of development appear quite normal e.g. cell division, cell differentiation and survival (White et al., 1994). Interestingly, *H99* mutants are not only deficient in normal cell death but are also protected against ectopic PCD caused by low-dose X-irradiation (~500 rads) or in *crumbs* mutant embryos. However, when *H99* deletion embryos are irradiated with very high doses of X-rays, some cell death with typical apoptotic morphology was detected (White et al., 1994). These results indicate that the cell death machinery is intact, but cannot be readily activated in embryos homozygous for *H99*. In addition, these results suggest that multiple signaling pathway for the activation of cell death may converge to induce cell death gene expression. According to this theory, the expression of cell death gene(s) would lead to the selective activation of cell death effector proteins that may be present but inactive in most cells (Steller and Grether, 1994). The activation of cell death effector proteins may occur either by stimulating the factors that promote cell death or by inhibiting the protective factors (Steller and Grether, 1994).

Molecular analysis of the *H99* deletion has led to the identification of the *reaper* (*rpr*) gene in *Drosophila melanogaster* (White, et al., 1994). In normal embryogenesis, *rpr* mRNA is specifically expressed in cells that are destined to die. Macrophages, which engulf dead cells, enclose phagocytosed corpses which contain *rpr* mRNA. The expression of *rpr* mRNA begins approximately 1-2 hours before any morphological features of PCD are apparent (White, et al., 1994). Therefore, *rpr* mRNA should be a useful marker for the identification of fated cells

before there is any morphological manifestation of PCD.

Expression of *rpr* under the heat-inducible *hsp70* promoter is sufficient to induce extensive apoptosis in both wild type and *Df(3L)H99* embryos (White et al., 1996). Ectopic overexpression of *rpr* in the developing retina results in eye ablation (White et al., 1996). Therefore, the *rpr* gene is likely responsible for the PCD-deficient phenotype of the *H99* deficiency and plays a central role in regulating the induction of cell death. The *rpr* gene encodes a small peptide of only 65 amino acids which shares some amino acid similarity with the "death domain" of the mammalian tumor necrosis factor receptor 1 (TNFR1) and Fas (Golstein et al., 1995). The baculovirus P35 protein, which has been shown to block cell death in both vertebrate and invertebrate cells, can also block cell death induced by *rpr* in *Drosophila* (Rabizadeh et al., 1993; Hay et al., 1994; Sugimoto et al., 1994; White et al., 1996). Since no single gene mutation in the *H99* interval could mimic the phenotypes of *Df(3L)H99* embryos, it was proposed that the cell death function may be encoded by two or more redundant genes in that genomic region (White et al., 1994).

Two additional cell death genes in the *H99* deficient region were identified and named *head involution defective* (*hid*; Grether et al., 1995) and *grim* (Chen et al., 1996). *hid* had been identified through its mutant phenotype, i.e. a pronounced defect in the morphogenetic movements of head involution (Abbott and Lengyel, 1991). *hid* mutant embryos have decreased levels of cell death and contain extra cells in their heads (Grether et al., 1995). It is possible that the *hid* phenotype results from inhibition of PCD. This notion is supported by the finding that a 401 amino acid protein encoded by the *hid* gene shares some limited

sequence similarity at the amino-terminus with the Reaper protein (Grether et al., 1995). *hid* mRNA is expressed in many different regions where cell death occurs. However, *hid* mRNA expression is limited to certain areas and does not correlate with PCD in the ventral nerve cord during late embryogenesis (Grether et al., 1995). This suggests that *hid* function may not correspond to PCD in the ventral nerve cord. Ectopic cell death was induced in both wild type and *H99* deficient embryos carrying the *hs-hid* construct following heat shock (Grether et al., 1995). Ectopic expression of *hid* in the *Drosophila* retina results in eye ablation and this effect is completely suppressed by coexpression of the baculovirus P35 gene (Grether et al., 1995). This further suggests a role for *hid* in PCD.

The *grim* gene encodes a 138 amino acid protein in which the amino terminal end shares notable similarity to Reaper and less similarity to Hid (Chen et al., 1996). The expression of *grim* mRNA is detected in regions and tissues where cell death occurs. Ectopic induction of *grim* triggers extensive cell death in embryos and cultured cells. This event required neither *rpr* nor *hid* function and could be prevented by coexpression of the baculovirus P35 protein (Chen et al., 1996). Therefore, *grim* is another cell death gene independent of *rpr* and *hid*.

It is commonly accepted that *rpr*, *hid* and *grim* encode cell death proteins since overexpression of any one of these genes, using heat shock or a strong tissue-specific promoter, results in an induction of PCD. Each gene can induce cell death without any of the others but mutations of any one of these genes cannot block all PCD. It appears that these three genes might encode partially redundant functions and act in a parallel switch that can

ultimately activate downstream apoptotic effectors. Interestingly, baculovirus P35 can block ectopic cell death triggered by *rpr*, *hid* and *grim* (White et al., 1996; Grether et al., 1995; Chen et al., 1996). This finding suggests that all three genes might ultimately activate a common pathway involving conserved ICE/CED-3 like proteases, which are targets for inactivation by P35 (Fig. 1C). This hypothesis was supported by recent studies which showed that Reaper-mediated apoptosis in *Drosophila* Schneider cells was completely blocked by Z-VAD-fmk (an ICE-like protease inhibitor; Pronk et al., 1996). In mammals, the cytotoxic signals of both Fas (CD95) and tumor necrosis factor receptor-1 (TNFR-1) can be blocked by ICE-like protease inhibitors as well (Enari et al., 1995; Los et al., 1995). Thus, the intracellular Rpr protein in *Drosophila* uses a signaling pathway for cell death similar to that used by the Fas and TNFR-1 in mammals; suggesting a high degree of conservation of this pathway among species.

Methods for detecting apoptosis in *Drosophila*

There are many methods for detection of PCD in *Drosophila* embryos, including DNA end labeling techniques, visualizing apoptotic cells in living embryos with vital dyes, the study of morphology, and flow cytometry. First, during apoptosis, the dying cells digest DNA into fragments that can be detected by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (Tunel) technique (Gavrieli et al., 1992; White et al., 1994). Second, acridine orange can rapidly and reliably stain dead cells and has been utilized to study PCD (Abrams et al., 1993). Third, morphological studies (light and electron microscopy with or without enhancer traps) are very useful for surveying apoptosis (Abrams et al., 1993; White et al.,

1994; Sonnenfeld and Jacobs, 1995). The use of enhancer traps allows us to detect MG during later stages of apoptosis due to perdurance of β -galactosidase (Sonnenfeld and Jacobs, 1995). The time course of PCD observed by using enhancer traps was similar to that observed by the TUNEL assay. In addition, enhancer trap-labeled small cell profiles are death cell profiles which are located outside the midline and appear to be contained within large macrophages (Sonnenfeld and Jacobs, 1995). Therefore, using enhancer traps is convenient for identifying the lineages of dead cells. This method is also useful for studying the origin and fate of the supernumerary cells in the embryos deficient for apoptosis. Fourth, *rpr* mRNA expression was proposed to have an advantage of revealing the cells bound for PCD at earlier stages than other methods (White et al., 1994). *In situ* hybridization signals for *rpr* mRNA can also be found in phagocytosed corpses within macrophages (White et al., 1994; Zhou et al., 1995; Dong pers. obs.). The *rpr* mRNA expression was used in this study to detect apoptotic cells at early stages. According to our knowledge, this study is the first to use this method to examine PCD in *Drosophila* and it proved to be useful.

Development of mesectoderm cells in *Drosophila*

In this study, the role of PCD has been examined in a well characterized embryonic lineage in *Drosophila*, the mesectoderm. The ventral midline in central nervous system (CNS) of *Drosophila* is an attractive system for investigating the mechanisms of cell determination, differentiation and cell death. This lineage model has been thoroughly investigated and characterized (Klambt et al., 1991; Bossing and Technau, 1994; Sonnenfeld and Jacobs, 1995;

Nambu et al., 1991). The neurons and glia of the midline are the progeny of mesectoderm cells (MECs). At the cellular blastoderm stage, the MECs originate from a small set of blastoderm precursor cells that form two stripes along the length of the anterior/posterior axis of the embryo. These stripes lie between the cells that will give rise to the mesoderm and the cells that will form the lateral CNS and ventro-lateral epidermis (Crews et al., 1988; Thomas et al., 1988; Klämbt et al., 1991). During gastrulation when the mesoderm invaginates, these stripes come together at the ventral midline and divide synchronously. Then the daughter cells of these stripes occupy the medial 2-3 rows of the ectoderm. In the course of germ band elongation, MEC cells become stretched along the longitudinal axis forming a single row. At the end of stage 9, all midline cell nuclei become progressively shifted interiorly, but still maintain a prominent cytoplasmic extension to the periphery. During late stage 11, all midline cells completely delaminate from the ectoderm (Crews et al., 1988; Bossing and Technau, 1994).

Mesectoderm lineages

Using different enhancer traps to study the mesectoderm lineages, a model has been proposed from the results obtained by Klämbt et al. (1991). The model describes 8 midline precursor cells giving rise to 4 cell lineages in each segment during embryogenesis. The anterior-most 3 midline precursors, which yield the midline glia (MG) lineage, divide once to produce 6 MG which are referred to as MGA, MGM and MGP (anterior, middle and posterior, respectively) according to their final positions. The nuclei of the MG are located at

the dorsal surface of the ventral nerve cord (VNC) and the cytoplasm ensheath the commissures (Jacobs and Goodman, 1989a). The fourth midline precursor, which yields the MP1 lineage, divides once to produce a bilateral pair of MP1 neurons (Thomas et al., 1984). Axons from the MP1 neurons extend posteriorly in ventral nerve cord segments and pioneer the longitudinal axon tracts (Jacobs and Goodman, 1989b). The other two or three midline precursors, posterior to the MP1 precursor, produce six ventral unpaired median neurons (VUM; Goodman et al., 1984). The VUM neurons are located medially and ventrally to the posterior commissure with their fibers running dorsally between the anterior and posterior commissures (Jacobs and Goodman, 1989b). The final midline precursor produces median neuroblasts (MNB) and possibly its support cells in each segment.

Another midline lineage model has been proposed that was generated by studying the fates of midline precursor cells after tracing each progenitor and its progenies with a lipophilic fluorescent tracer (DiI) in vivo (Bossing and Technau, 1994). This model suggests that seven midline progenitors give rise to 5 different lineages. Among these different lineages, 4 are of the same type as those previously characterized (Klämbt et al., 1991) with the addition of a novel cell type called the unpaired median interneuron (UMI). Since the authors observed variations in the number of progenitors between segments, they suggest a progenitor identified by its ordinal location might yield different progeny in different segments. The first model described by Klämbt et al. (1991) proposes that the midline precursors are a set of identifiable cells with an invariant fate. However, the second model proposed by Bossing and Technau (1994) suggests that the positional information within a segment is insufficient for specification

of midline precursors' identity. The experiment of the first model did not trace the precursors' fates and the experiment of the second model did not observe the relative position of progenies from different precursors. Therefore, the technological limits make it difficult to draw a clear conclusion. Data reported in this work will address this inconsistency.

Of the various lineages mentioned above, one is the MG lineage. Midline glia cells are distinct from neurons in a number of aspects. For example, during early development, glia cells have an elongated shape, with a high surface area and irregular nuclear profiles. They are located along the dorsal surface of the developing CNS, where the axon tracts will form. During later stages of embryogenesis, the MG increase their surface area and ensheath commissural axons. Their cytoplasm becomes more electron lucent and contains extensive rough endoplasmic reticulum (Jacobs and Goodman, 1989a). Midline glial cells play an important role during the development of the nervous system that is discussed below.

Interactions between midline cells and the axon commissures

A model for describing the interaction between midline cells and the CNS axon commissures has been proposed by Klämbt et al. (1991). According to the model, the formation of the two axon commissures takes place at stage 12 of embryogenesis. The first CNS growth cones that pioneer the posterior commissure can be detected at stage 12/5. These growth cones extend straight toward the most anterior pairs of VUMs. As they reach the midline, they change direction and grow anteriorly around the VUM cells (in the space that the MP1 had just migrated from) to contact their contralateral homologs and form the

posterior commissure (Klämbt et al., 1991).

At stage 12/3, the anterior commissure is pioneered by the growth cones which migrate toward the posterior edge of the MGA and then cross the midline. At this stage the anterior and posterior commissures are in close proximity to each other at the midline and are not yet separated from one another. The growth cones of the VUM neurons are located between the commissures while the cell bodies are migrating ventrally (Jacobs and Goodman, 1989b). The separation of the anterior and the posterior commissures takes place during stage 12/0; this separation correlates with the posterior migration of MGM and the medial migration of the RP1 and RP3 neurons (Patel et al., 1987; Jacobs and Goodman, 1989a,b). The MGM cells migrate over the MGA and move along the growth cones of the VUM neurons in between the two commissures in order to separate them. The pairs of MGM cells and cell bodies of RP1 and RP3 neurons adhere tightly, separating the commissures. The MGP migrate anteriorly into the previous segment but are not involved in commissure separation. By now the MG nuclei set their positions as anterior, middle and posterior with respect to the commissural axons and are termed MGA, MGM and MGP. Therefore, the model described above predicted that the MGA, MGM and VUM play a major role in the formation of the commissures.

The function and survival of MG require function of *spitz* group genes (Klämbt et al., 1991; Sonnenfeld and Jacobs, 1994). The *spitz* group genes, identified by Mayer & Nüsslein-Volhard (1988), include the following members: *single-minded (sim)*, *rhomboid (rho)*, *Star (S)*, *spitz (spi)*, and *pointed (pnt)*. Mutations in any of these genes cause embryonic lethality and similar cuticular phenotypes (Mayer & Nüsslein-Volhard, 1988). Embryos with *spi* group

gene mutations also affect axon tract formation. For example, the *sim* gene mutations result a collapse of the longitudinal axon tracts to a single midline tract. *spi*, *S*, *pnt* gene mutations cause incomplete separation of commissures (Mayer & Nüsslein-Volhard, 1988; Klämbt et al., 1991; Sonnenfeld and Jacobs, 1994). Previous reports showed that mutants of *spitz* group genes and *DER* (Drosophila Epidermal growth factor Receptor) have similar CNS phenotypes (Raz and Shilo, 1992), suggesting that they share a common signaling pathway.

To examine the effect of *spi* group genes mutations on midline cells, different midline lineages were analyzed by exploiting enhancer traps as cellular markers (Sonnenfeld and Jacobs, 1994; Klämbt et al., 1991; Klämbt, 1993; Nambu et al., 1991). *sim* functions early in the midline lineage. In *sim* mutations, the midline precursors fail to undergo synchronous postblastodermal cell division and fail to extend into the nerve cell layer. Additionally, they do not differentiate into neurons and glia and die (Nambu et al., 1991). In embryos of *S* mutants, the midline glial cells develop normally before stage 12/3, but the MG then fail to migrate and died at stage 13. This supports the hypothesis that the lack of MG function in *S* mutants can result in a failure of commissure separation (Sonnenfeld and Jacobs, 1994; Klämbt et al., 1991). Embryos mutant for *spi*, initially have midline glial cells present but the MGM do not properly migrate in between the two commissures as is also the case in *S* mutants. However, unlike the *S* mutants, in which the midline glia ultimately die, in *spi* mutants, the glia cells persist and lie abnormally on the dorsal surface of the CNS until the end of embryogenesis. All other midline cells appeared unaffected in *spi* (Klämbt et al., 1991). *spi* has a maternal contribution, this may be one of the reasons for MG survival (Mayer and Nüsslein-Volhard,

1988). In *pnt* mutants, the midline glial cells are still present. However, these glial cells do not migrate posteriorly along the cell processes of the VUM neurons. In later stages of embryogenesis, the misplaced midline glia cells degenerate and eventually die (Klämbt, 1993). Hence, different mutations affecting different MG result in diverse phenotypes which include failure of commissure separation and misplacement of midline glia cells.

PCD in the ventral midline of *Drosophila*

Abrams et al (1993) observed the spatial pattern and ultrastructural appearance of cell death in *Drosophila* embryos and has provided ground work for studying the genetic and molecular mechanisms of PCD. They found that a large number of cells die by apoptosis during embryonic development in *Drosophila*. The ultrastructural morphology of the dying cells resembles that of apoptosis in other systems, thus indicates that the dying cells in *Drosophila* embryos undergo PCD. Cell death begins at stage 11 of embryogenesis observed in the dorsal region of the head or inside the epidermal cell layer of the gnathal segments. Thereafter cell death spreads widely in many regions. As the VNC condenses, prominent cell death appears throughout the CNS (Abrams et al., 1993).

Recently, PCD at the mesectoderm was been reported by Sonnenfeld and Jacobs (1995; see also Zhou et al., 1995). They found apoptotic cells in the MGA, MGM and MGP lineages but not in the VUMs and MP1 lineages of the midline (Sonnenfeld and Jacobs, 1995). In wild type embryos, approximately 50% of midline glia cells die by apoptosis after the separation of commissural axon tracts. The death of MG has a spatial pattern which reveals

that the MGM have a greater probability of survival than either the MGA or MGP. After stage 13, apoptosis decreases the number of MG. Apoptotic MG are expelled from the CNS and are subsequently cleaned by phagocytic haemocytes.

In embryos deficient for apoptosis, *Df(3L)W4* or *Df(3L)H99*, PCD of MG is blocked, and thus leads to an increase in MG number in comparison to the wild type (Sonnenfeld and Jacobs, 1995; Zhou et al., 1995). Previous reports suggest that at stage 13 there are normally 6 MG; 3 of them die during development, so that at stage 17 only 3 MG survive (Sonnenfeld and Jacobs, 1995). If PCD is blocked by mutation, at stage 17 the expected number of MG should be 6. Surprisingly, 9-10 MG labeled by *P[slit1.0/lacZ]* were found in *Df(3L)W4* embryos within which virtually all apoptosis in *Drosophila* was blocked (Sonnenfeld and Jacobs, 1995). Where did the extra MG in embryos deficient for apoptosis come from? We consider the following possibilities. First, the extra MG may derive from MECs that undergo apoptosis before expressing specific MG markers. There are no specific markers for the MG lineage before stage 12, therefore any PCD in MG at that time is difficult to establish. Second, the absence of apoptosis may change the fate of the cells during development (Jones et al., 1995). Therefore cells of other lineages might divert to the MG fate when PCD is blocked. Third, a block of cell death may result in extra cell divisions, since cell activities, like proliferation, differentiation and apoptosis, are intimately coupled (Evan et al., 1995).

Based on the proposed possibilities, the objective of this thesis is to investigate the origin and fate of the supernumerary midline glia cells in *Df(3L)H99* embryos. Questions related to this objective will be addressed. Where do the supernumerary midline glia cells in

Df(3L)H99 embryos come from? When do the extra MG appear during development? What kinds of morphological and functional features do these extra MG possess? Can *Df(3L)H99* embryos rescue the phenotype of *spi* group gene mutations? Is the DER pathway involved in the survival of supernumerary midline glia cells in *H99* deficient embryos? Answers to these questions may reveal more about the function and differentiation of MG in embryos and the regulation of apoptosis during CNS development.

MATERIALS AND METHODS

1. *Drosophila* strains

Drosophila melanogaster strains were obtained from the Indiana Stock Center unless otherwise stated. All fly strains were grown on sucrose-agar-yeast medium at room temperature (22-25°C). The double mutants *Drosophila* or *Drosophila* carrying both a mutation and an enhancer trap were established as balanced stocks except for *S^{IN23}* and *Df(3L)H99* double mutant line.

1.1 Mutant flies

Df(3L)H99 was originally recovered by Mackay and Bewley (1989) as the result of γ -irradiation of flies carrying a *ri sbd e* chromosome to generate a deficiency which uncovers the genomic interval at 75C1,2. The *Df(3L)H99* obtained from H. Steller's laboratory was balanced with a *TM6B* chromosome carrying P[*Ubx lacZ w+*], which allowed independent identification of the homozygous *Df(3L)H99* embryos.

Star^{IN23} : an EMS-induced allele located at 2-1.3 (Nüsslein-Volhard et al., 1984; Lindsley and Zimm, 1992).

Df(2L)E55: an EMS-induced deficiency which maps at 37D2-E1, 37F5-38A1 (Wright et al., 1976). This deficiency uncovers the *spitz* mutation.

single-minded^{B13.4}: an EMS-induced protein null allele at 3-52.2 (Nüsslein-Volhard

et al., 1984; Thomas et al., 1988).

pointed^{Δ88}: a null allele located at 3-79 was generated by imprecise excision of P element insertion at the *pnt* locus (Scholz et al., 1993) and was provided by Dr. C. Klämbt.

1.2 Enhancer traps and gene fusion reporter elements

A large number of *lacZ* enhancer trap lines that express β -galactosidase in specific patterns of cells and tissues have been described in the literature (O'Kane and Gehring, 1987; Bier *et al.*, 1989; Bellen *et al.*, 1989; Rubin and Spradling, 1982). The following enhancer trap lines and reporter construct elements were used in this study:

AA142: P element insertion at cytological location 66D (Klämbt et al., 1991). This line expresses β -galactosidase strongly in the MGA, MGM and weakly in MGP after stage 14.

X55: This line carries a P element insertion at 56F. In this line, the VUM neurons, the MNB and its support cells, and the MGPs are clearly labeled from stage 12 (Klämbt et al., 1991).

P223: This line carries a P element insertion at cytological interval 98. P223 expresses β -galactosidase in the MP₁ neuron and two MP₂ neurons in each hemisegment from stage 11 until the end of the embryogenesis.

pointed^{Δ277}: Integration of a P element occurred in the middle of the P2 exonI of *pnt* results in a weak, homozygous viable P2-specific mutation. However, P2 transcription is only very slightly reduced in homozygous *pointed*^{Δ277} embryos by whole mount *in situ*

hybridization using P2 specific probe (Scholz et al., 1993). The enhancer trap *pnt*^{1277/+} is expressed in the MG from stage 13.

argos^{w11}: The *argos*^{w11} mutant was generated by a P-element-insert at a cytological position 73A3, 4 (Freeman et al., 1992). *argos*^{w11} heterozygous is expressed in the subset of MG, which do not die during embryogenesis (C. Stemerding and R. Jacobs submitted).

P[sim/lacZ](EE1): This gene fusion reporter consists of a P element transposon containing 7.8 kilobases of DNA from *sim* regulatory region fused to the β -galactosidase gene (Nambu et al., 1990). EE1 is used to label all midline precursors at stage eight. Subsequently the labeling is restricted to MG and a set of ventral neurons by stage 15.

P[slit1.0/lacZ]: This gene reporter construct consists a P element transposon containing a 1.0 kilobase restriction fragment of *slit* DNA (Wharton and Crews, 1993). This line expresses β -galactosidase in all midline glia cells from stage 12 until the end of the embryogenesis..

2. Antibodies

anti- β -galactosidase: This is a commercial rabbit polyclonal antibody (Cappel) used to identify expression patterns of enhancer traps and gene fusion reporter constructs. A dilution of 1:100 was used for whole mount embryo staining.

BP102: This is a mouse monoclonal antibody that recognizes an uncharacterized carbohydrate moiety present on the longitudinal and commissural axon tracts of the CNS (provided by C.S. Goodman, Berkeley). It was used at a 1: 2 dilution for embryo staining.

22C10: This antibody was used to identify the ventral unpaired median neurons at stage 14 and the MP1 and MP2 neurons at stage 12 of embryogenesis (Fujita et al., 1982). It was used at a 1:10 dilution for embryo staining. (This was a gift from S. Benzer Galtech).

anti-engrailed: This mouse monoclonal antibody recognizes the product of the nuclear engrailed protein during embryogenesis and was used to label the median neuroblast and its progeny during embryogenesis (kindly provided by C. S. Goodman, Berkeley). A dilution of 1:1 was used for embryo stains.

anti-elav: This is a mouse antisera obtained from the Developmental Hybridoma Bank for staining nuclei of all neurons (Robinow et al., 1988). A dilution of 1:5 was used for the whole mount embryo staining.

anti-BrdU: This is a mouse monoclonal antibody used at a 1:50 dilution. BrdU (Boehringer Mannheim) is a thymidine analogue which is incorporated into the DNA during S-phase of the cell cycle. Cell nuclei that have incorporated BrdU can be detected by this antibody (De Fazio et al., 1987; Grenier, 1982; Bodmer et al., 1989).

3. Embryo collection and staging

Embryos were collected with apple juice agar plates and the plates were smeared with live yeast paste (Fleischmann's dry yeast). The collecting plates were changed twice a day and stored at 4°C for a maximum of two days. These plates were warmed for two hours at room temperature to allow microtubule repolymerisation and restoration of axon morphology before antibody staining. Embryos were staged according to Campos-Ortega and Hartenstein

(1985). The length of the germband during stage 12 are subdivided according to Klämbt et al. (1991).

4. Immunocytochemistry in Whole-Mount *Drosophila* embryos

4.1 HRP Reaction

The embryos on the collecting plates were dechorionated with 50% commercial bleach (sodium hypochlorite) for five to ten minutes. Dechorionated embryos were rinsed thoroughly with distilled water onto a nitex sieve that was blotted dry on a kimwipe. The dechorionated embryos were immersed in a scintillation vial containing 5ml heptane, 4.5ml phosphate buffered saline (PBS, pH 7.4) and 0.5ml 37% formaldehyde. The embryos were fixed for 30 minutes at the interface of the two solvents on a rotator. The lower layer was removed with a pasteur pipette and an equal volume of methanol was added violently to the remaining heptane containing the fixed embryos. The vial was shaken vigorously for 30 seconds to split the vitelline membrane of the embryos. The devitellinated embryos fell to the bottom of the container and were transferred with a pasteur pipette to a glass test tube. Four subsequent methanol washes were done to remove the last traces of heptane. Embryos were either stored in methanol at 4°C until further use or were immediately processed for antibody staining.

In order to continue with antibody staining, the embryos were rehydrated by removing methanol and replacing with PBT (PBS containing 0.2% Triton X-100). Embryos were washed with PBT three times for five minutes and once for 30 minutes. After washing, the embryos were incubated in 100 µl PBT containing 7% normal goat serum (NGS) for 30 to

60 minutes. NGS was used to block nonspecific antibody binding sites. A specific dilution of primary antibody was added to the blocking solution and the embryos were incubated overnight at 4°C. The primary antibody was washed out with PBT three times for five minutes and four times for thirty minutes. The washed embryos were reblocked in PBT and NGS for 30 minutes. The secondary antibody [either goat anti-mouse IgG or donkey anti-rabbit IgG (Jackson immunoresearch) conjugated to horse-radish peroxidase HRP] was added to the solution at a dilution of 1:100. The embryos were incubated in secondary antibody for two hours at room temperature and were then washed three times for five minutes and four times for thirty minutes with PBT solution.

To visualize the antibody reaction, the embryos were incubated in 200µl PBT plus 100µl 1mg/ml diaminobenzidine (DAB) for two minutes. The reaction was started by adding 3µl 3% hydrogen peroxide (H₂O₂) and stopped with several washes of PBT. The brown signal was monitored with the dissecting microscope. Waste DAB was neutralized in bleach. Embryos processed for single antibody labeling were dehydrated through an ethanol series (50%, 70%, 90%, 95% and 100%) and preserved in methyl salicylate.

Embryos processed with double antibody HRP labeling were incubated with first primary and secondary antibody. An 8% cobalt chloride solution (CoCl₂.6H₂O; Fisher, lot 884698) was added to the diluted DAB solution to a final volume of 4 µl/ml to produce a purple to black color signal. Embryos were washed with PBT for one hour and then proceeded to the second primary and secondary antibody incubations. Subsequent processing is as described above. Stained embryos were examined and photographed with a Zeiss

Axiophot microscope.

4.2 Anti- β -galactosidase HRP and anti-*elav* fluorescent double labeling

Embryos were fixed and devitellinated as described above. After blocking, embryos were incubated overnight at 4°C in PBT+ NGS containing a 1:100 dilution of rabbit anti- β -galactosidase and a 1:10 dilution of mouse anti-*elav*. Antibodies were washed out by three times for five minutes and four times for thirty minutes with PBT. Then embryos were blocked thirty minutes in PBT+ NGS and incubated for two hours at room temperature in PBT+ NGS containing 1:100 dilution of goat anti-rabbit IgG conjugated to Texas Red (kindly provided by Dr. Nurse' lab McMaster University) plus 1:100 dilution of goat anti-mouse IgG conjugated to FITC (kindly provided by Dr. Campos' lab McMaster University). Secondary antibody was washed out by three times for five minutes and two times for thirty minutes with PBT.

Fluorescently stained embryos were washed with PBS once and replaced with PBS : glycerol (1:1). Embryos were kept at 70% glycerol with a pinch of p-phenylenediamine (anti-fade agent). The embryos can be stored at least three weeks at 4°C in the dark. Mounted embryos were examined on a Biorad Confocal Microscope (York University). Images for each fluorescence channel were merged and printed using Adobe Photoshop.

4.3 Anti- β -galactosidase HRP and anti-BrdU FITC double labeling

The embryos were dechorionated with 50% commercial bleach (sodium hypochlorite)

for five to ten minutes. Dechorionated embryos were rinsed thoroughly with distilled water onto a nitex sieve which was blotted dry on a kimwipe. The dechorionated embryos were immersed in a glass test tube containing octane equilibrated with 1X *Drosophila* Schiender's medium for four minutes on the rotator. The embryos were transferred with glass pipette with a minimum of octane to a scintillation vial containing 1mg/ml BrdU in 1X *Drosophila* Schiender's medium. Remaining octane was evaporated thoroughly by air and embryos were settled down to the bottom by air pressure with the glass pipette. After incubation in the medium for thirty minutes, the embryos were washed with distilled water for five times and collected onto a nitex sieve. Fixation, devitellination and anti- β -galactosidase HRP staining were described above. For anti-BrdU staining, the embryos were hydrolyzed with 2N HCl in PBT for 30 minutes on the shaker and then washed with PBT. After blocking (see above), embryos were incubated overnight at 4°C in PBT+ NGS containing a 1: 50 mouse anti-BrdU and then washed three times for five minutes and four times for thirty minutes with PBT. The embryos were reblocked 30 minutes and incubated for two hours at room temperature in PBT+ NGS containing 1:100 dilution of goat anti-mouse IgG conjugated to FITC. Secondary antibody was washed out three times for five minutes and twice for thirty minutes with PBT and cleared with glycerol as described above.

Visualization of the immunofluorescence and photography were performed with a Zeiss axioskop microscope equipped for epifluorescence.

5. Anti- β -galactosidase and *in situ* hybridization double labeling

5.1 Embryo fixation

Embryos were dechorionated with 50% bleach for five minutes. Dechorionated embryos were rinsed with embryo wash (solutions for *in situ* see 5.4) into a nitex sieve which was blotted dry on a kimwipe. Dechorionated embryos were then put into scintillation vial containing a fix mixture and shaken well. Embryos were fixed on the rotator for 20 minutes and sat for 10 minutes. The embryos were devitellinated as described above. The devitellinated embryos were transferred into a glass tube and washed with methanol four times and ethanol four times. Embryos were either stored in ethanol at -20°C or immediately processed for antibody staining.

5.2 Antibody staining prior to RNA *in situ* hybridization

To proceed with antibody staining, half of the ethanol in which embryos were stored was replaced with PBTH. Then, the embryos were washed three times for five minutes and once for thirty minutes with PBTH (PBT containing 0.05mg/ml Heparin). After washes, embryos were incubated in 100µl of blocking solution for 40 minutes at room temperature. A dilution of 1: 100 rabbit anti-β-galactosidase primary antibody was added to the blocking solution and the embryos were incubated overnight at 4°C. The primary antibody was washed three times for five minutes and four times for thirty minutes with fresh PBTH solution. Embryos were reblocked as above and incubated with a dilution of 1: 100 donkey anti-rabbit HRP for two hours at room temperature. The reaction was started when 300µl antibody staining solution was added and stopped with two washes of PBTH. After reaction, embryos

were post-fixed in PBTH: 10% formaldehyde mixture (4:1) for ten minutes and then rinsed three times with PBTH. Half of PBTH was removed and replaced with ethanol. Embryos were washed four times with ethanol and stored in ethanol at -20°C for further *in situ* hybridization.

5.3 *In situ* hybridization

Embryos which were stored in ethanol were rinsed once with ethanol : xylene (1:1) and soaked in xylene for 2-3 hours on shaker. The embryos were washed with ethanol : xylene (1:1, freshly made) once again. Four times of ethanol washes were performed and glass tubes were changed each time, so there were no traces of xylene. Prehybridization and hybridization solutions were preheated to 53°C . Embryos were washed with methanol : PBT (1:1) and transferred to eppendorf microfuge tube (1.5 ml). Then embryos were post-fixed for 10 minutes (tubes laying on their side on the shaker) and rinsed three times for two minutes with PBT. 1ml PBT was added to the tube followed by $40\mu\text{g}$ Proteinase K. The timing is critical for proteinase K digestion, usually three to four minutes. The tubes were agitated by hand and stopped about 20 seconds before time point is over to allow embryos to settle. Proteinase reaction was stopped by washing three times with 2mg/ml glycine in PBT. Embryos were rinsed two times with PBT and post-fixed again for ten minutes. The embryos were washed four times for two minutes with PBT and three times for two minutes with PBT : prehybridization (1:1) solution. After that, embryos were washed five minutes with prehybridization solution at room temperature and prehybridized in prehybridization

solution at 53°C for one hour with orbital rotation in the hybridization oven.

RNA probes for *in situ* hybridization were generated by *in vitro* transcription of linearized cDNA. Single-stranded digoxigenin-labeled antisense probes were synthesized by T3 RNA polymerase (Boehringer Mannheim). *rpr* cDNA was carried by a vector, Bluescript-SK, obtained from Dr. H. Steller. DER cDNA plasmid, Bluescript-DER, offered by Dr. E. Bier. RNA probes were prepared by Christian Smith and Christopher Stemerding of our laboratory.

The probe from the freezer was thawed on ice and diluted 1 to 10 in DEPC H₂O in another tube. This dilution probe was boiled 20 minutes at 60°C in the dry heating block and placed on ice immediately after heating. The prehybridization solution was removed and replaced with hybridization solution carefully. Then probe was added at a dilution of 1: 2000 to 1: 2500 and solution was mixed well. Tubes were placed on side in hybridization oven at 53°C overnight without shaking. The next day wash solutions were prepared first and allowed to equilibrate at 53°C. Wash solutions were made with different concentration of posthybridization solution and PBT. Embryos were first spun in the microcentrifuge for two minutes at 5000 rpm and washed 20 minutes with 80% posthybridization solution in PBT. Then embryos were washed 20 minutes 60%, 20 minutes 40%, 20 minutes 20% posthybridization solution in PBT and two times 20 minutes with PBT alone. After washes, embryos were digested with 20µg/ml RNase for 25 minutes at 37°C in a waterbath and then washed 3 times with PBT. Embryos were incubated with 1ml of 1: 2000 dilution of anti-DIG-antibody for one hour at room temperature on the shaker. Antibodies were washed out

with four times 20 minutes of PBT. Embryos were then washed three times five minutes with detection solution and could be stored overnight at 4°C. Embryos were transferred to glass test tube to do the reaction. The reaction was started by adding 1ml reaction mix and kept in the dark without shaking. The blue signal could be seen in one to two hours and the reaction usually need seven to nine hours. The reaction was stopped by three times PBT washes and washed three times more with PBT. Embryos were then washed with PBS, 30% and 50% glycerol in PBS and stored in 70% glycerol.

To observe the staining pattern, embryos were replaced with 50% glycerol and then washed several times with PBS. Then embryos were dehydrated through an ethanol series washes and kept in methyl salicylate.

5.4 Solutions

DEPC treated ddH₂O: ddH₂O 1000 ml and 1ml

shake well and incubate overnight at room temperature and autoclave.

Embryo wash: 7% NaCl

0.05% Triton X-100 (make from 10% first)

5X Fixation buffer: 800mM KCl

200mM NaCl

20mM Na₃EGTA (pH8.0)

5mM Spermadine Hcl

2mM Spermine

	150mM Pipes pH 7.4
	DEPC water
Fix mix:	2mL embryo wash
	2mL 10% formaldehyde
	1mL 5X Fixation buffer
	5mL heptane
PBT:	1X PBS with 0.1% Tween-20
	100mL 10XPBS
	10mL 10% Tween-20
	890mL ddH ₂ O
PBTH:	PBT containing 0.05mg/ml heparin (Gibco BRL, Cat# 15077-019)
PBTH with tRNA:	PBTH containing 0.25mg/ml tRNA (BMC, cat# 109525)
Blocking solution:	900µl PBTH with tRNA
	100 µl RNase free BSA (20mg/ml; Sigma, cat# 9048-46-8)
	1 µl RNase inhibitor
Antibody staining solution:	
	948µl PBTH
	50 µl DAB stock (10mg/ml)
	2 µl 30% peroxide
	1 µl RNase inhibitor
Post-fix solution:	80% PBT and 20% 10% formaldehyde

Prehybridization solution: 50% Formamide	25ml Formamide
300mM NaCl	3ml 5M NaCl
10mM Tris-HCl pH 6.8	0.5ml 1M Tris HCl
10mM Na Phosphate pH 6.8	5ml 0.1M Na Phosphate
1X Denhart's solution	1ml 50X Dendarts
5mM EDTA pH 8.0	0.5ml 0.5M EDTA
1 mg/ml Yeast tRNA	1ml 50mg/ml yeast tRNA
DEPC water	14ml DEPC water
Denhart's solution (Sigma, cat# D-9905-contains 1% solution of Bovine Serum Albumin, Ficoll, and Polyvinylpyrrolidone)	

Hybridization solution: same with prehybridization solution except there is no tRNA and 10% Dextran sulfate powder was added. This powder is difficult to dissolve so solution must first be at 53°C before adding the Dextran sulfate. Agitate periodically until fully dissolved. The solution is very viscous.

Posthybridization solution: same with prehybridization solution except there is no tRNA

Detection solution: 100mM NaCl
50mM MgCl₂
100mM Tris pH 9.5
0.1% Tween 20

Reaction mix: 10ml detection solution

45 μ l 4-Nitroblue tetrazolium chloride (NBT)

35 μ l 5-Bromo-4-Chlo-3-Indolyl-phosphate (4-toluidine salt)

6. Electron microscopy protocol

The dechorionated embryos were put in heptane equilibrated with fixative containing 25% glutaraldehyde for ten minutes on a rotator, then transferred onto a glass slide and blotted dry with filter paper. Embryos were gently stuck on double sided tape and the tape was placed within a silicone sealant well and then immersed in 0.1%M cacodylate buffer. Devitellinized by a dissection needle or a tungsten needle, the embryos were transferred into a small tube. Staining solution without bluogal (5-Bromo-3-indolyl- β -D-galactopyranoside) was added and left for at least 5 minutes. The staining solution consists of 10mM PO_4 buffer (pH7.2), 150 mM NaCl, 1mM MgCl_2 , 3.1 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$ and 3.1 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$. The staining solution was replaced with the complete staining solution to which 0.1% bluogal had been added and incubated overnight at 18°C. The stock bluogal is 8% bluogal in dimethylsulphoxide (DMSO). The embryos were washed with 0.1%M cacodylate buffer (pH 7.4). After that the embryos were post-fixed in 1% Osmium tetroxide in cacodylate buffer and washed with cacodylate buffer followed by distilled water. The embryos were stained in 5% uranyl acetate for 30 minutes and washed in distilled water.

Following staining the embryos were dehydrated with a series of washes in 50%, 70% and 90% ethanol for 10 minutes each, and 95% and 100% ethanol twice each for 10 minutes and then 100% ethanol once 30 minutes. The following plastic mixture for embryo embedding

was made: 4.4g of araldite, 6.2g of Epon, 12.0g of DDSA and 0.3mL of DMP*30 (catalyst). For infiltration the embryos were incubated in absolute ethanol: plastic (1:1) with catalyst for one hour and a half, followed by absolute ethanol: plastic (1:3) with catalyst for one hour and a half. The embryos were placed in 100% plastic with catalyst 48 hours in a desiccator and changed the plastic once. In order to get the embryos in the middle of the block, half-filled molds were cooked for four hours to polymerize. The embryos were placed in the semi-polymerized plastic, the remainder of the wells were filled with plastic. The embryos were oriented to get sagittal sections. The plastic was allowed to polymerize for two days in the oven at 70°C. Sections (0.1µm) from blocks were collected on top of slot grids coated with 0.3% formvar film and stained in distilled water with lead citrate for 5 minutes. After staining, the grids were washed with boiled distilled water and viewed with transmission electron microscope.

7. Cell counting

Cell numbers in each MEC lineages at different stages in wild type and *H99*-deficiency embryos had been counted with image tracing methods. Embryos which were preserved on methylsalicylate were mounted into permanent slides using permount. The number of cells of segment A1 to A7 from each embryo was counted with camera lucida. This number was then divided by 7 giving the average cell number for each segment of one embryo. The averages from different embryos were used to get the mean value and standard error of mean (SEM). All data are expressed as mean±SEM per segment.

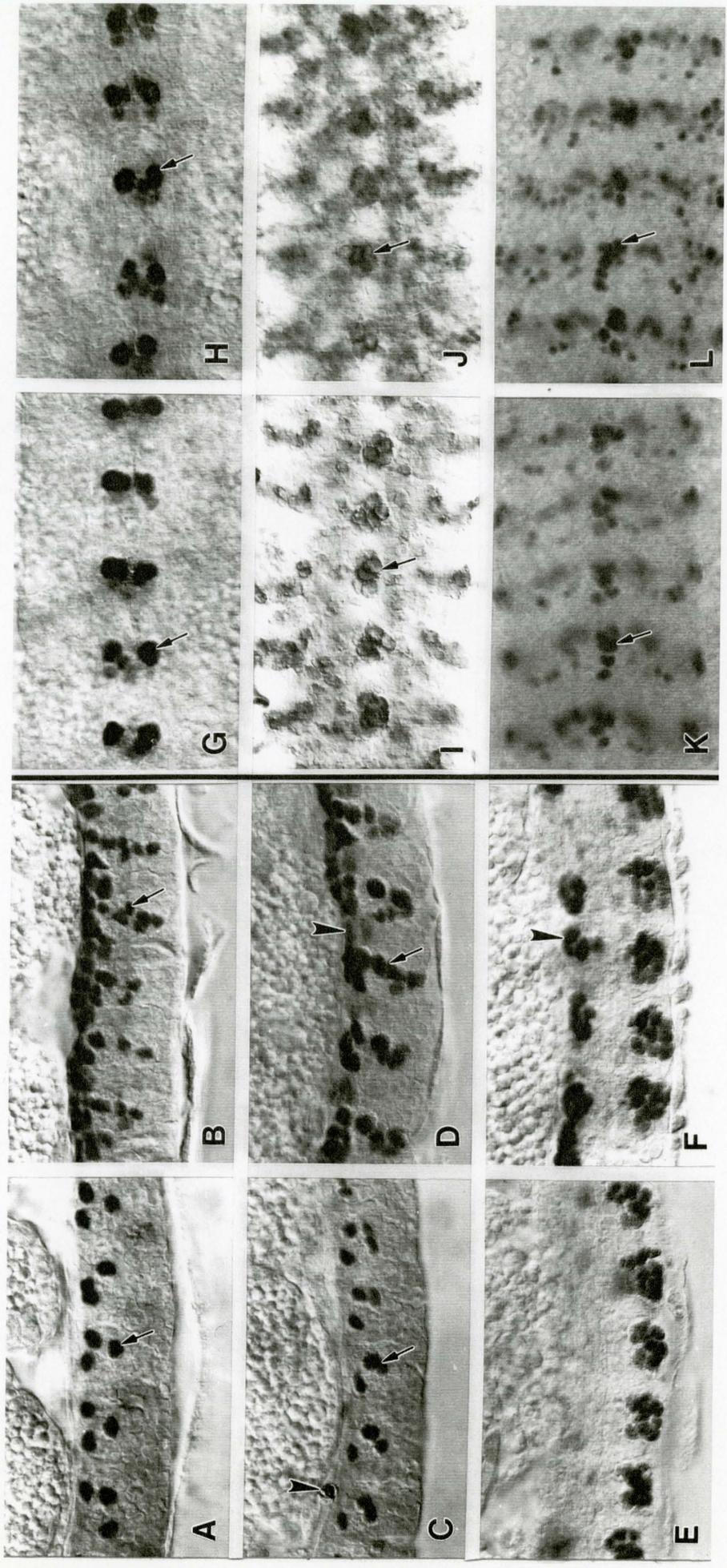
RESULTS

Lineage analysis of the mesectoderm in embryos deficient for apoptosis

Df(3L)H99 uncovers the genomic interval at 75C1, 2 which is required for all programmed cell death in *Drosophila* embryos. In order to study the developmental role of midline cell death, several enhancer traps and antibodies were used to trace the fate of different cell lineages in the embryonic MEC in embryos deficient for apoptosis. We have observed the cell number within different MEC lineages at stage 16. *P[slit1.0/lacZ]* and the AA142 enhancer trap were used to identify MG. All data presented here are numbers of cells per segment. Details are given in Table (Appendix) which summarizes all the counts. In wild type embryos, there were 3.2 ± 0.1 cells labeled by *P[slit1.0/lacZ]* (n=6, Fig. 2A) and 3.3 ± 0.1 cells labeled by AA142 (n=7, Fig. 2C); while in *Df(3L)H99* embryos, there were 12.3 ± 0.4 cells labeled by *P[slit1.0/lacZ]* (n=7, Fig. 2B) and 11.3 ± 0.4 cells labeled by AA142 (n=8, Fig. 2D). Therefore, in *Df(3L)H99* embryos there were supernumerary cells labeled by MG specific markers.

The number of MGP was analysed using the enhancer trap X55 which is expressed in the MGP, the VUMs and the MNB (see methods). At stage 16, wild type embryos have 0.07 ± 0.04 X55 labeled cells (n=8, Fig. 2E); the number of MGP in *Df(3L)H99* embryos was 3.5 ± 0.2 (n=5, Fig. 2F). In the embryos containing the AA142 enhancer trap, extra MGP were detected as faintly stained cells labeled in *Df(3L)H99* embryos, as visualized in the posterior region of each segment (Fig. 2D). These cells were included in the total MG counts. In terms

Figure 2. The fate of different MEC lineages in *Df(3L)H99* embryos. Embryos containing the *P[slit/lacZ]1.0* reporter construct (A, B), AA142 enhancer trap (C, D) and X55 enhancer trap (E, F) were labeled with antibodies to β -galactosidase. In wild type embryos, 3 MG cells were labeled (A, C arrow) and dead cell profiles could also be seen (arrowhead). No MGP remain at stage 16 in wild type embryos (E). In *Df(3L)H99* embryos, supernumerary MG cells were labeled by the *P[slit1.0/lacZ]* reporter construct and AA142 (B, D arrow). Among them, 3-5 MGPs also express X55 (F, arrowhead) and faintly labeled by AA142 (D, arrowhead). Embryos containing the P223 enhancer trap (G, H) were labeled with antibodies to β -galactosidase. 2 MP1 neurons per segment were labeled by P223 in both wild type (G, arrow) and *Df(3L)H99* embryo (H, arrow). MP2 neurons were also labeled by P223 (arrowhead). The VUMs were identified in wild type (I) and in *Df(3L)H99* embryos (J) by using the monoclonal antibody 22C10. There was no apparent difference in the number of VUMs between wild type (I, arrow) and *Df(3L)H99* mutant embryos (J, arrow). Anti-engrailed antibody was used to label the MNB and its progeny in wild type (K) and *Df(3L)H99* mutant embryos (L). No differences were detected in the MNB lineage between wild type (K, arrow) and *Df(3L)H99* mutant embryos (L, arrow). Embryos are at stage 16 with anterior to the left. A-F are sagittal views with the dorsal facing up and G-L are frontal views.



of the number of neurons labeled by X55, 7.7 ± 0.2 neurons were labeled in wild type embryos (n=8, Fig. 2E) while 8.5 ± 0.3 neurons were labeled in *Df(3L)H99* embryos (n=5, Fig. 2F).

The fate of MP1 are traced by the enhancer trap P223 while the VUMs and MNBs were further characterized by the monoclonal antibody 22C10 and antibody to engrailed respectively. No difference was observed in the number of MP1 between wild type (2.0 ± 0.03 , n=7, Fig. 2G) and *Df(3L)H99* embryos (2.0 ± 0.04 , n=4, Fig. 2H). There was no apparent difference in the number of labeled VUMs between wild type (4.0 ± 0.1 , n=8, Fig. 2I) and *Df(3L)H99* embryos (4.1 ± 0.1 , n=9, Fig. 2J). No difference was detected in the MNB lineage between wild type (5.8 ± 0.1 , n=8, Fig. 2K) and *Df(3L)H99* embryos (5.8 ± 0.2 , n=8, Fig. 2L). The above data show that supernumerary midline cells in *Df(3L)H99* embryos express genes of the MG lineage, suggesting that virtually all midline PCD occurs in the MG lineage, although there is approximately one additional neuron labeled by X55 in *Df(3L)H99* embryos.

Time course of the effects of *Df(3L)H99* on the number of midline cells

Were the extra MG in *Df(3L)H99* embryos persistent cells or were new MG recruited at different stages? We used *P[*sim/lacZ*]* and *P[*slit1.0/lacZ*]* gene fusion reporter constructs to address this question. *P[*sim/lacZ*]*, a good marker for early stages of embryogenesis, was used to label all midline precursors from stage 8 to stage 13. In stage 8, 9 and 10 wild type embryos, the midline cells labeled by *P[*sim/lacZ*]* were 13.6 ± 0.3 (n=8), 13.7 ± 0.4 (n=5) and 14.1 ± 0.5 (n=6) respectively. By stage 11 and 12, the number of cells increased to 15.3 ± 0.5 (n=6) and 19.4 ± 0.5 (n=10). Comparing the number of midline cells labeled by *P[*sim/lacZ*]*

in mutant with wild type embryos, there was no obvious difference before stage 12 (Fig. 3). In stage 8, 9, 10, 11 and 12 *Df(3L)H99* embryos, the midline cells labeled by *P[*sim/lacZ*]* were 13.8 ± 0.4 (n=3), 14.1 ± 0.3 (n=3), 14.5 ± 0.3 (n=5), 16.0 ± 0.6 (n=5) and 20.1 ± 0.5 (n=8) respectively. By stage 13, 19.7 ± 0.8 cells were labeled by *P[*sim/lacZ*]* in wild type (n=6) and 22.6 ± 0.7 cells in *Df(3L)H99* embryos (n=6, Fig. 3).

The *P[*slit1.0/lacZ*]* gene fusion reporter construct expresses β -galactosidase in all MG cells from stage 12 until the end of the embryogenesis. The expression of *P[*slit1.0/lacZ*]* begins from stage 12/5 (Fig. 4A, 4B). In wild type stage 12/0 and early stage 13 embryos, the number of cells labeled by *P[*slit1.0/lacZ*]* was 9.3 ± 0.3 cells in wild type embryos (n=9, Fig. 4C). By late stage 13, the number of MG was decreased (7.1 ± 0.3 , n=8, Fig. 4E) with many dead cell profiles in wild type embryos. These small *P[*slit1.0/lacZ*]* and AA142 expressing profiles were MG that had died and were expelled from the VNC (Sonnenfeld and Jacobs, 1995). The number of MG was 5.1 ± 0.1 (n=6), 3.7 ± 0.1 (n=6, Fig. 4G) and 3.2 ± 0.1 (n=6) at stage 14, 15 and 16, respectively. By stage 17, only 3.1 ± 0.1 MG remained in wild type embryos (n=6, Fig. 4I). The time course of expression of *P[*slit1.0/lacZ*]* in the MG lineage is shown on Figure 3. The results suggest that in the wild type embryos, approximately 6 *P[*slit1.0/lacZ*]* expressing cells die between early stage 13 and stage 17. In *Df(3L)H99* embryos, the number of MG was 10.2 ± 0.3 (n=6, Fig. 4D) at early stage 13, 11.1 ± 0.3 (n=6, Fig. 4F) at stage late 13 and 12.1 ± 0.3 (n=8) at stage 14. Approximately 12 MG were observed at stage 15 (12.1 ± 0.3 , n=8, Fig. 4H), stage 16 (12.0 ± 0.3 , n=8) and stage 17

Figure 3. Time course of P[*sim/lacZ*] and P[*slit1.0/lacZ*] expression in the mesectoderm during embryogenesis. The expression of P[*sim/lacZ*] in the midline precursors from stage 8 to stage 13 are presented by the circles (○ wild type; ● *Df(3L)H99*). From stage 8 to stage 12 there was no obvious difference between mutant and wild type in the number of midline cells labeled by P[*sim/lacZ*]. By stage 13, there were approximately 3 additional cells labeled by P[*sim/lacZ*] in *Df(3L)H99* embryos comparing with wild type.

The expression of P[*slit1.0/lacZ*] in the MG are presented by the triangles (▽ wild type; ▼ *Df(3L)H99*). The expression of P[*slit1.0/lacZ*] begins from stage 12/5 and the number of P[*slit1.0/lacZ*] expressing cells decreased dramatically during embryogenesis in wild type embryos, while the number of P[*slit1.0/lacZ*] expressing cells shows no reduction in *Df(3L)H99* embryos. Each point represents mean cell number per segment from 5-10 embryos and for some of the data points, the standard errors were smaller than the symbol size and therefore do not appear on the graph.

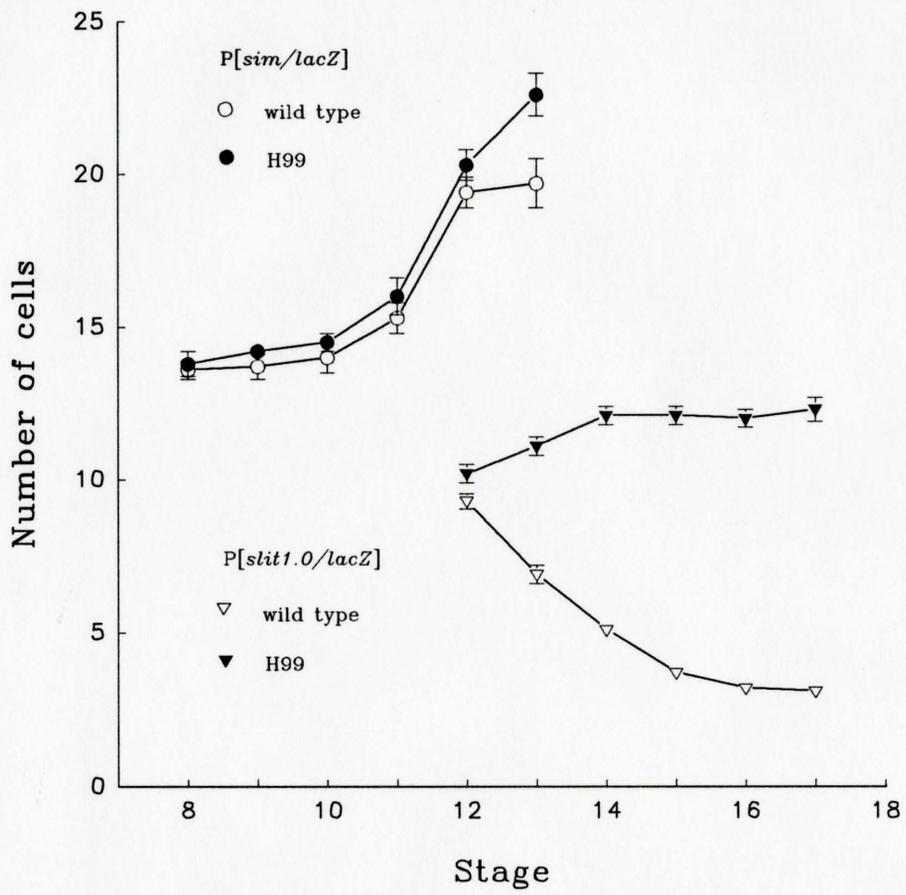
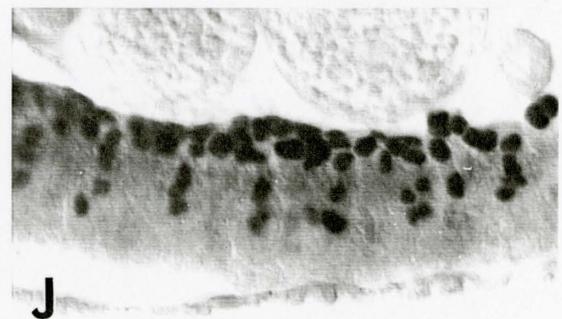
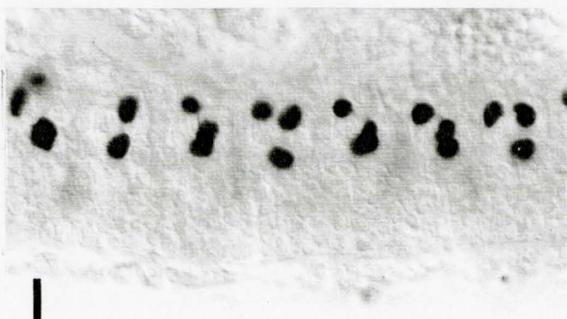
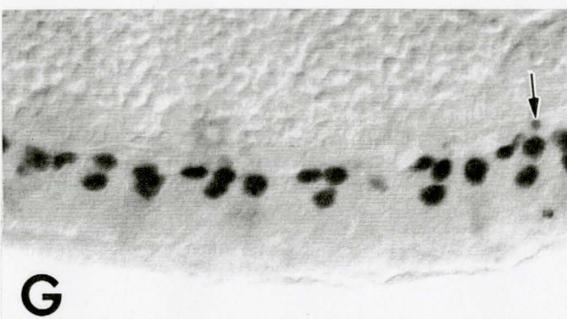
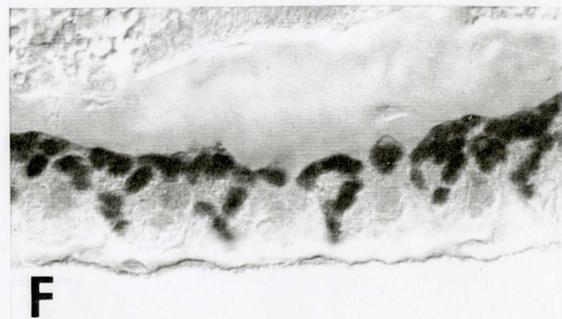
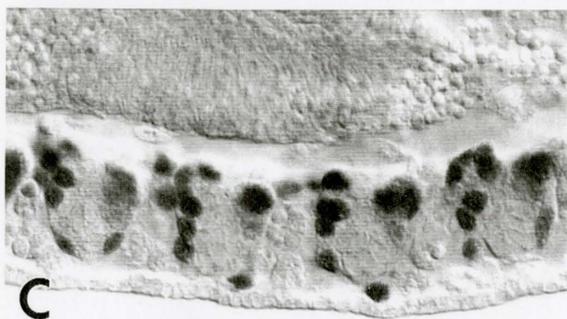
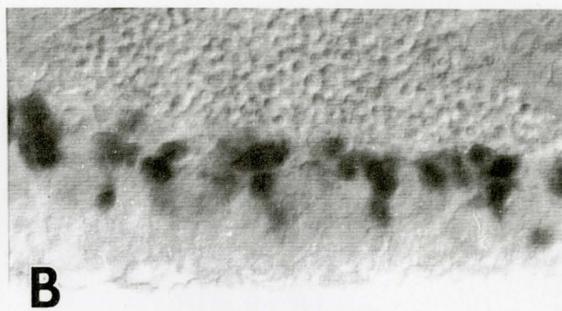
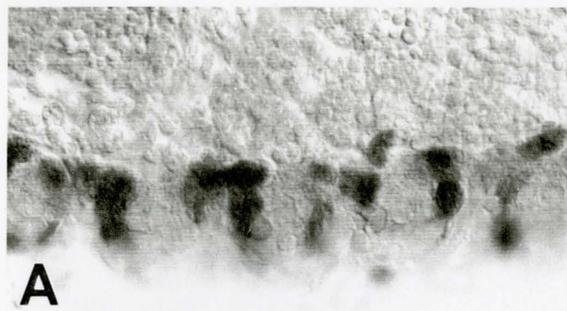


Figure 4. Early appearance of supernumerary MG in *Df(3L)H99* embryos. Embryos contain the *P[slit1.0/lacZ]* reporter construct expressing β -galactosidase in the MG, visualized with an antibody to β -gal. The left column 1 (panels A, C, E, G, I) represents wild type and the right column 2 (panels B, D, F, H, J) represents *Df(3L)H99* embryos. The expression of *P[slit1.0/lacZ]* begins at stage 12 and panels (A, B) are stage 12/4 embryos. At early stage 13, there are no obvious differences in the number and location of MG between wild type and *Df(3L)H99* embryos (C, D). The number of MG has decreased in wild type embryos in late stage 13 (E) and stage 15 (G) and dead cell profiles can also be seen (E, G arrow), while there is no reduction in cell numbers in *Df(3L)H99* embryos (F, H). At stage 17, most segments retain 3 MG in wild type embryos (I) and 12 MG in *Df(3L)H99* embryos (J). Views are sagittal with anterior to the left and dorsal at the top.



(12.3 ± 0.4 , $n=7$, Fig. 4J) in *Df(3L)H99* embryos. Unlike wild type embryos, there was no reduction observed in the number of MG labeled by *P[slit1.0/lacZ]* and no apoptotic profiles outside of the nervous system between early stage 13 to stage 17 in *Df(3L)H99* embryos. The above data show that *Df(3L)H99* embryos have an additional 4 cells at late stage 13 and 9 additional cells at stage 17 compared with wild type embryos. This suggests that cell death naturally occurs from late stage 12 up to stage 17.

The MGP, VUMs and MNBs can be labeled by the enhancer trap X55. At stage 13, it is difficult to distinguish MGP from neurons by their location and morphology (eg. size), but from stage 14, the MGP are located more dorsally and are larger than the more ventrally positioned neurons. Therefore, we can identify MGP from stage 14 to stage 17. At stage 13, there were 9.4 ± 0.2 cells expressing X55 in wild type embryos ($n=4$) while in *Df(3L)H99* embryos there were 11.1 ± 0.3 cells ($n=6$). Therefore *Df(3L)H99* had ~2 extra X55 labeled cells. By stage 14, there were 1.3 ± 0.1 ($n=6$) MGP labeled by X55 in the wild type embryos and 3.2 ± 0.2 cells ($n=7$) in *Df(3L)H99* embryos; ~2 extra MGP had been identified in *Df(3L)H99* embryos at this stage. There was no difference in the number of neurons between wild type (8.4 ± 0.2 , $n=6$) and *Df(3L)H99* embryos (8.5 ± 0.2 , $n=7$) at stage 14. Therefore, we infer the 2 extra cells found at stage 13 in *Df(3L)H99* embryos are MGP. By stage 17, no MGP ($n=5$) were seen in the wild type due to PCD while 3.3 ± 0.2 MGP ($n=5$) still exist in *Df(3L)H99* embryos. This result is consistent with the *P[slit1.0/lacZ]* data.

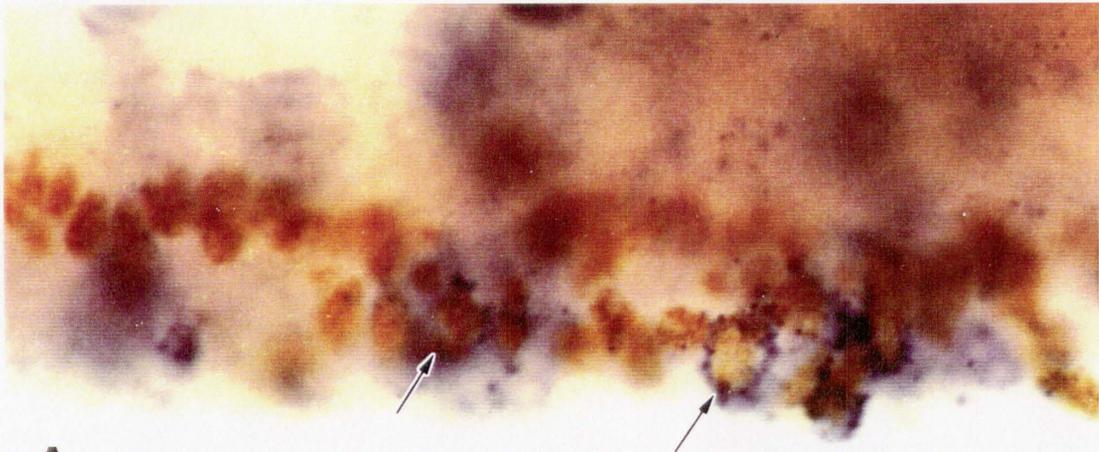
The number of neurons labeled by X55 at stage 15, stage 16 and stage 17 in wild type embryos were 8.4 ± 0.1 ($n=5$), 7.7 ± 0.2 ($n=8$) and 7.3 ± 0.2 ($n=5$) respectively; while there were

8.3±0.2 (n=6), 8.5±0.3 (n=5) and 8.5±0.2 (n=5) X55 labeled neurons in *Df(3L)H99* embryos at stage 15, stage 16 and stage 17 respectively. This indicates there is one neuron lost in wild type after stage 15. There were, however, no obvious differences between wild type and mutant, in terms of the number of VUM and MNB cells using the monoclonal antibody 22C10 and anti-engrailed. It is possible that the additional neuron labeled by X55 belongs to a mesectodermal neuronal lineage other than the VUM and MNB lineages, for instance, the UMI.

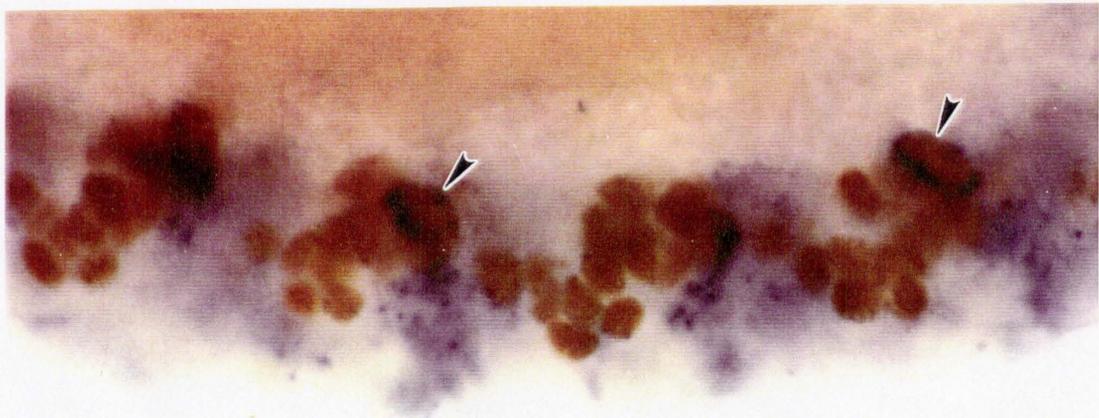
Expression of *reaper* in the mesectoderm

We assumed that extra cells persisting in *Df(3L)H99* embryos result from a block of cell death. Therefore, in wild type embryos, these cells should express cell death *reaper* gene and die (White et al., 1994). We used *in situ* hybridization with digoxigenin-labeled RNA probes to examine *reaper* expression during embryonic development. The earliest evidence of *reaper* expression in the midline cells of wild type embryos was at late stage 11 at the midline cells identified by *P[sim/lacZ]* (data not shown). By stage 12, the labeling became more dense and more cells were labeled ranging from 1-4 cells per segment (Fig. 5A). These *reaper* expressing cells die during stage 12 and 13. Since there were extra cells labeled by MG specific markers in late stage 13 *Df(3L)H99* embryos (see Fig. 4F), it is likely that these *reaper* expressing cells in wild type embryos are MG. To confirm the cell death occurred at stage 12 and 13 in the MGP, we observed *reaper* expression in X55 labeled cells. At late stage 12, 1 to 2 cells expressing *reaper* were observed (Fig. 5B). These *reaper* expressing

Figure 5. *reaper* mRNA expression in the mesectoderm. Wild type embryos carrying the *P[*sim/lacZ*]* reporter construct (A) were labeled for β -galactosidase (brown) and *reaper* mRNA (blue). At stage 12/3, 1-4 midline cells per segment were labeled with both *reaper* mRNA and the *P[*sim/lacZ*]* reporter construct (A, arrows). Embryos which express X55 enhancer trap (B) are double labeled for β -galactosidase (brown) and *reaper* mRNA (blue). At stage 12/0, 1-2 cells are double labeled per segment (B, arrowheads). These cells are larger and located dorsally at the posterior of each segment. These are saggital views with anterior to the left and dorsal to the top.



A



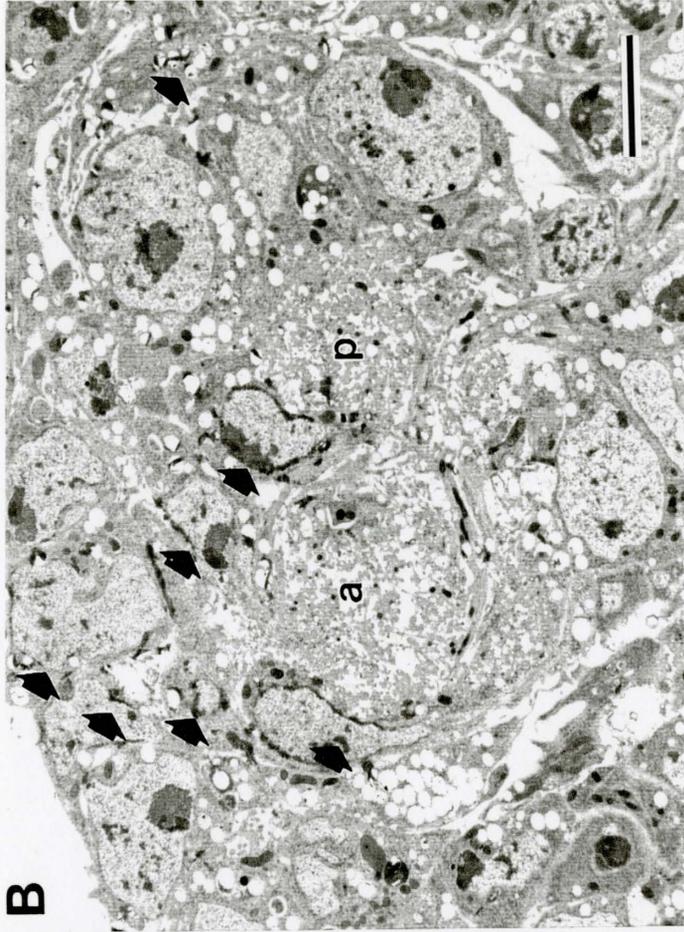
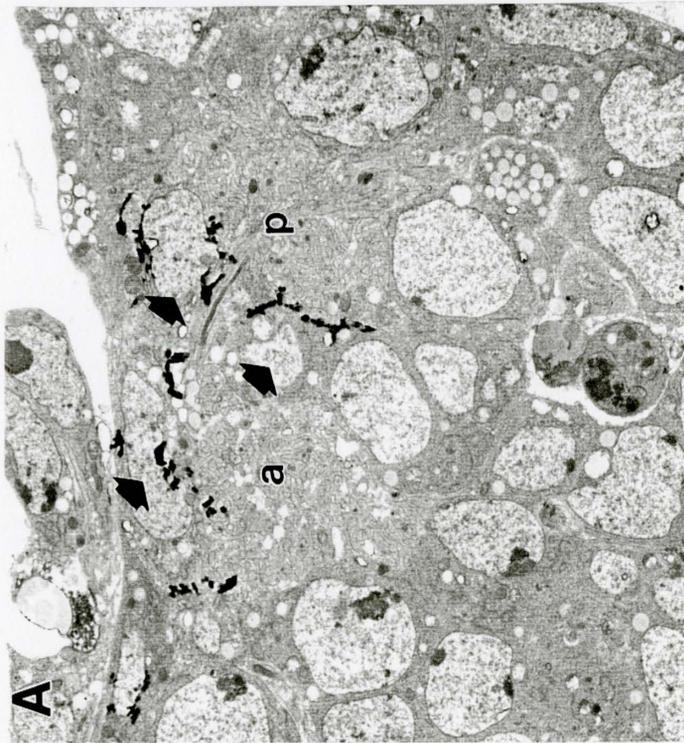
B

cells are larger and located dorsally at the posterior of each segment, which is characteristic of MGP. Furthermore, in *Df(3L)H99* embryos there were two extra MGP labeled by X55 during stage 14, suggesting that these *reaper* expressing cells in wild type X55 embryos are likely the MGP. These *reaper* expressing cells die during late stage 12 and 13. *reaper* expressing cells were also observed in X55 labeled cells at later stages (14, 15 and 16) and other MG markers (eg. *P[slit1.0/lacZ]*, AA142 labeled cells; data not shown). In wild type embryos, one neuron was double labeled by X55 and *reaper* mRNA at stage 15 and 16. This indicate approximately one neuron will die at corresponding stages during embryogenesis which is consisted with my X55 cell counts. No *reaper* mRNA hybridization was detected in *Df(3L)H99* embryos (data not shown).

Supernumerary midline cells in *Df(3L)H99* embryos are MG

In wild type embryos, MG ensheath the axon commissures (Jacobs and Goodman, 1989a). We have used electron microscopy to examine whether supernumerary MG maintain their function or differentiate like glia. Wild type and *Df(3L)H99* embryos carrying the enhancer trap AA142 were stained with blueo-gal, an electron dense substrate for β -galactosidase, in order to mark the MG. In stage 14 wild type embryos, 3-4 MG enwrap the commissures (Fig. 6A). In *Df(3L)H99* embryos of the same age, 4-5 MG extend ensheathing lamellipodia, yet several blueo-gal labeled MG are close to or in contact with axons of the commissures. The additional cells are also smaller. Blueo-gal labeling is darker in MG that actually ensheath axons (Fig. 6B). This indicate that the extra cells in *Df(3L)H99* embryos are

Figure 6. Supernumerary midline cells in *Df(3L)H99* contact the commissural tracts. Saggital views of the CNS at stage 14 are shown for a wildtype (A) and a *Df(3L)H99* embryo (B). Cells expressing the AA142 enhancer are labeled with electron dense substrate for β -galactosidase (bluo-gal; see arrowheads). Position of the anterior (a) and posterior (p) commissures are indicated. Three MG are identified in wildtype (A), while at least 7 AA142 expressing cells are seen in *Df(3L)H99* (B). Scale: 4.0 μ m.



MG.

An alternative hypothesis is that the extra MG found in *Df(3L)H99* embryos are neurons which misexpress glial genes, therefore, they might be labeled by both neuronal and glial markers. To assess whether extra MG are neurons expressing MG markers, wild type and *Df(3L)H99* embryos which contain AA142 were immunolabeled by anti- β -gal and anti-elav. If the extra MG have neuron identity, they should be double labeled by anti- β -gal and anti-elav. At stage 16, approximately three AA142 expressing cells (MG) were identified in wild type (Fig. 7A) but nine in *Df(3L)H99* embryos (Fig. 7B). Anti-elav which labels neurons were expressed in both wild type and *Df(3L)H99* embryos. In mutant embryos, the overall size of the central nervous system was increased (not shown) as well as the number of neurons labeled by anti-elav (Fig. 7B). Similar results have been reported by White and colleagues (1994). Interestingly, no co-expressing cells were observed in either wild type or *Df(3L)H99* embryos. Therefore, extra AA142 expressing midline cells do not have neuronal identity. As mentioned above, no decrease in the cell number of other midline lineages in *Df(3L)H99* embryos have been found, hence supernumerary MG are less likely derived from these lineages.

Absence of extra cell divisions in the mesectoderm of *Df(3L)H99* embryos

It is possible that an altered pattern of cell division in *Df(3L)H99* embryos give rise to supernumerary MG. BrdU labeling of replicating cell, and subsequent detection by immunofluorescence, was used to test whether supernumerary MG in *Df(3L)H99* embryos

Figure 7. Expression of *elav* and AA142 in the CNS midline of wildtype and *Df(3L)H99* embryos. Confocal images of AA142 expression (red) and *elav* immunolabeling (green) in the CNS identifies approximately three MG in wildtype (A) and nine AA142 expressing cells in *Df(3L)H99* embryo (B). No co-expressing cells in either embryo are seen.

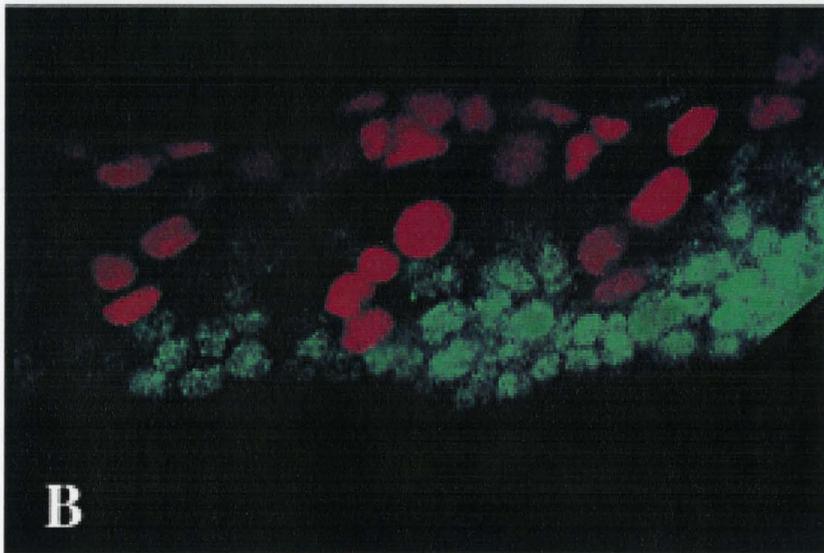
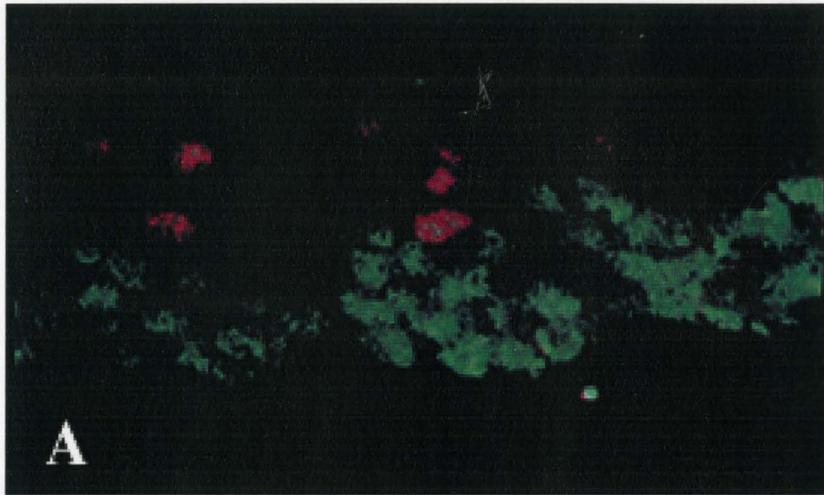
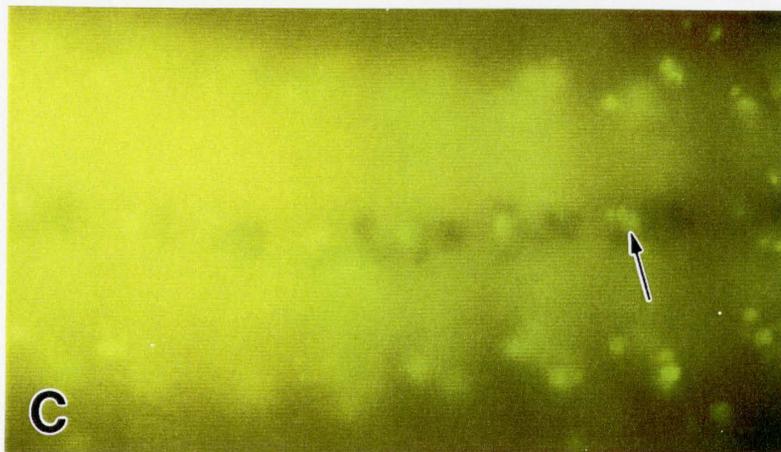
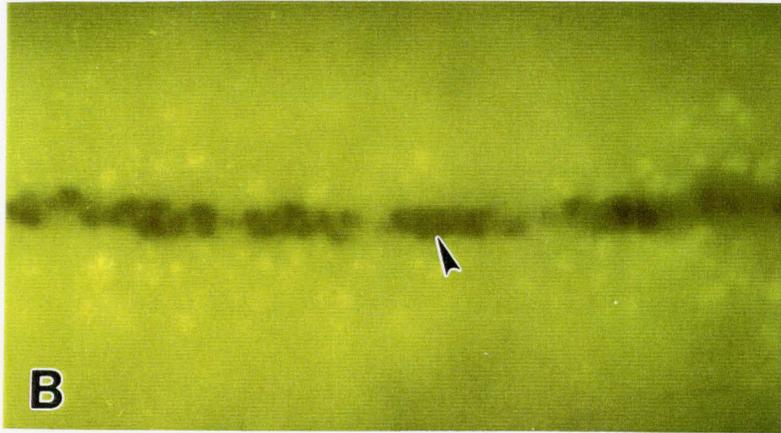


Figure 8. Immunofluorescence labeling of BrdU in wild type embryo mesectoderm. Embryos containing the *P[*sim*/*lacZ*]* reporter construct (A and B) and the *P[*slit1.0*/*lacZ*]* reporter construct (C) were labeled for β -galactosidase (dark brown) and anti-BrdU immunofluorescence labeling (green). At late stage 8 or early stage 9, all midline cells which are labeled by the *P[*sim*/*lacZ*]* reporter construct are also labeled with BrdU (A, arrow). By stage 10, there are no midline cells labeled for BrdU incorporation (B, arrowhead). At stage 13, there are 2 cells labeled with BrdU, which are not double labeled by MG marker *P[*slit1.0*/*lacZ*]* reporter construct (C, long arrow).



are from extra cell divisions. BrdU is a thymidine analogue which is incorporated into the DNA during S-phase of the cell cycle. At late stage 8 or early stage 9, midline cells labeled by the *P[sim/lacZ]* reporter construct are BrdU positive in wild type embryos (Fig. 8A). No BrdU labeling was detected in midline cells at stage 9 and 10 in wild type embryos (Fig. 8B). BrdU labeling in the midline was observed between stage 12 and 13, but these cells were not labeled by the MG marker *P[slit1.0/lacZ]* reporter construct (Fig. 8C). A similar pattern of cell division was also observed in *Df(3L)H99* embryos (data not shown). Therefore, it appears that supernumerary midline cells present at stage 12 and 13 do not arise from extra cell divisions and do not represent aberrant reporter expression in neurons. Because cell numbers are equivalent in wild type and *Df(3L)H99* embryos until the onset of PCD during stage 12, we can only conclude that there is a precursor population of 12 cells competent to express genes of the MG lineage.

Expression of *DER*, *pnt* and *argos* in *Df(3L)H99* embryos

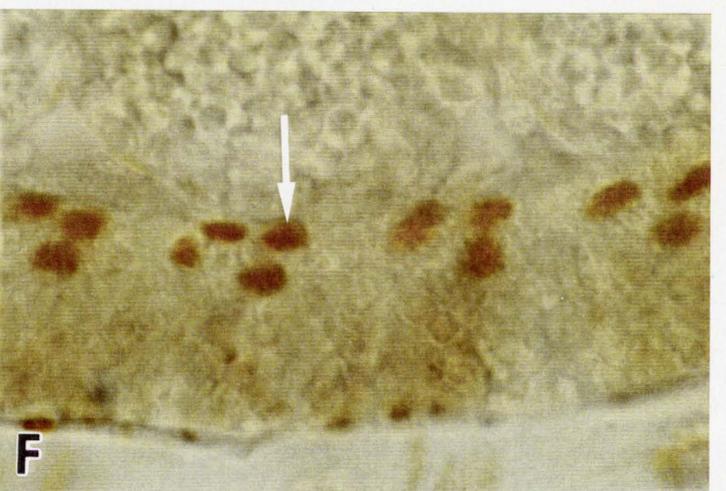
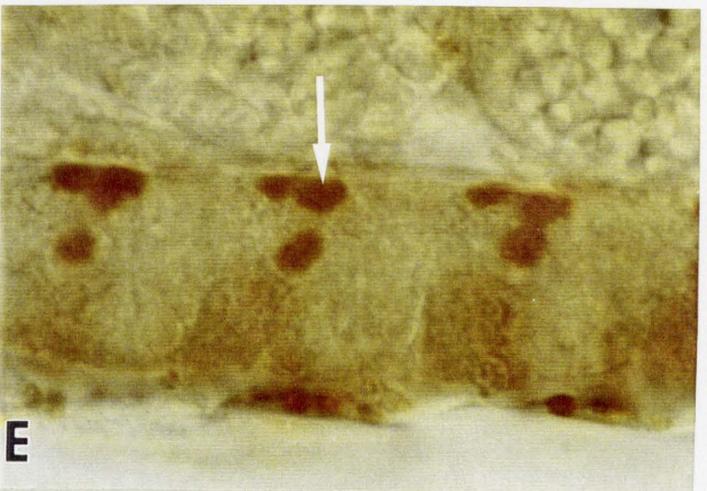
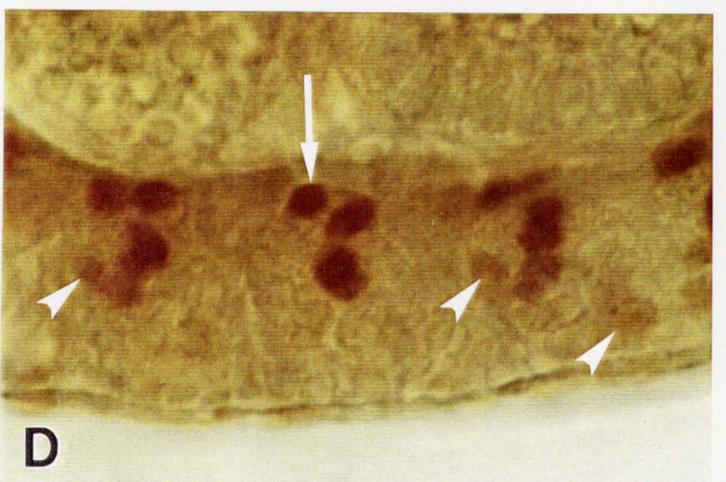
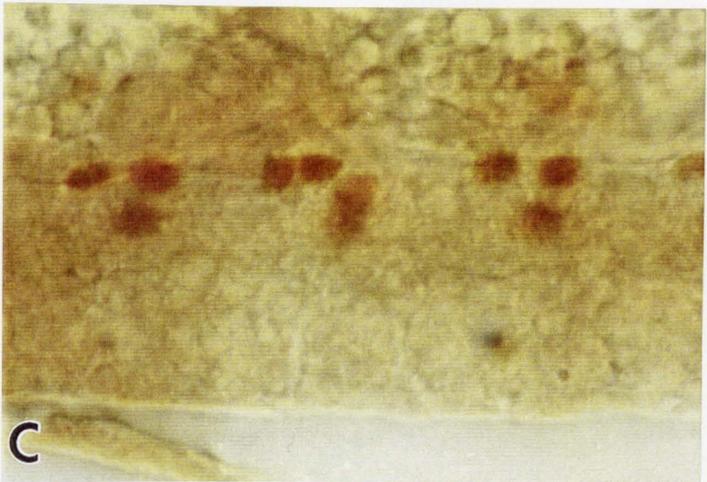
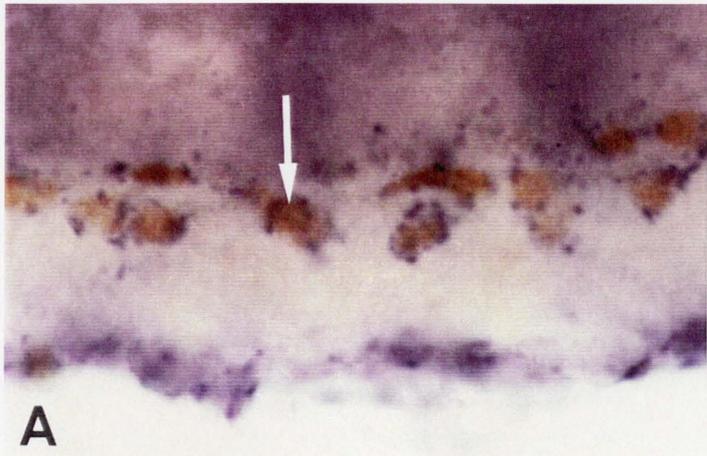
MG survival requires the function of *DER* and *pnt* (Sonnenfeld and Jacobs, 1994). The *pnt* gene encodes two putative transcription factors (P1 and P2) of the ETS family; the *pntP2* is expressed only in the midline glial cells (Klämmt, 1993). The *argos* gene encodes a secreted protein that regulates cell fate decisions (Freeman et al., 1992). The *pnt* and *argos* are downstream targets of *DER* and require *DER* signaling to be expressed (Brunner et al., 1994; Golembo et al., 1996). In wild type embryos, only the MG that survive express *argos* (Stemerink and Jacobs, submitted). In the absence of PCD, is this pathway required for

continued MG gene expression? We have observed *DER*, *pnt* and *argos* expression in *Df(3L)H99* embryos using enhancer traps and *in situ* hybridization. The onset of *DER* mRNA expression was at stage 12. In wild type stage 13 embryos, *DER* was expressed in the MG labeled by *P[slit1.0/lacZ]* and the expression was maintained in the surviving MG to later stages (Fig. 9A). In *Df(3L)H99* stage 13 embryos, the expression of *DER* was similar to wild type (not shown). In *Df(3L)H99* stage 15 and 16 embryos, *DER* was expressed in the supernumerary MG labeled by *P[slit1.0/lacZ]*. However, some of these cells labeled weakly for *DER* expression (Fig. 9B).

The onset of *pnt* gene expression labeled by the *pnt¹²⁷⁷/+* enhancer trap occurred in wild type embryos at stage 13 and by stage 14, it was evident that only a subset of the MG were labeled by *pnt* gene expression (3.0 ± 0.03 , $n=4$). The *pnt* expression level was maintained in these cells through stage 15 (2.9 ± 0.07 , $n=5$) and stage 16 (2.9 ± 0.04 , $n=8$, Fig. 9C). The results suggest that in wild type embryos, *pnt* is expressed in the MG that do not die during embryogenesis. As in wild type *pnt* expression began at stage 13 in *Df(3L)H99* embryos, and by stage 14 3.6 ± 0.2 MG were labeled by *pnt* at wild type level ($n=5$) and by stage 15 3.5 ± 0.3 MG were labeled at stage 15 ($n=3$). During stage 16, there were 3.3 ± 0.1 cells expressing *pnt* at wild type levels in *Df(3L)H99* embryos and up to 3 additional MG per segment were labeled less intensely ($n=8$, Fig. 9D). In *Df(3L)H99* embryos there is a slight increase in the number of MG expressing the *pnt* enhancer trap and some additional weakly labeled cells were also observed.

argos expression as labeled by the *argos^{W11}/+* enhancer trap began at stage 13. During

Figure 9. Expression of genes of the DER pathway in *Df(3L)H99* embryos. At left panels (A, C, E) represent wild type and at right panels (B, D, F) represent *Df(3L)H99* embryos. Embryos carrying the *P[slit1.0/lacZ]* reporter construct were double labeled for β -galactosidase (brown) and *DER* mRNA (blue) (A, B). At stage 15, *DER* is expressed in all the MG labeled by *P[slit1.0/lacZ]* in both wild type (A, arrow) and *Df(3L)H99* embryos (B, arrow). Embryos containing the enhancer trap *pnt*¹²⁷⁷/+, *pnt* expressing MG can be visualized with an antibody to β -gal (C, D). At stage 16, *pnt* is expressed in 3 MG in wild type embryos (C). In *Df(3L)H99* embryos, 3 MG express *pnt* at wild type levels (D, arrow), and up to 3 MG per segment label less intensely (D, arrowheads). Embryos containing the enhancer trap *argos*¹¹/+, *argos* expressing MG can be visualized with an antibody to β -gal (E, F). At stage 16, *argos* is expressed in 3-4 MG per segment in both wild type and *Df(3L)H99* embryos (E, F arrow).

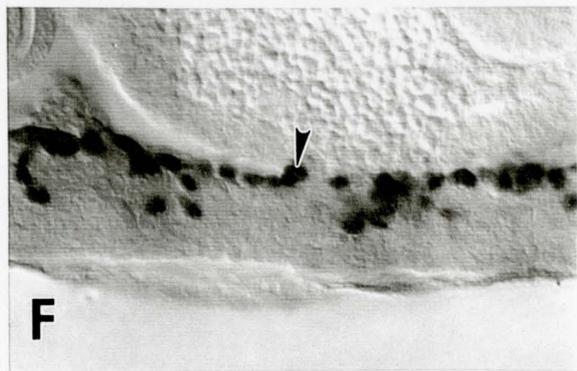
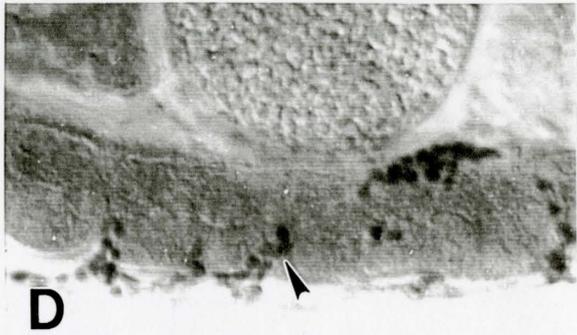
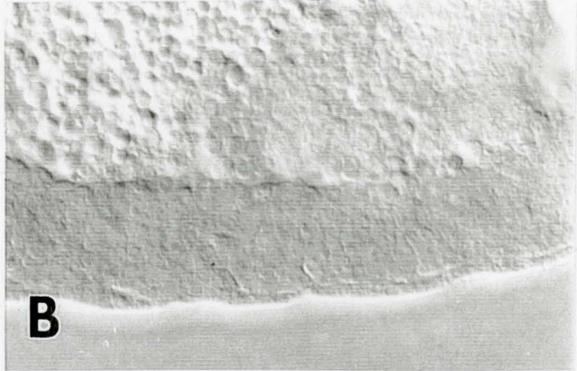


stage 14 3.3 ± 0.1 MG (n=8) and by stage 15 3.3 ± 0.1 MG (n=10) were expressing *argos* in wild type embryos. In stage 16 wild type embryos, 3.2 ± 0.1 MG (n=10) were expressing *argos* (Fig. 9E). Therefore, *argos* is also expressed in a subset of MG in wild type embryos. In *Df(3L)H99* embryos, *argos* was expressed in 3.3 ± 0.1 MG at stage 14 (n=7) and 3.4 ± 0.1 MG at stage 15 (n=8). By stage 16, 3.4 ± 0.1 MG were expressing *argos* in *Df(3L)H99* embryos (n=9; Fig. 9F). There was not a significant increase in the number of cells labeled by *argos* in *Df(3L)H99* embryos compared with wild type.

***Df(3L)H99* rescues some aspects of *spitz* group mutant phenotype**

The MG require *spitz* group gene function to differentiate or survive; otherwise they enter apoptosis (Sonnenfeld and Jacobs, 1994; Klämbt et al., 1991). In double mutants for the *spitz* group genes and *Df(3L)H99*, is some aspect of the *spitz* group phenotype rescued? In *sim^{B13.4}* mutant embryos, no P[*slit1.0/lacZ*] expressing cells were observed at stage 16 (Fig. 10A). In embryos mutant for *S^{IN23}* and *Df(2L)E55* (uncovering *spitz*), there were no P[*slit1.0/lacZ*] expressing cells by stage 16, and only apoptotic profiles were observed (Fig. 10C and 10E). Few surviving MG were detected in *pnt^{A88}* mutant embryos (Fig. 10G). Therefore, MG died in the embryos mutant for *sim^{B13.4}*, *S^{IN23}*, *Df(2L)E55* and *pnt^{A88}*. In stage 16 embryos double mutant for the *sim^{B13.4}* and *Df(3L)H99*, no P[*slit1.0/lacZ*] expressing cells were observed (Fig. 10B). In *S^{IN23}*, *Df(3L)H99* double mutant embryos, extra cells were labeled by P[*slit1.0/lacZ*] and these cells were smaller and displaced dorsally and ventrally (Fig. 10D) in comparison with *Df(3L)H99* embryos (see Fig. 2B). At stage 16, extra MG

Figure 10. The fate of MG in embryos mutant for the spitz group genes or double mutants for the spitz group genes and *Df(3L)H99*. All embryos are at stage 16 and contain the *P[slit1.0/lacZ]* reporter construct expressing β -galactosidase in the MG, visualized with an antibody to β -gal. The left column represents embryos mutant for genes in the spitz group and the right column represents spitz group-*Df(3L)H99* double mutant embryos. There are no *P[slit1.0/lacZ]* expressing cells in either *sim^{B13,4}* mutant (A) or *sim^{B13,4}, Df(3L)H99* double mutant embryos (B). In embryos mutant for *S^{HN23}* (C), *Df(2L)E55*, uncovering *spitz* (E) and *pnt^{A88}* (G), the MG undergo cell death; dead cell profiles are present and indicated with arrows. In *S^{HN23}, Df(3L)H99* double mutant embryos (D), extra MG are displaced dorsally and ventrally (arrowhead). In *Df(2L)E55, Df(3L)H99* double mutant embryos (F) and *pnt^{A88}, Df(3L)H99* double mutant embryos (H), extra MG are detected and some dorsal displacement of MG is evident (arrowhead).

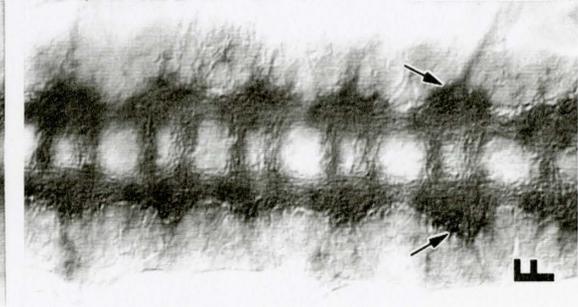
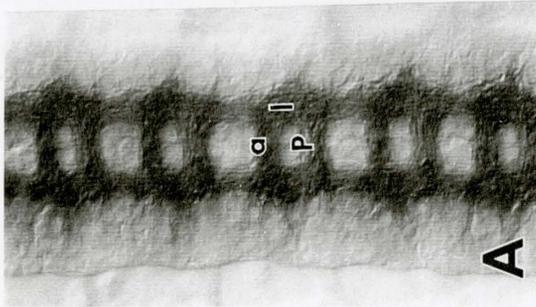
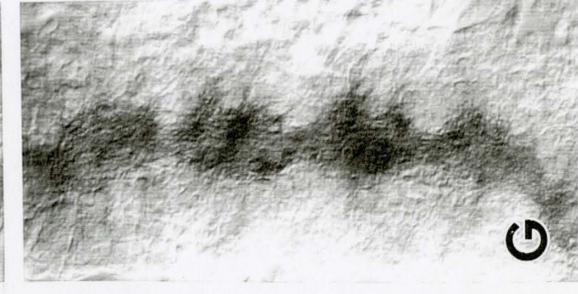
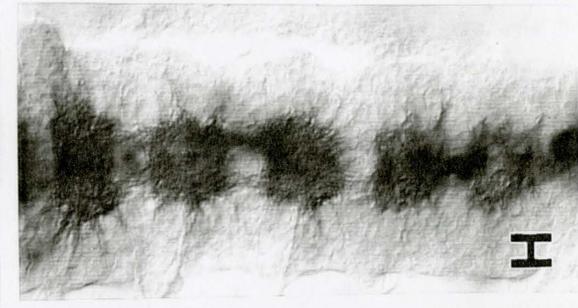
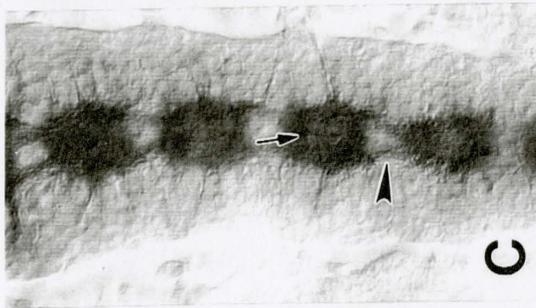
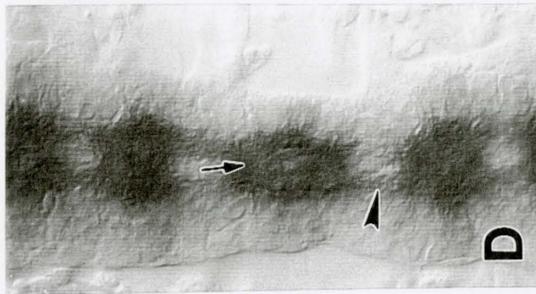
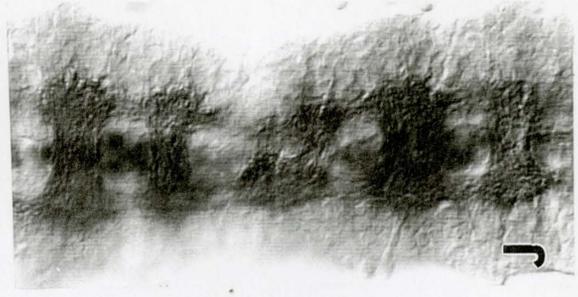
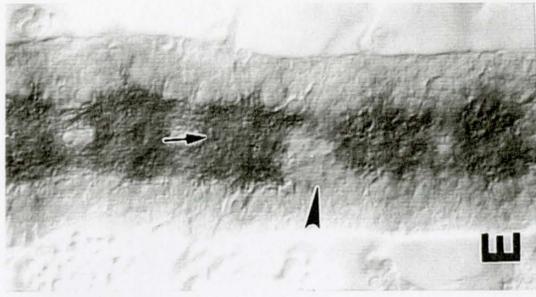


compared with wild type were observed in *Df(2L)E55*, *Df(3L)H99* double mutant embryos (Fig. 10F) and *pnt^{A88}*, *Df(3L)H99* double mutant embryos (Fig. 10H); some dorsal displacement of the MG was evident. Therefore, the MG cells which die in spitz group gene single mutants can survive in double mutant embryos for spitz group gene and *Df(3L)H99*.

In spitz group mutants the MG die. It has been suggested that lack of MG function in spitz group mutants results in fused commissures (Sonnenfeld and Jacobs, 1994; Klämbt et al., 1991). *Df(3L)H99* could suppress the MG phenotype of spitz group mutants. It would be interesting to know the effects of *Df(3L)H99* on the phenotype of axon tract morphology of spitz group gene mutants. At stage 16, the anterior and posterior commissures and the longitudinal axon tracts comprise a ladder like structure in wild type embryos (Fig. 11A). In *sim^{B13.4}* mutant embryos, the longitudinal axon tracts were collapsed at the midline into a single, fused tract (Fig. 11B). In embryos mutant for *S^{IN23}*, *Df(2L)E55* and *pnt^{A88}*, the CNS is narrower; the commissures are thicker and incompletely separated; the longitudinal connectives are thinner or missing in some segments (Fig. 11C, 11D and 11E respectively). A ladder-like arrangement of two commissures and longitudinal axon tracts was observed in *Df(3L)H99* embryos (Fig. 11F) comparable to wild type. In *Df(3L)H99* embryos, the junctions of the longitudinal and commissural bundles were thicker (Fig. 11F). In embryos double mutant for *sim^{B13.4}* and *Df(3L)H99*, there was no obvious changes in the collapsed longitudinal axon tracts (Fig. 11D) as compared with *sim^{B13.4}* mutant embryos (see Fig. 11C). In *S^{IN23}*, *Df(3L)H99* double mutant embryos (Fig. 11F), the CNS was a little wider than *S^{IN23}* mutant embryos (Fig. 11E). In *Df(2L)E55*, *Df(3L)H99* double mutant embryos (Fig. 11H) and

pnt^{Δ88}, *Df(3L)H99* double mutant embryos (Fig. 11J), the width of the CNS was closer to wild type. However, commissure separation remains incomplete, as in spitz group gene mutants. Therefore, spitz group genes are required for wild type axon tracts and MG morphology of the CNS although supernumerary MG in double mutant embryos can partially rescue the CNS width phenotype.

Figure 11. Axon tract morphology in embryos mutant for the spitz group genes or double mutant for the spitz group genes and *Df(3L)H99*. CNS axon tracts were stained with the monoclonal antibody BP102 and visualized with HRP-conjugated secondary antibody. Top row represents spitz group genes mutants and bottom one represents double mutants for spitz group genes and *Df(3L)H99*. Panels G-J are double labeled with BP102 and anti- β -galactosidase of P[*slit1.0/lacZ*] reporter construct. Frontal views of stage 16 embryos are with anterior to the top. The anterior (a) and posterior (p) commissures and the longitudinal axon tracts (l) comprise a ladder like structure in wild type embryo (A). In *Df(3L)H99* embryo (F), the junctions of the longitudinal and commissural bundles are thicker (arrows). In *sim^{B13,4}* mutant embryos (B), the longitudinal axon tracts collapse at the midline. In embryos mutant for *S^{MIN23}* (C), *Df(2L)E55* (D) and *pnt^{A88}* (E), the CNS is narrower; the commissures are thicker and incompletely separated (arrows). The longitudinal connectives are thinner or missing (C, D and E, arrowhead). In *Df(3L)H99* and spitz group double mutant embryos (G, H, I and J), the width of the CNS is close to wild type. However, commissure separation remains incomplete, as in spitz group gene mutants.



DISCUSSION

This study examined the role of PCD in the midline lineages of the embryonic *Drosophila* CNS. In addition, we investigated the origin and fate of supernumerary cells in *Drosophila* embryos deficient for apoptosis.

PCD in the mesectoderm of *Drosophila*

PCD occurs during development in the ventral midline of the CNS resulting in the elimination of extra cells (Sonnenfeld and Jacobs, 1995; Zhou et al., 1995; Oppenheim, 1991). In the *Drosophila* midline there are at least 4 lineages. It would be interesting to determine in which of these lineages PCD occurs. Mutants which can block PCD are useful tools for the study of this process (Wolff and Ready, 1991; Sonnenfeld and Jacobs, 1995; Zhou et al., 1995; Fischbach and Technau, 1984). In PCD deficient mutants, cells which normally are programmed to die survive. In these embryos, we can map cell death to certain lineages by examining cell number. These mutants offer an opportunity to study the features of those cells which normally die in wild type. In this way we can trace the origin and function of these cells. In addition, analysis of these mutants may provide insight into the mechanisms of cell death.

In this study we found that some cells labeled by MG specific markers are eliminated during embryogenesis in wild type embryos. In *Df(3L)H99* embryos, in which PCD has been blocked, there are extra midline cells (also see Sonnenfeld and Jacobs, 1995; Zhou et al.,

1995). These extra midline cells are labeled by MG specific markers (*P[slit1.0/lacZ]*, AA142) and located dorsally in the VNC. These cells demonstrate ultrastructural characteristics of the MG. There is little evidence suggesting PCD in other midline lineages. In the *Df(3L)H99* embryos, there are no detectable changes in the number of the MP1, VUM neurons or the MNBs labeled by P223, monoclonal antibody 22C10 and antibody to Engrailed, respectively. However, there is one more neuron labeled by X55 in these mutants. This study did not pursue the identity of this extra neuron. Therefore, the PCD occurs mainly in the MG lineage and this lineage is the focus of this study.

The results show that from stage 8 to 11 there is no reduction in the number of cells labeled by *P[sim/lacZ]* and no death cell profiles can be detected in wild type embryos. It is therefore unlikely that PCD occurs during this period. These results agree with published observations (Zhou et al., 1995). Our data also shows that at late stage 12 and early stage 13, there is one extra cell labeled by *P[slit1.0/lacZ]* in *Df(3L)H99* than in wild type embryos, suggesting that PCD occurs at this stage. *In situ* hybridization reveals that the onset of *rpr* mRNA expression in the MG is during late stage 11. An increase in the number of cells expressing *rpr* mRNA is observed at stage 12. This further indicates that PCD occurs at late stage 12 and early stage 13 since the delay between *rpr* mRNA expression and cell death morphology is about 1-2 hours (White et al., 1994). If this is true, we should expect to see dead cell profiles at late stage 12. However, death cell profiles were rarely seen at this stage. The reason for the failure to detect the death profiles may be that the cells die before MG lineage markers (like AA142) can be visualized by the methods employed here. According to

our results we propose that initial PCD in the mesectoderm occurs from late stage 11 to late stage 12.

In late stage 13 wild type embryos, approximately 7 MG cells are labeled by *P[slit1.0/lacZ]* and labeled dead cell profiles are obvious at this time. This and the above data indicate that in addition to the 7 surviving labeled MG cells, some MG cells have already died, bringing the total number of MG cells at late stage 13 to more than 7. By stage 17, approximately 3 MG survive in wild type embryos, while 12 MG survive in *Df(3L)H99* embryos. If supernumerary MG in *Df(3L)H99* embryos result from blocking PCD, it is estimated that approximately 9 MG die during embryogenesis in the wild type.

Source of supernumerary MG in *Df(3L)H99* embryos

The results demonstrate that from stage 12 to 14 in *Df(3L)H99* embryos, the number of MG increase from 10 to 12. It is of interest to determine the origin of these 2 cells.

The extra cells in *Df(3L)H99* embryos may come from the altered division of MG. Using BrdU to mark dividing cells, we do not see any MG cell division between stage 12 to 13. This result is consistent with previous reports (White et al., 1994; Zhou et al., 1995). Also, preliminary data from *HSrho* experiments in our laboratory indicate that there is no MG cell division at that period (Lanoue and Jacobs, unpublished data). Other reports demonstrate that in addition to the first postblastodermal division of the midline precursors, the second and third midline cell divisions occur during stage 11-13 and stage 14-16, respectively. The second and third cell divisions in the midline are restricted to the MNB lineage (Bossing and

Technau, 1994). Midline lineage analysis using enhancer traps also suggests that no further MG cell division occurs after the first postblastodermal division (Klambt et al., 1991). In brief, our results as well as those of others suggest that the extra MG are unlikely to come from MG divisions during stage 12-14.

The extra cells in *Df(3L)H99* embryos may come from other midline lineages or lineages outside the midline. Our results suggest that extra midline glia cells do not come from midline neurons since the number of midline neurons labeled by different neuronal markers does not decrease. Previous reports have shown that differentiated or undifferentiated cells can migrate to their functional location (Jacobs et al., 1989; Jacobs and Goodman, 1989b). The extra MG may come from undifferentiated cells or other cell lineages outside of the midline; which migrate to the midline and take up the MG fate. In this case, we would expect embryos double mutant for *sim* and *Df(3L)H99* would still express MG *slit* gene. Since *sim*, which controls the expression of midline genes (e.g. *slit*) is expressed only in the progenitors of the MEC (Nambu et al., 1991; Crews et al., 1988), *slit* expression in any cells migrating from outside of the midline should not depend on *sim* expression. We did not see any cells labeled with *P[slit1.0/lacZ]* in embryos for mutant for *sim* or double mutant for *sim* and *Df(3L)H99*. Hence, this possibility is unlikely.

Another possibility is that the cells derived from MEC already reside on the midline but do not express MG specific markers. In wild-type embryos, these cells are induced to die before they turn on MG specific markers and therefore can't be detected. In *Df(3L)H99* embryos, we found 10 cells labeled by *P[slit1.0/lacZ]* at stage 12. If PCD is blocked, this cell

number would remain constant at later stages of embryogenesis. According to our observations, the number of MG at stage 14 is 12, which is greater than we expected. We suggest that there is a cell pool of approximately 12 midline glia cells at stage 12; among these 12 cells, 10 of them are already expressing MG markers at this stage, while the other 2 cells begin MG gene expression during stage 13 and 14 respectively. Other preliminary data from our laboratory indirectly supports this notion. When flies carrying the *rhomboid* transgene driven by the *hsp70* promoter were heat shocked at stage 9, an increase in the number of MG cells was observed at stage 12 (Sonnenfeld and Jacobs, 1994; Dong pers. obs.). This indicates that the MG cells have a latent ability to enlarge their cell pool. Hence, a MG cell pool with 12 cells is possible in *Df(3L)H99* embryos if these additional cells do not die during embryogenesis. We hypothesize that there is a progenitor population of cells which are competent to become MG and express MG markers; some of the MG progenitor cells die before they ever differentiate into MG and express specific markers. In embryos deficient for apoptosis, these cells survive and generate supernumerary midline glia cells.

Midline glia progenitor pool

In late stage 12 and early stage 13 wild type embryos, we observe 9 MG cells labeled by *P[slit1.0/lacZ]*, while Zhou et al., (1995) found 8 MG cells labeled by *P[slit1.0/lacZ]*. In stage 17 *Df(3L)H99* embryos, the numbers of MG reported from different laboratories are not identical. In this study, we find 12 MG cells labeled by *P[slit1.0/lacZ]*. Previous studies report 9-10 MG cells (Sonnenfeld and Jacobs, 1995), or 8 MG cells labeled by the same

marker (Zhou et al., 1995). The reasons for the different results from these studies are unknown. They may be partially due to methodology e.g. the time of cell counting, and/or different antibody sensitivity.

Our results indicate that there is a MG cell pool of approximately 12 cells in the MEC at stage 12. Up to now, there are two major models for the generation of MEC lineages.

In the first model, based on enhancer traps studies, it is proposed that eight midline progenitors give rise to 4 midline lineages (Klämbt et al., 1991). From anterior to posterior, the progenitors are: the three midline glia progenitors, the MP1, 1-3 VUM progenitors and the MNB progenitor. The glia progenitors and the MP1 progenitor were reported to divide once to give rise to three pairs of midline glia (MGA, MGM and MGP) and two MP1 neurons, respectively. The fifth midline precursor gives rise to the most anterior VUM neurons. The other VUM neurons probably arise from the sixth and seventh midline precursors. The most posterior progenitor divides more than once to give rise to the MNB and its support cells (Fig. 12A; Klämbt et al., 1991). By stage 12, there are 6 MG cells per segment in wild type embryos.

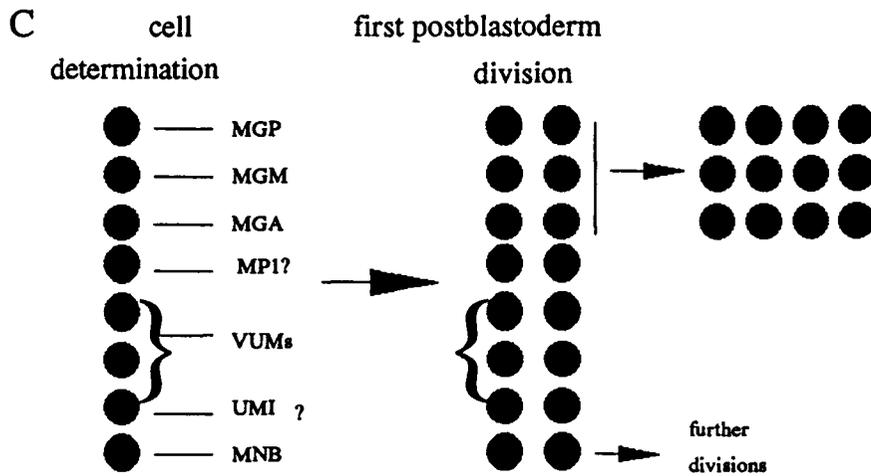
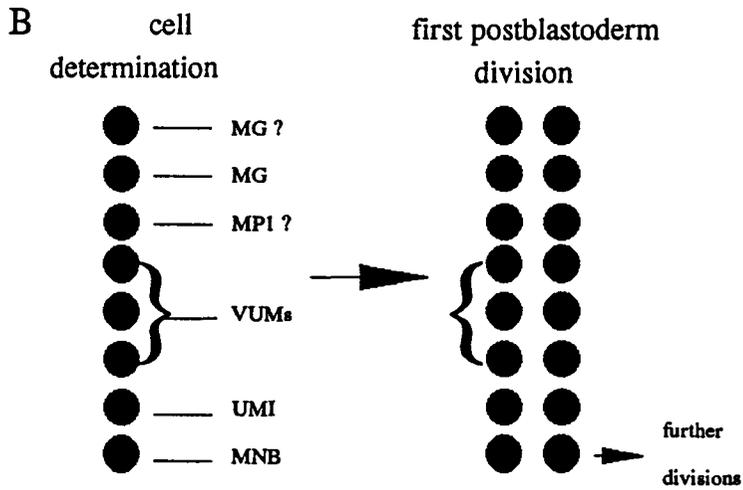
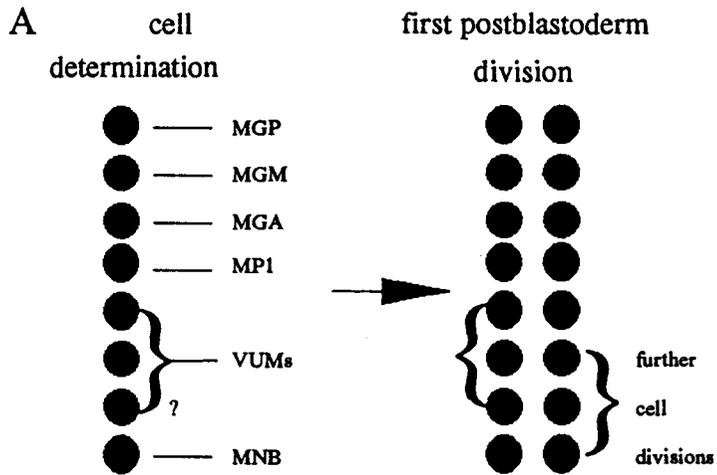
The second model was generated after the analysis of different midline clones whose progenitors have been labeled by DiI in vivo (Bossing and Technau, 1994). This model is shown in Figure 12B. Midline progenitors of thoracic segment were selected for this analysis. The labeled progenitors produce five different types of clones including the VUM, UMI, MP1, MG and MNB. In a few cases (3.5%), the mixed composition of clones were detected. These don't seem to be a random mixture of clonal types, since only three out of ten possible

combinations of different classes of clones were observed. In theory, the ratios of the frequency of the different clonal types should correspond to the number of progenitors giving rise to each particular type for each segment. According to this hypothesis, 7 midline progenitors give rise to the five different types of midline clones described above (Bossing and Technau, 1994). There are three progenitors per segment for the VUM clones (~40%) and one progenitor (~14%) for each of the other four clonal types (MP1, MG, UMI, and MNB). UMI (Unpaired Median Interneuron) has not previously been described in *Drosophila*. Surprisingly, there is only one MG progenitor implied from the above results and this MG progenitor produces a MG clone of 2 cells which are considered, according to their position, to be the MGM. The segmental position of the MEC progenitor is not a strong predictor of the identity of the progeny. Instead, the frequency with which different consecutive progenitors produce a certain type of clone is distributed in a periodic manner along the length of the segment. The distribution of these peak frequencies for the different clonal types allows the deduction of a likely sequence of progenitors from anterior to posterior as following: the MG progenitors would be followed by the VUM progenitors and then by the UMI and the MNB progenitors. The MNB progenitor appears to form the posterior segment border. As the relative position of the MP1 is not clear, we place it posterior to the MG progenitor in concurrence with earlier studies (see Fig. 12B). Since the numbers of midline progenitors per segment and the position of the segmental borders are variable, a progenitor labeled at a certain distance from the border may not give rise to a fixed type of midline clone. A high variability would not be surprising. Their assumption that there

is only one midline progenitor per segment for the midline glia lineage is in conflict with previous reports (Jacobs and Goodman 1989a; Klämbt et al., 1991). The authors then examined the number of MG using the reporter construct $P[*sim/lacZ*]$ labeling. The average number of labeled MG per thoracic segment is 3 to 4, corresponding to 2 MG progenitors. This result is in conflict with that of DiI labeling. It is possible that the presumed second glial progenitor resides apart from the midline and therefore has not been labeled in their experiments. Alternatively, cell death may have led to a reduced number of midline glia and might partially account for the DiI labeling (27%) which did not yield labeled progenies in the late embryos.

When mutant embryos in which PCD had been blocked are examined, the results indicated that a pool of 12 MG cells exist in the MEC at stage 12. It has been commonly accepted that 7-8 midline progenitors generate 4-5 midline lineages (Klämbt et al., 1991; Bossing and Technau, 1994). Mechanisms regarding the origin of these 12 cells from progenitor(s) should be considered. Six midline progenitors could divide once giving rise to the 12 MG seen in *Df(3L)H99* embryos, leaving only 2 other progenitors for the generation of other 4 midline lineages. This is improbable, since 1 progenitor is unlikely to give rise to 2 different midline lineages (Klämbt et al., 1991; Bossing and Technau, 1994). Although a neuroectodermal precursor previously described as NB1-1 gives rise to both neurons and glia in the abdominal CNS, no common precursors for glia lineage(s) and/or neuronal lineage(s) have been identified in the mesectoderm (Udolph et al., 1993; Bossing and Technau, 1994). According to the model proposed by Bossing and Technau (1994), all MG derive from 1 or

Figure 12. Schematic models of mesectoderm lineages. A. The Klämbt et al. (1991) model. This is a frontal representation with anterior at top. Eight midline precursors are determined to give rise to 4 lineages. From anterior to posterior the anteriormost three precursors give rise to MGP, MGM and MGA. The fourth gives rise to MP1 neurons. These anterior four midline precursors divide only once and the posterior four midline precursors divide more than once. The posteriormost precursor gives rise to the MNB. The others (two to three precursors) give rise to VUM neurons (modified from Klämbt et al., 1991). **B. The Bossing and Technau (1994) model.** There are seven midline precursors giving rise to 5 lineages. The anteriormost precursor is MG followed by three VUM and one UMI. The posteriormost progenitor is MNB. We assume MP1 following MG since the position of MP1 is not clear. All these precursors divide only once except MNB has further divisions (Drawn with data from Bossing and Technau, 1994). **C. The model proposed here.** The assumed relative position of the eight midline precursors are: three midline precursors giving rise to MGP, MGM, and MGA, one MP1 precursor, two VUM precursors, one UMI and one MNB. MG precursors divide twice to give rise to 12 MG cells. Except MNB the other precursors divide once giving rise to corresponding lineages (Drawn with data from this study).



2 progenitors at stage 9. In this situation, the progenitor divides 3-4 times to generate 12 MG. At stage 9, we observe only the first progenitor cell division and no others until stage 12. Our results did not support this hypothesis, since it is unlikely that we miss all the second, third and fourth divisions. Alternatively, 3 midline progenitors could divide twice yielding 12 MG. Although, we rarely see secondary division at stage 9, it is possible that the second division is too fast to be detected. This model is more rational (see Fig. 12C). The techniques of DiI in vivo labeling of the midline cells in embryos deficient for apoptosis may be very important to resolve this question. Using this method, early cell divisions of midline progenitors and lineage alterations in the midline can be detected. Since there is no cell death, all the MG progenitor(s) can be traced to their mature clones.

Function of supernumerary MG in mutant for *Df(3L)H99* and double mutants for spitz group genes and *Df(3L)H99*

In wild type embryos, some MG die by PCD while others survive. The surviving MG ensheath the commissures and express the MG genes *pnt* and *argos* (Jacobs and Goodman, 1989a; Stemerink and Jacobs, submitted). The axon tracts of *Df(3L)H99* embryos are similar to those seen in wild type embryos even though there are extra midline glia cells in the mutants. The supernumerary MG are close to or in contact with the commissural axons. Three to four of these cells express MG genes such as *pnt* and *argos* and are labeled heavily for AA142 enhancer trap expression. These cells extend lamellipodia to ensheath the commissures. The other MG cells are smaller and show lower levels of AA142 labeling.

These weakly labeled MG are closely associated with the commissures but do not actually ensheath them. Therefore, supernumerary MG in *Df(3L)H99* embryos consist of two different types. One group is composed of functionally mature MG which express *pnt* and *argos* like the MG in wild type. The other group is functionally immature and do not express *pnt* and *argos* genes. The functionally immature MG cells do not have counterparts in wild type due to apoptosis.

The MG play an important role in the formation of commissure tracts (Klämbt et al., 1991). *spitz* group gene mutants show fused commissures and a decreased distance between the two longitudinal axon tracts due in part to MG displacement and death (Klämbt et al., 1991; Sonnenfeld and Jacobs, 1994; Klämbt, 1993). In embryos double mutant for *spitz* group genes and *Df(3L)H99*, supernumerary MG cells survive. These supernumerary MG cells can not rescue the axon tract phenotype of *spitz* group gene mutants. For example, in double mutants for *pnt* and *Df(3L)H99* or *spitz [Df(2L)E55]* and *Df(3L)H99*, the supernumerary MG cells are located dorsally, similar to the *Df(3L)H99* mutant. The space between two longitudinal axon tracts is wider and closer to wild type, but the commissures remain fused, similar to those seen in *spitz* group mutants. In double mutants for *S* and *Df(3L)H99*, the supernumerary MG cells are displaced ventrally and dorsally. The axon tract phenotypes do not show obvious changes in comparison to the *S* single mutant. This indicates that *spitz* group function is not required to establish commissures but is required to separate and ensheath them, and the *spitz* group genes are required to produce 'mature' MG.

DER pathway

This study demonstrates that in stage 13 and 16 wild type embryos, the number of MG labeled by *P[slit1.0/lacZ]* are 7 and 3 respectively, whereas only 3 MG cells express *pnt* or *argos* at either stage. It is known that *pnt* and *argos* expression are critical for MG survival and only expressed in surviving MG cells in wild type embryos (Stemerdink and Jacobs, submitted). It is of considerable interest to determine the pathway by which *pnt* and *argos* regulate MG survival. Published data show that mutants of *DER*, *spitz* and *pnt* have similar phenotypes, implying the existence of a common pathway (Mayer and Nüsslein-Volhard, 1988; Raz and Shilo, 1992; Klämbt, 1993; Raz and Shilo, 1993). Secreted Spitz can trigger DER and its downstream signaling pathway which includes Ras/Raf/MAPK (Schweitzer et al., 1995b). The product of the *pnt* P2 transcript is a target of MAPK in the Sevenless signal transduction pathway in the developing eye of *Drosophila* and thus may act at the end of the pathway (Brunner et al., 1994; O'Neill et al., 1994). There is evidence suggesting that the DER signaling cascade can also induce *argos* transcription, which is then able to form an inhibitory feedback loop in the embryonic ventral ectoderm (Golembo et al., 1996; Schweiter et al., 1995a). Thus, Spitz and Argos proteins act as ligands to modulate DER activation. It has been proposed that during the development of the MG, the DER signaling pathway activates *pnt* expression and the latter triggers *argos* expression. Secreted Argos forms an inhibitory feedback pathway which inhibits the DER signaling cascade in surrounding cells but does not down regulate signaling in the Argos secreting cells (Stemerdink and Jacobs, submitted). Thus it is proposed that Argos contributes to the establishment of MG fate by

eliminating those MG cells that are not induced to produce it.

Our results from embryos deficient for apoptosis showed that there are 12 MG cells labeled by $P[slit1.0/lacZ]$ at stage 16, while there are only 3.3 cells expressing *pnt* at wild type level and another 3-4 cells expressing *pnt* weakly. We also found that 3.48 MG express *argos* in *Df(3L)H99* embryos. According to their location, these cells correspond to those labeling strongly for *pnt*. All MG in the midline were double labeled by *DER* and *slit* in stage 13 *Df(3L)H99* embryos; while at stage 15 the *DER* signal became very weak in some of the MG. This decrease in *DER* mRNA may be due to the reception of *Argos* signaling which restricts the duration and level of *DER* signaling via an inhibitory feedback loop (Stemerink and Jacobs, submitted). This mechanism has been demonstrated in the embryonic ventral ectoderm (Golembo et al., 1996). In this experiment, we were unable to make any quantitative analysis, since the signals coming from the *DER* mRNA *in situ* hybridization were weak and it was difficult to tell the difference between a weak signal and a negative signal. In *Df(3L)H99* embryos, some of the supernumerary MG cells can express the *pnt* enhancer weakly but do not express the *argos* enhancer. This suggests that there is a threshold level of Pnt protein required for *argos* expression. Briefly, in embryos deficient for apoptosis, the *DER* signaling cascade is able to activate *pnt* and *argos* which are important for MG survival. *Argos* may regulate, at least in part, the survival of the MG through an inhibitory pathway. Expression of MG specific genes (eg. *slit*) does not require *DER* signaling. Instead, the *DER* pathway appears to function to specify which and how many MEC progenitors do not enter apoptosis.

In summary, the results of this study show that there are up to ~12 MG in *Df(3L)H99* embryos and indicate that the additional MG cells in *Df(3L)H99* mutants come from MG progenitors. This raises new questions. How many progenitors give rise to these supernumerary MG cells? How many cell divisions do these progenitors undergo to produce their progenies? Using DiI in vivo labeling the midline cells of *Df(3L)H99* embryos might resolve these questions in the near future.

REFERENCE

- Abbott, M. K. and Lengyel, J. A.** (1991). Embryonic head involution and rotation of male terminalia require the *Drosophila* locus *head involution defective*. *Genetics* 129,783-789.
- Abrams, J.M., White, K., Fessler, L.I. and Steller, H.** (1993). Programmed cell death during *Drosophila* embryogenesis. *Dev.* 117,29-43.
- Arends, M. J. and Wyllie, A. H.** (1991). Apoptosis: Mechanisms and role in pathology. *Int. Rev. Exp. Pathol.* 32,223-254.
- Bate, M., Goodman, C. S. and Spitzer, N. C.** (1981). Embryonic development of identified neurons: segment-specific differences in the H cell homologues. *J. Neurosci.* 1,103-106.
- Bellen, H. J., O'Kane, C. J., Wilson, C., Grossniklaus, U., Pearson, R. K. and Gehring, W. J.** (1989). P-element-mediated enhancer detection: a versatile method to study development in *Drosophila*. *Genes & Dev.* 3,1288-1300.
- Bier, E., Vaessin, H., Shepherd, S., Lee, K., McCall, K., Barbel, S., Ackerman, L., Carretto, R., Uemura, T., Grell, E., Jan, L. Y. and Jan, Y. N.** (1989). Searching for pattern and mutation in the *Drosophila* genome with a P-*lacZ* vector. *Genes & Dev.* 3,1273-1287.
- Bodmer, R., Carretto, R. and Jan, Y. N.** (1989). Neurogenesis of the peripheral nervous system in *Drosophila* embryos: DNA replication patterns and cell lineages. *Neuron* 3,21-32.
- Bossing, T. and Technau, G.M.** (1994). The fate of the CNS midline progenitors in *Drosophila* as revealed by a new method for single cell labelling. *Dev.* 120,1895-1960.

Brunner, D., Ducker, K., Oellers, N., Hafen, E., Scholz, H. and Klambt, C. (1994). The ETS domain protein Pointed-P2 is a target of MAP kinase in the Sevenless signal transduction pathway. *Nature* 370,386-389.

Bump, N. J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferenz, C., Franklin, S., Ghayur, T., Li, P., Licari, P., Mankovich, J., Shi, L., Greenberg, A. H., Miller, L. K. and Wong, W. W. (1995). Inhibition of ICE family proteases by Baculovirus antiapoptotic protein p35. *Science* 269,1885-1888.

Campos-Ortega, J. and Hartenstein, V. (1985). The embryonic development of *Drosophila melanogaster*. 227.

Chen, P., Nordstrom, W., Gish, B. and Abrams, J. M. (1996). *grim*, a novel cell death gene in *Drosophila*. *Genes & Dev.* 10,1773-1782.

Cowan, W. M., Fawcett, J., O'leary, D. and Stanfield, B. B. (1984). Regressive events in neurogenesis. *Science* 225,1258-1265.

Crews, S. T., Thomas, J. B. and Goodman, C. S. (1988). The *Drosophila* single-minded gene encodes a nuclear protein with sequence similarity to the per gene product. *Cell* 52,143-151.

DeFazio, A., Leary, J. A., Hedley, D. W. and Tattersall, M. H. N. (1987). Immunohistochemical detection of proliferating cells in vivo. *J. Histochem. Cytochem.* 35,571-577.

Ellis, R. E. and Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode *Caenorhabditis elegans*. *Cell* 44,817-829.

- Ellis, R. E., Yuan, J. and Horvitz, H. R. (1991).** Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.* 7,663-698.
- Enari, M., Hug, H. and Nagata, S. (1995).** Involvement of an ICE-like protease in Fas-mediated apoptosis. *Nature* 375,78-81.
- Evan, G. I., Brown, L., Whyte, M. and Harrington, E. (1995).** Apoptosis and the cell cycle. *Curr. Opin. Cell Biol.* 7,825-834.
- Fischbach, K. F. and Technau, G. (1984).** Cell degeneration in the developing optic lobes of the sine oculis and small-optic-lobes mutants of *Drosophila melanogaster*. *Dev. Biol.* 104,219-239.
- Freeman, M., Klambt, C., Goodman, C. and Rubin, G. (1992).** The *argos* gene encodes a diffusible factor that regulates cell fate decisions in the *Drosophila* eye. *Cell* 69,963-75.
- Fujita, S. C., Zipursky, S. L., Benzer, S., Ferrus, A. and Shotwell, S. L. (1982).** Monoclonal antibodies against the *Drosophila* nervous system. *Proc. Natl. Acad. Sci. USA* 79,7929-7933.
- Fujisawa, T. and David, C. N. (1984).** Loss of differentiating nematocytes induced by regeneration and wound healing in *Hydra*. *J. Cell Sci.* 68,243-255.
- Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. (1992).** Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* 119,493-501.
- Gehring, W. J. (1989).** P-element-mediated enhancer detection: a versatile method to study development in *Drosophila*. *Genes & Dev.* 3,1288-1300.
- Golembo, M., Schweitzer, R., freeman, M. and Shilo, B.-Z. (1996).** *argos* transcription

is induced by the *Drosophila* EGF receptor pathway to form an inhibitory feedback loop. *Dev.* 122,223-230.

Golstein, P., Marguet, D. and Depraetere, V. (1995). Homology between Reaper and the cell death domains of Fas and TNFR1. *Cell* 81,185-186.

Goodman, C. S., Bastiani, M. J., Doe, C. Q., du Lac, S., Helfand, S. L., Kuwada, J. Y. and Gratzner, H. G. (1982). Monoclonal antibodies to 5'-bromo and 5'-iododeoxyuridine: a new reagent for detection of DNA replication. *Science* 218,474-475.

Grether, M., Abrams, J. M., Agapite, J., White, K. and Steller, H. (1995). The *head involution defective* gene of *Drosophila melanogaster* and its role in programmed cell death. *Genes & Dev.* 9,1694-1708.

Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in *Drosophila*. *Dev.* 120,2121-2129.

Hengartner, M. O., Ellis, R. E. and Horvitz, H. R. (1992). *C. elegans* gene *ced-9* protects cells from programmed cell death. *Nature* 356,494-499.

Hengartner, M. O. and Horvitz, H. R. (1994a). Programmed cell death in *Caenorhabditis elegans*. *Curr. Opin. Genet. Dev.* 4,581-586.

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

Hengartner, M. O. (1996). Programmed cell death in invertebrates. *Curr. Opin. Genet. Dev.* 6, 34-38.

Hinchliffe, J. R. and Thorogood, R. V. (1974). Genetic inhibition of mesenchymal cell

death and the development of form and skeletal pattern in the limbs of *talpid*³ mutant chick embryos. *J. Embryol. Exp. Morphol.* 31,747-760.

Jacobs, J. R., Hiromi, Y., Patel, N. and Goodman, C. S. (1989). Lineage, migration and morphogenesis of the longitudinal glia in the *Drosophila* CNS as revealed by a molecular lineage marker. *Neuron* 2,1625-1631.

Jacobs, J. R. and Goodman, C. S. (1989a). Embryonic development of axon pathways in the *Drosophila* CNS: I. A glia scaffold appears before the first growth cones. *J. Neurosci.* 9,2404-2411.

Jacobs, J. R. and Goodman, C. S. (1989b). Embryonic development of axon pathways in the *Drosophila* CNS II. Behaviour of pioneer growth cones. *J. Neurosci.* 9,2412-2422.

Jones, B. W., Fetter, R. D., Tear, G. and Goodman, C. S. (1995). *glial cells missing*: a genetic switch that controls glial versus neuronal fate. *Cell* 82,1013-1023.

Klämbt, C., Jacobs, J. R. and Goodman, C. S. (1991). The midline of the *Drosophila* central nervous system: a model for the genetic analysis of cell fate, cell migration and growth cone guidance. *Cell* 64,801-815.

Klämbt, C. (1993). The *Drosophila* gene *pointed* encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development* 117,163-176.

Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G. and Earnshaw, W. C. (1994). Cleavage of poly(ADP-ribose)polymerase by a proteinase with properties like ICE. *Nature* 371,346-347.

Lindsley, D. L. and Zimm, G. G. (1992). The genome of *Drosophila melanogaster*. San

Diego: Academic Press, Inc.

Los, M., Van de Graen, M., Penning, L. C., Schenk, H., Westendorp, M., Baeuerle, P. A., Proge, W., Krammer, P. H., Fiers, W. and Schulze-Osthoff, K. (1995). Requirement of an ICE-CED-3 protease for Fas/APO-1-mediated apoptosis. *Nature* 375,81-83

Mackay, W. J. and Bewley, G. C. (1989). The genetics of catalase in *Drosophila melanogaster*: isolation and characterization of acatalasemic mutants. *Genetics*, 122,643-652.

Martin, D. P., Schmidt, R. E., DiStefano, P. S., Lowry, O. H., Carter, J. G. and Johnson, E. M. (1988). Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.* 106,829-844.

Mayer, U. and Nüsslein-Volhard, C. (1988). A group of genes required for pattern formation in the ventral ectoderm of the *Drosophila* embryo. *Genes Dev.* 2,1496-1511.

Nambu, J. R., Franks, R. G., Hu, S. and Crews, S. T. (1990). The *single-minded* gene of *Drosophila* is required for the expression of genes important for the development of CNS midline cells. *Cell* 63,63-75.

Nambu, J. R., Lewis, J. O., Wharton, JR. K. A. and Crews, S. T. (1991). The *Drosophila single-minded* gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell* 67,1157-1167.

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y.A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T., Yu, V. L. and Miller, D. K. (1995). Identification and

- inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376,37-43.
- Nüsslein-Volhard, C., Wieachaus, E. and Kluding, H. (1984).** Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster* I. Zygotic loci on the second chromosome. *Roux's Arch. Dev. Boiol.* 193,267-282.
- O'Kane, C. and Gehring, W. J. (1987).** Detection in situ of genomic regulatory elements in *Drosophila*. *Proc. Natl. Acad. Sci. USA.* 84,9123-9127.
- O'Neill, E.M., Rebay, I., Tijan, R. and Rubin, G.M. (1994).** The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. *Cell* 78,137-147.
- Oppenheim, R. W., Prevette, D., Tytell, M. and Homma, S. (1990).** Naturally occurring and induced neuronal death in the chick embryo in vivo requires protein and RNA synthesis: evidence for the role of cell death genes. *Dev. Biol.* 138,104-113.
- Oppenheim, R. W. (1991).** Cell death during development of the nervous system. *Annu. Rev. Neurosci.* 14,453-501.
- Patel, N. H., Snow, P. M. and Goodman, C. S. (1987).** Characterization and cloning of Fasciclin III: A glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. *Cell* 48,975-988.
- Pronk, G. J., Ramer, K., Amiri, P. and Williams, L. T. (1996).** Requirement of an ice-like protease for induction of apoptosis and ceramide generation by reaper. *Science* 271,808-810.
- Rabizadeh, S., LaCount, D. J., Friesen, P. D. and Bredesen, D. E. (1993).** Expression of the baculovirus P35 gene inhibits mammalian neural cell death. *J. Neurochem.* 61,2318-2321.

- Raff, M. C.** (1992). Social controls on cell survival and cell death. *Nature* 356,397-400.
- Raz, E. and Shilo, B.-Z.** (1992). Dissection of the faint little ball (*flb*) phenotype: determination of the development of the *Drosophila* central nervous system by early interactions in the ectoderm. *Development* 114,113-23.
- Raz, E. and Shilo, B.-Z.** (1993). Establishment of ventral cell fates in the *Drosophila* embryonic ectoderm requires DER, the EGF receptor homolog. *Genes & Dev.* 7,1937-1948.
- Robinow, S., Campos, A. R., Yao, K. M. and White, K.** (1988). The *elav* gene product of *Drosophila*, required in neurons, has three RNP consensus motifs. *Science* 242,1570-1572.
- Rubin, G. M. and Spradling, A. C.** (1982). genetic transformation of *Drosophila* with transposable element vectors. *Science* 218,348-353.
- Scholz, H., Deatrick, J., Klea, A. and Klämbt, C.** (1993). Genetic dissection of *pointed*, a *Drosophila* gene encoding two ETS-related proteins. *Genetics* 135,455-468.
- Schweitzer, R., Howes, R., Smith, R., Shilo, B.-Z. and Freeman, M.** (1995a). Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos. *Nature* 376,699-702.
- Schweitzer, R., Shaharabany, M., Seger, R. and Shilo, B.Z.** (1995b). Secreted spitz triggers the DER signaling pathway and is a limiting component in embryonic ventral ectoderm determination. *Genes & Dev.* 9,1518-1529.
- Shankland, M.** (1984). Positional determination of supernumerary blast cell death in the leech embryo. *Nature* 307,541-543.
- Sonnenfeld, M. J. and Jacobs, J. R.** (1994). Mesectodermal cell fate analysis in *Drosophila* midline mutants. *Mech. Dev.* 46,3-13.

- Sonnenfeld, M. and Jacobs, J. (1995).** Apoptosis of the midline glia during *Drosophila* embryogenesis: a correlation with axon contact. *Development* 121,569-578.
- Steller, H. and Grether, M. E. (1994).** Programmed cell death in *Drosophila*. *Neuron* 13,1269-1274.
- Sugimoto, A., Friesen, P. D. and Rothman, J. H. (1994).** Baculovirus P35 prevents developmentally regulated cell death and rescues a *ced-9* mutant in the nematode *Caenorhabditis elegans*. *EMBO J.* 13,2023-2028.
- Tata, J. R. (1966).** Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. *Dev. Biol.* 13,77-94.
- Thomas, J. B. (1984).** Cell recognition during neuronal development. *Science* 225,1271-1279.
- Thomas, J. B., Bastiani, M. J., Bate, M. and Goodman, C. S. (1984).** From grasshopper to *Drosophila*: a common plan for neuronal development. *Nature* 310,203-207.
- Thomas, J. B., Crews, S. T. and Goodman, C. S. (1988).** Molecular genetics of the single-minded locus: A gene involved in the development of the *Drosophila* nervous system. *Cell* 52,133-141.
- Truman, J. W. (1984).** Cell death in invertebrate nervous systems. *Annu. Rev. Neurosci.* 7,171-188.
- Udolph, G., Prokop, A., Bossing, T. and Technau, G. M. (1993).** A common precursor for glia and neurons in the embryonic CNS of *Drosophila* gives rise to segment-specific lineage variants. *Dev.* 118,765-775.

- Vaux, D. L., Weissman, I. L. and Kim, S. K. (1992).** Prevention of programmed cell death in *Caenorhabditis elegans* by human *bcl-2*. *Science* 258,1955-1957.
- Wharton, K. A. J. and Crews, S. T. (1993).** CNS midline enhancers of the *Drosophila* slit and Toll genes. *Mech. of Dev.* 40,141-154.
- White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K. and Steller, H. (1994).** Genetic control of programmed cell death in *Drosophila*. *Science* 264,677-683.
- White, K., Tahaoglu, E. and Steller, H. (1996).** Cell killing by the *Drosophila* gene *reaper*. *Science* 271,805-807.
- Wolff, T. and Ready, D. F. (1991).** Cell death in normal and rough eye mutants of *Drosophila*. *Dev.* 113,825-839.
- Wright, T. R. F., Hodgetts, R. B. and Sberald, A. F. (1976).** The genetics of dopa decarboxylase in *Drosophila melanogaster*: isolation and characterization of deficiencies that delete the dopa-decarboxylase-dosage-sensitive region and the α -methyl-dopa-hypersensitive locus. *Genetics* 84,267-285.
- Wyllie, A. H., Kerr, J. F. R. and Currie, A. R. (1980).** Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68,251-306.
- Yuan, J., Shaham, S., Ledoux, S., Ellis, R. E. and Horvitz, H. R. (1993).** The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 β converting enzyme. *Cell* 75,641-652.
- Zhou, L., Hashimi, H., Schwartz, L. M. and Nambu, J. R. (1995).** Programmed cell death in the *Drosophila* central nervous system midline. *Curr. Biol.* 5,784-790.

APPENDIX

Table: Cell Numbers of Different Midline Lineages in wild type and *Df(3L)H99* embryos

Strain	stage 8	stage 11	stage 12	stage 13	stage 14	stage 15	stage 16	stage 17
P[<i>sim/lacZ</i>]/+/+	13.6±0.3(8)	15.3±0.5(6)	19.4±0.5(10)	19.7±0.8(6)				
<i>H99</i>	13.8±0.4(5)	16.0±0.6(5)	20.1±0.5(8)	22.6±0.7(6)				
P[<i>sli/lacZ</i>]/+/+			9.3±0.3(9)#	7.1±0.3(8)	5.1±0.1(6)	3.7±0.1(6)	3.23±0.1(6)	3.1±0.1(6)
<i>H99</i>			10.2±0.3(6)#	11.1±0.3(6)	12.1±0.3(8)	12.1±0.3(8)	12.0±0.3(8)	12.3±0.4(7)
AA142 +/+				5.6±0.2(7)	4.6±0.1(7)	3.5±0.1(6)	3.3±0.1(7)	3.1±0.1(5)
<i>H99</i>				10.4±0.4(7)	10.9±0.4(7)	11.5±0.2(9)	11.3±0.4(8)	11.2±0.3(8)
X55 +/+					1.3±0.1(6)	0.7±0.1(7)	0.07±0.04(8)	0.0±0.0(5)
(MGP) <i>H99</i>					3.2±0.2(7)	3.5±0.2(6)	3.5±0.2(5)	3.3±0.2(5)
X55 +/+			10.3±0.3(5)*	9.4±0.2(4)*	8.4±0.2(6)	8.4±0.1(5)	7.7±0.2(8)	7.3±0.2(5)
(neurons) <i>H99</i>			10.6±0.4(3)*	11.1±0.3(6)*	8.5±0.2(7)	8.3±0.2(6)	8.5±0.3(5)	8.5±0.2(5)
<i>pnt</i> ¹²⁷⁷ /+ +/+					3.0±0.03(4)	2.9±0.07(5)	2.9±0.04(8)	
<i>H99</i>					3.6±0.2(5)	3.5±0.3(3)	3.3±0.1(8)	
<i>argos</i> ^{w11} /+ +/+					3.3±0.1(8)	3.3±0.1(10)	3.2±0.1(10)	
<i>H99</i>					3.3±0.1(7)	3.45±0.1(8)	3.48±0.1(9)	
P223 +/+				2.0±0.06(5)	2.0±0.1(5)	2.0±0.1(4)	2.0±0.03(7)	2.01±0.03(3)
<i>H99</i>				2.03±0.05(6)	2.0±0.03(5)	1.9±0.1(7)	2.0±0.04(4)	2.0±0.02(4)
22C10 +/+							4.0±0.1(8)	
<i>H99</i>							4.1±0.1(9)	
engrailed +/+							5.8±0.1(8)	
<i>H99</i>							5.8±0.2(8)	

#: total number of P[*sli/lacZ*] expressing cells at stage 12/0 and early stage 13.

*: total number of cells labeled by X55 (MGP and neurons).

The results are represented by mean±S.E. (n=number of embryos).

+/+ represents wild type and *H99* represents *Df(3L)H99* embryos.