

**ARGOS AND SPITZ GROUP/DER PATHWAY FUNCTION IN THE MG**

**ARGOS AND THE SPITZ GROUP/DER PATHWAY FUNCTION TO  
REGULATE MIDLINE GLIAL CELL NUMBERS IN *DROSOPHILA* EMBRYOS**

By

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## ABSTRACT

The midline glia (MG) perform an active role in the pioneering and morphogenesis of the commissural axons of the central nervous system (CNS) during *Drosophila* embryogenesis. Following the establishment of these commissural axon pathways, a subset of the MG undergoes apoptosis and the surviving MG remain to ensheath the commissures. Previous studies demonstrated that the pattern of this developmental apoptosis is stochastic. The surviving MG exhibit intersegment variability in their final number and position relative to the commissures. Interestingly, *argos*, a gene which encodes a diffusible extracellular factor with an epidermal growth factor (EGF) motif, is only expressed in MG which survive. *argos* expression in this subset of MG is initiated at the onset of apoptosis, reflecting the temporal pattern of cell death of the other MG. In *argos* loss-of-function mutants, there are extra surviving MG in each segment while ectopic over-expression of *argos* results in increased apoptosis among the MG. Therefore, Argos has a negative regulatory effect on MG survival. Its effects are opposite to the *spitz* group/*Drosophila* EGF receptor (DER) pathway, a cassette of genes required for MG survival. However, *argos* is hypostatic to the *spitz* group/DER pathway function and its expression requires a certain threshold of *spitz* group and DER pathway activity. Argos is postulated to act as an EGF receptor antagonist. It attenuates signaling of the DER pathway. Therefore, the regulation of MG survival is mediated by a balance



of extracellular inductive and inhibitory signals. How can this signaling pathway be reconciled with the stochastic pattern of MG survival and the observation that only *argos* expressing MG survive? We propose a model in which the adherens junction along with possible accessory proteins like Rhomboid and Star mediate the close apposition of MG and promote intense Spitz mediated DER pathway signaling. The MG with the most concentrated coupling of this adhesion and signaling pathway network achieve sufficient DER signaling to express *argos*. These MG are in turn fated to assume a final differentiated identity and will remain to ensheath the commissures. Argos is secreted and it mediates the apoptosis of MG with reduced levels of adhesion contacts and lower DER signaling (MG not expressing *argos*). The other mesectodermal cell (MEC) lineages appear to develop independently of *argos* function.

The repercussions of HS*argos* elicited MG loss to the commissural axons and CNS cytoarchitecture was also examined. Removal of the MG through ectopic expression of *argos* results in a loss of ensheathment and the commissural axon tracts are exposed to the haemolymph. Furthermore, the commissural axons are wider and they misexpress Fasciclin II, a phenotype reminiscent of mutations in *roundabout*.

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## **CONTRIBUTIONS**

Dr. J.R. Jacobs prepared and processed tissue for electron microscopy. All electron microscopy was performed by Dr. J.R. Jacobs. Figure 4 was generated by Dr. J.R. Jacobs.

## INTRODUCTION

The development of the central nervous system (CNS) requires a complex sequence of coordinated molecular and cellular events. During CNS development, two basic but monumental tasks are performed: glial and neuronal cell fates are established and growth cones must locate many of these established glial and neuronal cells to form axon tracts, and subsequent synaptic connections. The midline of the CNS is a dynamic model system, offering the developmental neurobiologist an abundance of regulatory mechanisms (for review, see Colamarino and Tessier-Lavigne, 1995; Goodman and Shatz, 1993; Tear *et al.*, 1993). From invertebrates to vertebrates, the CNS midline is strategically located, imparting bilateral symmetry to the nervous system. Therefore, it presents a natural boundary and serves as an organisation centre for the two sides. CNS neurons actively cross or avoid the midline, in response to signals emanating from it. The midline glia (MG) of the *Drosophila* embryonic CNS are an important group of cells within the midline which are thought to communicate midline positional signals (Harris *et al.*, 1996; Tear *et al.*, 1996). They provide a strong model system for the genetic analysis of cell-cell signaling, cell differentiation and survival, and growth cone guidance (Klämbt *et al.*, 1991).

Classical genetic and molecular genetic analysis has elucidated a cell signaling pathway which is involved in the regulation of differentiation and survival of the MG

(Klämbt *et al.*, 1991; Raz and Shilo, 1992; Klämbt *et al.*, 1993; Sonnenfeld and Jacobs, 1994). This mechanism is implemented in several contexts throughout fly development (Bier *et al.*, 1990; Rutledge *et al.*, 1992; Sturtevant *et al.*, 1993; Kolodkin *et al.*, 1994; Schweitzer *et al.*, 1995a,b; Golembo *et al.*, 1996a,b; Freeman, 1996). This is the *spitz* group of genes and they participate in a signaling pathway involving DER, the *Drosophila* epidermal growth factor (EGF) receptor homolog. Mutations in various components of this pathway lead to perturbations in the MG lineage and in turn affect the formation of CNS axon commissures (Klämbt *et al.*, 1991; Raz and Shilo, 1992; Sonnenfeld and Jacobs, 1994). Therefore, separate from serving as a tool in elucidating a general cell signaling mechanism, these mutations help dissect the cellular interactions involved in axon tract morphogenesis.

This introduction will focus briefly on the origins of the MG. It will then comment on MG functions and discuss the circumstances of their continued development in which the *spitz* group/*DER* pathway serves a role. This review will then introduce the recently characterised gene *argos* which encodes a DER antagonist (Freeman *et al.*, 1992a; Schweitzer *et al.*, 1995b). The potential for modulation of DER signaling by Argos will be discussed, providing the context for such a mechanism in the development of the MG.

The advantages of the MG system is that the MG lineage is well documented (Jacobs and Goodman, 1989; Klämbt *et al.*, 1991; Sonnenfeld and Jacobs, 1994; Sonnenfeld and Jacobs, 1995a). A number of MG specific markers exist including enhancer traps (Klämbt *et al.*, 1991; Freeman *et al.*, 1992a), reporter fusion constructs



(Wharton and Crews, 1993) and *in vivo* dye labeling (Bossing and Technau, 1994). Furthermore, through exploiting the powerful genetic tools offered by *Drosophila*, a battery of mutants were isolated (Nüsslein-Volhard *et al.*, 1984; Jürgens *et al.*, 1984) which were later found to perturb the MG lineage (Nambu *et al.*, 1990; Klämbt *et al.*, 1991; Raz and Shilo, 1992, Klämbt *et al.*, 1993; Sonnenfeld and Jacobs, 1994). Further mutations specific to the MG were also isolated (Rothberg *et al.*, 1990; Seeger *et al.*, 1993; Anderson *et al.*, 1995; Harris *et al.*, 1996).

## 1.0 ORIGIN OF THE MG

There exists developmental homology between the fly midline and the vertebrate floor plate cells (Tessier-Lavigne *et al.*, 1988). For example, homologs of the vertebrate Netrins (Kennedy *et al.*, 1994) which are diffusible chemotropic factors serving as guidance factors for commissural axons, have been isolated in *Drosophila* (Harris *et al.*, 1996). The D-Netrins will be reviewed below.

The MG is a discrete lineage which originates from a morphologically distinct group of cells termed the mesectodermal cells (MEC). The MEC occupy the ventral midline of the embryonic CNS (Crews *et al.*, 1988; Thomas *et al.*, 1988; Klämbt *et al.*, 1991). The MEC precursors undergo numerous mitotic divisions to generate a specialised population of glial and neuronal cells (Klämbt *et al.*, 1991; Bossing and Technau, 1994; Dong and Jacobs, in prep.). One of the sublineages within the MEC is the 12 MG (Dong and Jacobs, in prep.). This is opposed to the hypothesis that 6 MG are determined from the MEC as proposed by Klämbt *et al.*, (1991). There are 3 types based

on their positions relative to the commissures in the mature CNS. They are the anterior, middle, and posterior MG, termed MGA, MGM, and MGP respectively (Jacobs and Goodman, 1989). The other sublineages include two midline precursor 1 (MP1) neurons, six ventral unpaired median neurons (VUMs) and a median neuroblast (MNB) with its support cells. Bossing and Technau (1994) also described two unpaired median interneurons (UM1), putatively derived from the MEC. Using DiI, a lipophilic fluorescent tracer to label cells and their progeny *in vivo*, Bossing and Technau (1994) demonstrated that there was intersegment variability in the number of the various midline precursors. Furthermore, it was possible for a particular midline progenitor to give rise to any of the MEC sublineage types. Hence, determination solely dependent on cell position as proposed by Klämbt *et al.*, (1991) was believed unlikely. Specific cell-cell interactions between the midline cells in time and space might also lead to the establishment of unique cell fates.

The genetic mechanisms governing the determination of the midline sublineages are largely unknown. However, the earliest indication of determination is the expression of *single-minded (sim)* in the midline (Nambu *et al.*, 1990). Mutations in the *sim* locus result in a loss of the midline precursor cells (Thomas *et al.*, 1988). It is not clear whether these cells assume the fate of other unidentified cell types (Nambu *et al.*, 1990). The *sim* gene encodes a nuclear protein with putative DNA binding and protein dimerization domains in common with the basic-helix-loop-helix (bHLH) family of transcription factors (Crew *et al.*, 1988; Nambu *et al.*, 1991). Indeed, it is believed *sim* is a master regulator of midline determination and as a transcription activator, it interacts

directly with promoter elements of CNS midline genes (Nambu *et al.*, 1991; Franks and Crews, 1994).

## **1.1 INTERACTIONS BETWEEN MIDLINE CELLS AND PIONEERING COMMISSURAL AXON TRACTS – EARLY TRANSIENT MG FUNCTIONS**

Many of the MG are transient cells which function to establish commissural axon tract position and morphology (Klämbt *et al.*, 1991; Sonnenfeld and Jacobs, 1995a). The presence of early MG at the target regions of pioneering commissural growth cones is demonstrative of a highly stereotyped scaffold (Jacobs and Goodman, 1989). It was proposed that the MG present active guidance cues to the first growth cones of the CNS during stage 12. Indeed, prior to commissural axon tract establishment, up to 12 MG express a number of genes which encode structural proteins thought to provide directional cues, like Slit, Commissureless, and D-Netrins (Dong and Jacobs, in prep.; Rothberg *et al.*, 1990; Tear *et al.*, 1996; Harris *et al.*, 1996).

The *slit* gene encodes an extracellular matrix protein which contains two structural motifs traditionally associated with protein-protein interactions (Rothberg *et al.*, 1990). They are a leucine-rich repeat (LRR) and a series of epidermal growth factor (EGF)-like repeats. Slit is expressed and secreted by the MG and it becomes localised to the commissural axons. Mutant analysis suggests that *slit* is not required for MG differentiation and survival but is essential for proper commissure formation and overall CNS assembly (Sonnenfeld and Jacobs, 1994). However, Slit is not required for

commissural axon fasciculation but is likely involved in mediating communication between the commissures and its ECM environment (Rothberg *et al.*, 1990).

*Drosophila netrins* (*A* and *B*) encode chemotropic guidance signals produced by the MG (Harris *et al.*, 1996). In embryos deficient for Netrin function, phenotypes ranging from lack of commissure formation to partial commissures are exhibited. Ectopic expression of *D-netrins* result in phenotypic perturbations similar to the loss-of-function phenotype. Hence, it was proposed that a localised Netrin source is essential for proper guidance of the commissural axon growth cones.

*commissureless* (*comm*) encodes an axon guidance molecule presented by the MG to the commissural axons and *comm* mutants lead to an absence of commissural axons crossing the midline (Seeger *et al.*, 1993; Tear *et al.*, 1996). This is a positive attractant and can be juxtaposed with negative repulsive cues like the putative ligand for Roundabout (*Robo*) (C. Goodman, pers. communication; Seeger *et al.*, 1993). A mutation in *robo*, a gene which encodes a transmembrane receptor expressed on the commissural axons, results in a loss of inhibition and axons which are normally ipsilaterally directed, cross the midline.

Following the initial pioneering of the commissural axon tracts, approximately 6 of the MG act to delineate the anterior and posterior commissures (Sonnenfeld and Jacobs, 1995a). This separation of the commissures is partly mediated by the posterior migration of the MGM over top of the MGA (Klämmt *et al.*, 1991). Next, the MGP relocate across the anterior segment boundary to a position posterior to the posterior commissure of the next anterior segment (Klämmt *et al.*, 1991). The final positions of the

MGA and MGM are anterior to the anterior commissure and between the two commissures, respectively. These transverse commissures connect the two halves of the CNS and are repeated segment to segment along the VNC. They are bounded laterally by the longitudinal connectives. The mature VNC comprises a ladder-like structure and is revealed immunocytochemically with the monoclonal antibody BP102 (Klämbt *et al.*, 1991).

Recently, a novel carbohydrate-binding protein (a lectin) termed Gliolectin was identified and it is expressed by the MGA (Tiemeyer and Goodman, 1996). Cell surface carbohydrates function in cell-cell recognition events. Interestingly, *gliolectin* expression in the MGA occurs immediately prior to its encounter with the pioneering growth cones of the anterior commissure. Gliolectin expression continues as the MG migrate, mediating the physical separation of the anterior and posterior commissures within each neuromere. As previously described, the MGM migrates posteriorly over top of the MGA (Klämbt *et al.*, 1991). To date, the mechanism governing this migration event are not clear, but it is possible that Gliolectin contributes to the mediation of this glia-glia interaction. Further confirmation of such a role would come from the observation of disruption of commissural axon formation by *gliolectin* mutants (Tiemeyer and Goodman, 1996).

Another molecular contributor to the mechanism of MG migration might be Drifter (Anderson *et al.*, 1995). In *drifter* mutants, the MG do not die, but follow an aberrant migration route. As a result, the commissures are not separated and remain merged together. Interestingly, *drifter* expression in the midline is specific to the MGA

and MGM, that subset of MG which mediate commissure separation. *drifter* encodes a POU-domain transcription factor and might regulate the expression of cell surface protein involved in cell-cell interactions.

Following initial commissural axon formation and separation, only a subset of the MG assume the role of ensheathing glia. As axonogenesis proceeds, these MG increase their surface area and degree of contact with the commissural axons through extensive lamellipodial protrusions (Jacobs and Goodman, 1989).

## 1.2 ONLY A SUBSET OF THE MG SURVIVE

To summarise, up to 12 MEC express MG specific genes (Dong and Jacobs, in prep.). Most, if not all of these MG likely participate in the guidance of commissural axon growth cones (Tear *et al.*, 1996; Harris *et al.*, 1996). About half of these MG function in commissural axon separation (Sonnenfeld and Jacobs, 1995a). Subsequently, a further half of these MG survive to ensheath the commissural axons while the remainder undergo apoptosis. After this apoptotic event, an average of 3.2 MG per segment remain at the end of embryogenesis (Sonnenfeld and Jacobs, 1995a). Apoptosis is a natural phenomenon within the MG lineage as cell death was blocked in embryos deficient for the gene *reaper*. Reaper is postulated to be involved in the initiation of the cell death cascade. It has been proposed to converge various cell death signals (e.g. extracellular or cell autonomous lineage dependent signals) onto the downstream effectors to initiate apoptosis (White *et al.*, 1994).

An excess of MG are produced in early development, but not all survive.

Previous studies suggest that the number and position of the surviving MG are regulated by external cues.

### **1.3 MECHANISMS REGULATING MG SURVIVAL – THE AXON CONTACT HYPOTHESIS**

MG survival is positively correlated with the magnitude of commissural axon contact (Sonnenfeld and Jacobs, 1995a). It's hypothesised that the commissures provide a trophic cue to the MG, signaling them to survive. The amount of survival factor reaching the MG is proportional to the extent of axonal contact and this ultimately delegates which MG survive and which MG die. This hypothesis is supported by quantitative analysis in observing the variation in the number and position relative to the commissures of the MG within the nerve cord segments. The pattern of MG survival is stochastic. In 100% of the VNC segments observed, at least one MGM survived to the end of embryogenesis. In 89.2% of VNC segments, both MGM survived. Note this in comparison to an 18.2% survival rate for two MGA and a virtual 0% survival rate among the MGP. It follows that the MGM have the most axonal contact relative to the other MG, in that they are interposed between the two commissures. Furthermore, if the ratio of MG to commissural axon space is altered through certain mutant and transgenic perturbations, this ratio is in turn corrected through changes in the number of MG undergoing apoptosis. This axon contact model has also been applied to vertebrates. In the rat optic nerve, 50% of the oligodendrocytes die in order to correct the ratio of glia to

axons requiring myelination (Raff *et al.*, 1993). How might axons communicate survival cues?

#### 1.4 MECHANISMS REGULATING MG SURVIVAL – THE SPITZ GROUP/DER PATHWAY

Mutations in the genes of *spitz* (*spi*), *Star* (*S*), *rhomboid* (*rho*), and *pointed* (*pnt*), collectively termed the *spitz* group, result in similar defects in the embryonic ventral ectoderm (Mayer and Nüsslein-Volhard, 1988; Kim and Crews, 1993). Furthermore, *spitz* group mutants lead to a classic CNS phenotype in which the anterior and posterior commissures have been incompletely separated and remain merged together (Klämbt *et al.*, 1991; Klämbt, 1993; Sonnenfeld and Jacobs, 1994). In all *spitz* group mutants, the early pioneering of the commissural axon growth cones is relatively normal. In the case of *rho*, *S*, and *spi* mutants, the MG are determined and express midline specific genes like *AA142* and *slit*. However, they fail to migrate and subsequently die (Klämbt *et al.*, 1991; Sonnenfeld and Jacobs, 1994). In *pnt* mutants, the MG appear to erroneously migrate along the commissural axons and which is soon followed by premature MG death (Klämbt, 1993). Loss of function lethal mutations at the *faint little ball* (*flb*) locus which encodes the *Drosophila* Epidermal Growth Factor Receptor (DER), share the hallmark CNS phenotypes, characterised by the *spitz* group mutants (Raz and Shilo, 1992). In the CNS, the only cells expressing significant levels of DER are the MG (Zak *et al.*, 1990).

The gene products of *spi*, *S*, and *rho* are thought to interact with DER in a highly regulated fashion (Rutledge *et al.*, 1992; Sturtevant *et al.*, 1993; Schweitzer *et al.*, 1995a).



In turn, this specific interaction is thought to mediate cell-cell communication and activate a signal transduction pathway to impart various developmental choices throughout fly development. The observations documented above suggest that the *spitz group/DER* genes are required for MG survival. This putative signaling pathway might play a role in the developmental reduction from 12 to 3 MG per segment and specify which and how many MG do not enter apoptosis.

## 1.5 EXPRESSION AND MOLECULAR CHARACTERISATION OF SPITZ GROUP/DER GENES

To understand how the various elements of the *spitz/DER* cassette contribute to a signaling pathway, their molecular nature, expression patterns and molecular and cellular function will be reviewed below.

### 1.51 *spitz (spi)*

The *spitz* gene encodes a protein containing a putative signal sequence, a potential transmembrane domain, a dibasic amino acid sequence which might serve as a proteolytic cleavage site, and most significantly, an EGF domain (Rutledge *et al.*, 1992). *spitz* is believed to be the *Drosophila* homolog of the growth factor TGF- $\alpha$ .

*spitz* expression is ubiquitous but is enhanced in the mesoderm and procephalic region in germ-band extended embryos and in the brain, the ventral midline and the peripheral nervous system (PNS) in germ-band retracting embryos. *spitz* is also expressed in the morphogenetic furrow of the developing eye disc (Tio *et al.*, 1994). *spitz* function is required for the ventral specification of ectoderm, MG migration and survival,

sensory organ development, muscle development, and the formation of all photoreceptors except R8 (Klämmt *et al.*, 1991; Rutledge *et al.*, 1992; Freeman, 1994b; Tio *et al.*, 1994; Freeman, 1996). Spitz is a diffusible factor (Schweitzer *et al.*, 1995a) with a diffusion range of 3-4 cell diameters, as assayed by mosaic analysis in the eye (Freeman, 1994b).

### **1.52 faint little ball (*flb*)**

The *faint little ball* locus encodes DER (Price *et al.*, 1989; Schejter and Shilo, 1989). It's homologous to the vertebrate EGF receptor which is encoded by *erbB-1* (Ullrich *et al.*, 1984). It is a transmembrane protein containing a tyrosine kinase domain at the cytoplasmic region and an extracellular ligand binding domain (Livneh *et al.*, 1985). DER exhibits tyrosine kinase activity (Schejter and Shilo, 1989; Schweitzer *et al.*, 1995b). Furthermore, the capacity of DER autophosphorylation and downstream MAPK activation was stimulated by secreted, but not membrane-associated Spitz (Schweitzer *et al.*, 1995a).

Like *spitz*, DER protein is predominantly expressed in the mesoderm and head of germ band extended embryos and is enhanced in muscle insertion sites and along the ventral midline in germband retracted embryos (Zak *et al.*, 1990). The distribution of DER mRNA is very similar and it's postulated that the gene is regulated primarily at the level of transcription (Katzen *et al.*, 1991).

Mutations in *flb* lead to a complex phenotype as multiple embryonic tissues are perturbed (Schejter and Shilo, 1989). Because of this pleiotropic nature of the *flb* embryonic lethal mutant, Raz and Shilo (1992) used a *flb* temperature sensitive allele to dissect the different *flb* embryonic defects. They demonstrated that DER function is

required during germband retraction in the MG, as absence of DER function resulted in apoptosis. In turn, the commissures remained fused. DER is also expressed throughout the developing eye disc (Schejter *et al.*, 1986). Indeed, DER is reiteratively required for the development of all ommatidial cell types: photoreceptor, cone, and pigment cells (Freeman, 1996).

### **1.53 rhomboid (*rho*)**

*rhomboid* encodes a putative integral membrane protein as it is predicted to contain a number of membrane-spanning regions (Bier *et al.*, 1990). An ATTTA sequence in the cDNA and a PEST sequence in the gene product implicate high mRNA and protein turnover, respectively (Rogers *et al.*, 1986 and Shaw and Kamen, 1986 as cited in Bier *et al.*, 1990).

*rho* is expressed throughout the mesectoderm from this tissue's putative initiation at the cellular blastoderm stage (Bier *et al.*, 1990). During germband extension, *rho* expression occurs in the tracheal pit cells and in the chordotonal organ precursor cells. During germband retraction, *rho* continues to be expressed in the ventral midline. *rho* expression is also observed in the presumptive wing vein primordia (Sturtevant *et al.*, 1993) and in the R8, R2, and R5 photoreceptors of the developing ommatidium in the eye (Freeman *et al.*, 1992b). Rhomboid is concentrated in patches at the apical cell surface and in some cells, Rhomboid colocalises with adherens junction accessory proteins where EGF-R signaling is believed to occur (Sturtevant *et al.*, 1996).

### 1.54 *Star (S)*

*Star* encodes a single pass (typeII) transmembrane protein containing two PEST sequences (Kolodkin *et al.*, 1994). Like *rhomboid*, *Star* expression in the early CNS is restricted to the midline in the germband extended embryo. This expression becomes refined to the MG after germband retraction. Transient *Star* expression is also observed in ectodermal stripes and in the optic lobe anlagen of the embryonic brain. *Star* expression is also observed in the developing R8, R2, and R5 photoreceptor cells in the eye disc (Heberlein *et al.*, 1993; Kolodkin *et al.*, 1994).

The temporal and spatial expression patterns of the *spitz* group/DER genes outlined thus far are comparable. However, it is the similar expression patterns of *rhomboid* and *Star* that is most striking. *Star* interacts genetically with the EGF-R in the eye (Kolodkin *et al.*, 1994) and *rhomboid* function requires the EGF-R signaling pathway during oogenesis (Ruohala-Baker *et al.*, 1993). Moreover, *rhomboid* and *Star* exhibited especially strong genetic interactions with each other and interacted genetically with other components of the *spitz* mediated DER signaling pathway as analysed during wing vein development (Sturtevant *et al.*, 1993). These genetic interactions support models in which *rhomboid* and *Star* act synergistically with DER signaling component genes. Overexpression of *rhomboid* and *Star* result in supernumerary photoreceptor cells (Freeman *et al.*, 1992b; Kolodkin *et al.*, 1994). Furthermore, ectopic expression of *rhomboid* leads to increased wing venation tissue and an increase in MG numbers (Sturtevant *et al.*, 1993; Sonnenfeld and Jacobs, 1995a). All these tissues normally

require DER signaling. These observations support the notion that the tight regulation of *rhomboid* and *Star* is required for localised hyperactivation of DER signaling to appropriate cells. Rhomboid and Star might provide spatial and temporal cues to specify or amplify the signal exchanged at the cell membrane (Ruohala-Baker *et al.*, 1993; Sturtevant *et al.*, 1993; Kolodkin *et al.*, 1994). The inference from sequence analysis that both Rhomboid and Star have short half-lives is consistent with this hypothesis (Bier *et al.*, 1990; Kolodkin *et al.*, 1994). Regulation might also be achieved by Rhomboid and Star serving as the processing apparatus to prepare secreted Spitz from the membrane-associated Spitz precursor (Schweitzer *et al.*, 1995a; Golembo *et al.*, 1996b).

### ***1.55 pointed (pnt)***

The *pointed* gene encodes two different transcription factors, *P1* and *P2*, through differential splicing (Klämmt *et al.*, 1993). The region of homology is the ETS DNA-binding domain and it's related to a number of vertebrate transcription factors (Karim *et al.*, 1990).

*pointed P1* transcripts are observed during the cellular blastoderm stage in two broad stripes in the lateral neurogenic region (Klämmt *et al.*, 1993). Upon gastrulation, *P1* expression is graded with higher to lower levels from the neurogenic region to the presumptive mesectoderm. During gastrulation and through to germband extension, *P1* expression gradually becomes more refined to a narrow strip in the lateral neurectoderm and completely excluded from the flanking mesectoderm. Upon germband retraction, *P1* expression is no longer detected in the ventral ectoderm but is observed in the tracheal pit precursor cells. *P1* expression is first detected in the CNS at stage 12 in the longitudinal

glial cells which are glial cells supporting the longitudinal connectives (Jacobs and Goodman, 1989a). Concurrently, *P1* expression occurs in the VUMs. In contrast to *P1*, *P2* is expressed in the mesoderm in early embryos. Upon germband retraction however, mesoderm specific *P2* expression ends and *P2* is observed in the MG where it is maintained until all MG die during metamorphosis (Perz, 1994). Post-embryonically, *P1* was not detected in the eye disc, but *P2* was expressed in virtually all cells in the developing eye disc (O'Neill *et al.*, 1994). *P1* is a constitutive activator of transcription, while *P2* is positively regulated by the Ras/MAPK pathway (O'Neill *et al.*, 1994).

*P2* is required for MG survival and proper photoreceptor development (Klämbt, 1993; O'Neill *et al.*, 1994). Expression of vertebrate *ets* transgenes in *pointedP2* mutants exhibited substantial rescue of the MG and photoreceptor mutant phenotypes (Albagli *et al.*, 1996). These results indicate that function is conserved in that vertebrate ETS proteins are able to transactivate at least a subset of the PointedP2 target genes.

Pointed is believed to represent a nuclear downstream target of the DER mediated signal transduction cascade (Klaes *et al.*, 1994). Components of the cytoplasmic signaling cassette between the cell surface and the nucleus have all been isolated in *Drosophila* (for review, see Dickson and Hafen, 1994), through mutant screens affecting eye development. This biochemical pathway transmits the signal from the cell membrane to the nucleus and includes Drk, Sos, Ras1, Gap1, Raf, MEK, and MAPK. The *Drosophila* mitogen-activated protein kinase (MAPK) is encoded by *rolled* and is termed ERK-A (Biggs *et al.*, 1994). It enters the nucleus to phosphorylate PointedP2 at a MAP-kinase phosphorylation site, termed the POINTED domain (Brunner *et al.*, 1994).

As mentioned previously, the MG phenotype of *pointed* mutants differs slightly from that observed in other *spitz* group mutants. The other *spitz* group mutants have MG which fail to migrate and they subsequently die (Klämbt *et al.*, 1991; Sonnenfeld and Jacobs, 1994). Migration while aberrant, does occur in *pointed* mutants before the MG undergo apoptosis (Klämbt, 1993). These differences in MG phenotypes suggest that there are other downstream nuclear targets of the *spitz* group mediated DER signaling cascade which mediate MG development and migration. One candidate is *drifter* (Anderson *et al.*, 1995).

## 1.6 HOW IS DER SIGNALING MODULATED?

### 1.6.1 Negative Regulators - Yan

Another downstream target of MAPK and the EGF signaling pathway is Yan (Brunner *et al.*, 1994; Gabay *et al.*, 1996). *yan* like *pointed*, encodes an ETS-related protein (Lai and Rubin, 1992). However, *yan* serves as a negative regulator of the signal transduction pathway. Its activity is highly regulated through PEST sequence mediated degradation and MAPK mediated inhibition (Lai and Rubin, 1992; Brunner *et al.*, 1994). Furthermore, MAPK phosphorylation of Yan lead to a shift in subcellular localisation from the nucleus to the cytoplasm, followed by rapid degradation (Rebay and Rubin, 1995). A model was proposed in which unphosphorylated Yan resides in the nucleus. It is therefore active and serves as a transcriptional repressor of specific genes required for a particular developmental program. In response to the “correct” intercellular trigger as mediated by intense DER signaling, an intracellular signal is propagated, activating

MAPK which in turn phosphorylates Yan. Yan is removed from the nucleus and is immediately degraded (Rebay and Rubin, 1995).

### 1.62 Negative Regulators – Argos

There exists another negative regulator, but unlike Yan, it appears to be integrated with the extracellular inductive signals to impart an appropriate developmental decision. It is the gene product of *argos*, a putative secreted protein containing a single EGF domain (Freeman *et al.*, 1992a; Okano *et al.*, 1992; Kretzschmar *et al.*, 1992).

Embryonically, *argos* is expressed in the dorsal ectoderm anlagen at the cellular blastoderm stage (Freeman *et al.*, 1992a; Okabe *et al.*, 1996). At gastrulation, *argos* expression is limited to two cell rows in the ventral ectoderm as it borders the mesectoderm. Upon mesectoderm invagination, *argos* expression occupies the entire ventral ectoderm. Transient *argos* expression occurs in cells of the tracheal pits and cells surrounding the chordotonal organ precursors. Germband extended embryos have *argos* expressing cells in the head region. After germband retraction, *argos* is expressed in the MG and in terminal sensory organs. Post-embryonically, *argos* is expressed in all ommatidial cells of the developing eye disc as they differentiate (Freeman *et al.*, 1992a). *argos* is also expressed in the developing lamina, inner optic analage, the wing disc of third instar larvae, wing veins of the adult wing, and the antennae region of the adult eye (Kretzschmar *et al.*, 1992; Okano *et al.*, 1992). Interestingly, *argos* expression mimics the expression patterns of components of the *spitz* group/DER pathway (Bier *et al.*, 1990; Rutledge *et al.*, 1992; Raz and Shilo, 1992; Klämbt, 1993; Kolodkin *et al.*, 1994).



Through mutant analysis, *argos* function has been previously characterised in a number of tissues. Hypomorphic mutations resulted in extra photoreceptors and cone cells in the developing eye, extra wing veins, expansion of the embryonic ventral ectoderm, and extra chordotonal organs in the embryonic PNS (Freeman *et al.*, 1992a; Kretschmar *et al.*, 1992; Okano *et al.*, 1992; Sawamoto *et al.*, 1994; Golembo *et al.*, 1996a; Okabe *et al.*, 1996). Conversely, over-expression heat shock constructs of *argos* resulted in a reduction in the number of these same cells (Freeman, 1994a; Sawamoto *et al.*, 1994; Brunner *et al.*, 1994; Golembo *et al.*, 1996a; Okabe *et al.*, 1996). From these data, *argos* is believed to act as a negative regulator of DER pathway mediated determination and differentiation in these tissues. Indeed, recent studies have shown that *argos* functions through the DER pathway. The DER pathway induces *argos* expression and is inhibited by Argos protein, thereby forming an inhibitory feedback loop (Golembo *et al.*, 1996a; Schweitzer *et al.*, 1995b). Mosaic analysis in the adult eye has shown that Argos acts non cell autonomously and has a diffusion range of 10-12 cell diameters (Freeman *et al.*, 1992a).

To date, homologs of *argos* in other animals have not been isolated. However, the *lin-15* locus of *Caenorhabditis elegans* encodes two negative regulators of vulval development (Huang *et al.*, 1994; Clark *et al.*, 1994). While there exists no sequence homology between between *lin-15* and *argos*, they might share functional similarity in that *lin-15* gene products are believed to negatively regulate an EGF-R signaling pathway (Let-23). Because there are no clear consensus signal sequences, it is unlikely however that the *lin-15* gene products are secreted (Huang *et al.*, 1994). However, like *argos*,

genetic analysis suggests that Lin-15 functions non cell autonomously (Herman and Hedgecock, 1990). The high degree of evolutionary conservation between EGF-R signaling pathways of invertebrates and vertebrates, suggest that *argos* homologs exist in these other systems.

## 1.7 RATIONALE FOR THESIS

Previous studies have shown that *argos* is expressed by the MG (Freeman *et al.*, 1992a). However, a role for *argos* within the MG lineage has not been characterised. Careful analysis revealed that the temporal and spatial expression patterns of *argos* reflects the timecourse and stochastic nature of the apoptotic event in the MG. With the knowledge of *argos* functioning as a negative regulator in other tissues, it follows that it might play a similar role in the developmental reduction of MG numbers. We discovered that loss of function and misexpression of the *argos* gene confers an increase and decrease in MG number, respectively. These data further a role for *argos* in mediating the reduction of MG numbers. There's been some ambiguity relating to the function of the *spitz* group/DER pathway class of genes in the context of MG development. Through a number of misexpression constructs in various elements of this pathway, I further support the hypothesis that the *spitz* group mediated DER signaling cascade is required for MG survival and that it functions to specify which and how many MG do not enter apoptosis. In turn, I characterise *argos* function in relationship to this pathway. I present a model exhibiting the delicate balance between positive and negative factors in the regulation of MG survival.

Finally, through *HSargos* elicited loss of the MG, I present and discuss the repercussions to the development of the commissural axons.

## MATERIALS AND METHODS

### 2.0 *Drosophila melanogaster* Strains

Unless stated otherwise, individual mutants and wildtype lines were obtained from the Indiana Stock Centre. All fly stocks were maintained on a sucrose-salt-agar-yeast based medium at room temperature. Wildtype *Drosophila melanogaster* was from the CS-P stock, a line provided by A. Campos. Most double mutants and mutants and transgenics with enhancer trap or reporter construct markers were established as stable balanced lines. Double mutant analysis of *flb*<sup>2E07</sup>, *S*<sup>IN23</sup>, and *DfE55* with *argos*<sup>w11</sup> was performed through a quick cross and stable balanced lines were not established. *argos*<sup>A7</sup>, *Ellipse*<sup>B1</sup>, and the double mutant recombinants of *rho*<sup>38</sup>-*argos*<sup>w11</sup> and *pnt*<sup>9J31</sup>-*argos*<sup>w11</sup> were balanced over balancer chromosomes containing a P[*actin-lacZ*] reporter transposon which was provided by C. Goodman. Therefore, mutant homozygotes could be detected embryonically through lack of *actin-lacZ* labeling. *slit1.0lacZ* was incorporated with four copies of *HS-argos*. The *HS-argos* dosage was monitored by the maintenance of a uniform rough eye phenotype even when *HS-argos* flies were raised at room temperature. The *HS-argos* inserts on the second chromosome were believed to be integrated close to an enhancer element which constitutively promotes *argos* transcription (Sawamoto *et al.*, 1994).

## 2.01 Mutants and Transgenics

***argos*<sup>A7</sup>**: a amorphic third chromosome allele created by the imprecise excision of the *argos*<sup>w11</sup> transposon (see below) which has a cytological position at 73A3-4 (Freeman *et al.*, 1992a) and was provided by C. Klämbt.

***rhomboid*<sup>A38</sup>**: a null third chromosome allele created by the imprecise excision of the *rhomboid*<sup>v81</sup> transposon which has a cytological position at 62A1-2 (Freeman *et al.*, 1992b).

***Star*<sup>IN23</sup>**: a second chromosome EMS-induced allele which has a cytological position at 21E1-2 (Nüsslein-Volhard *et al.*, 1984).

***Df(2L)E55***: an EMS induced second chromosome deficiency which uncover the *spitz* locus and has a cytological position at 37D2-E1, 37F5-38A1 (Lindsley and Zimm, 1992).

***faint little ball*<sup>2E07</sup>**: a second chromosome weak hypomorphic allele of *DER* which has a cytological position at 57F1 (Nüsslein-Volhard *et al.*, 1984).

***pointed*<sup>9J31</sup>**: a third chromosome loss of function EMS induced allele which has a cytological map position of 94F (Jürgens *et al.*, 1984; Klämbt, 1993) and was provided by S. Crews.

***Ellipse*<sup>B1</sup>**: a second chromosome hypermorphic EMS induced allele of *DER* which has a cytological position at 57F1 (Baker and Rubin, 1989) and was provided by N. Baker.

***HS-rhomboid1B***: an ectopic expression construct on the X chromosome in which a full length *rho* cDNA clone under the control of the *hsp70* promoter was placed in the *hs-CaSpeR* P-element transformation vector to make the transformant line (Sturtevant *et al.*, 1993). *HS-rho1B* was provided by E. Bier.

***HS-argos***: these flies have four copies of the *HS-argos* ectopic expression construct (homozygous for these inserts on the second and third chromosomes). The *argos* cDNA was placed into a P-element vector containing a *hsp70* promoter to make the transformant line (Sawamoto *et al.*, 1994). *HS-argos* was provided by H. Okano.

***UAS-D-raf***: this transposon is found on the third chromosome and contains the target gene *D-raf* (Brand and Perrimon, 1994) which is activated via upstream GAL4 binding sites (UAS – upstream activating sequences) when crossed to a GAL4-expressing line.

The target gene is then transcribed in a GAL4-dependent pattern (Brand and Perrimon, 1993). *UAS-D-raf* was provided by A. Brand.

## 2.02 Enhancer traps and reporter gene fusion constructs

P-element transposons containing the *lacZ* reporter gene have been useful in elucidating tissue and cell specific gene expression patterns and have been important in identifying genes required for various aspects of *Drosophila* development (Bier *et al.*, 1989; Bellen *et al.*, 1989). P-element vectors can contain the *E. coli lacZ* gene which is under the control of a weak P-element promoter and which can not initiate *lacZ* expression on its own. If a P-element can be inserted into the regulatory elements of a given gene, *lacZ* will be transcribed in a pattern mimicking that flanking gene (Wilson *et al.*, 1989; O’Kane and Gehring, 1987). The following enhancer traps and reporter constructs were used in these studies:

*argos<sup>w11</sup>*: an enhancer trap containing a P element insertion at the cytological position 73A3-4, the locus of which is the *argos* gene. *argos<sup>w11</sup>* is a recessive hypomorphic but viable allele and was originally isolated as a rough eye mutation from an enhancer trap screen (Freeman *et al.*, 1992a). *argos<sup>w11</sup>* is expressed in numerous tissues throughout embryogenesis (Freeman *et al.*, 1992a) and heterozygous *argos<sup>w11</sup>* (wildtype) is expressed in a subset of the MG, the subset which survive past embryogenesis (this study).

**AA142:** an enhancer trap containing a P element insertion at the cytological position 66D, the putative gene of which has not been molecularly characterised (Klämbt *et al.*, 1991). AA142 is expressed strongly in the MGA and MGM and weakly in the MGP (Klämbt *et al.*, 1991; Sonnenfeld and Jacobs, 1994).

**X55:** an enhancer trap containing a P element insertion at the cytological position 56F, the putative gene of which has not been molecularly characterised. X55 is expressed in the VUMs, in the MNB and its progeny, and in the MGP (Klämbt *et al.*, 1991).

**P[*slit1.OlacZ*]:** a gene reporter construct consisting of a P element containing a restriction fragment of *slit* DNA (Wharton and Crews, 1993). *slit1.OlacZ* is expressed throughout the MG lineage from stage 12.

**C38-GAL4:** an enhancer trap located on the second chromosome in which the yeast transcriptional activator GAL4 mimics the expression pattern of the yet to be characterised gene C38 (Yeh *et al.*, 1995). C38 is expressed in a subset of MEC lineages among other tissues.



## 2.1 Antibodies

**anti- $\beta$ -galactosidase:** a commercial rabbit polyclonal antibody (Cappel) used to identify gene expression patterns of lines carrying a P[*lacZ*] element. A dilution of 1:100 – 1:200 was used for whole mount embryo staining.

**BP102:** a mouse monoclonal antibody for an uncharacterised carbohydrate moiety, a surface antigen located on the longitudinal connective and commissural axon tracts of the CNS. BP102 was generated by A. Bieber and N. Patel and was provided by C. Goodman. It was used at a dilution of 1:3 for whole mount embryo staining.

**22C10:** a mouse monoclonal antibody which recognises a moiety located on the VUM cell bodies and axons by stage 14 (Fujita *et al.*, 1982). 22C10 was generated and provided by the S. Benzer lab. It was used at a dilution of 1:10 for whole mount embryo staining.

**anti-engrailed (4D9):** a mouse monoclonal antibody which recognises Engrailed, a nuclear protein expressed by the MNB and its progeny (Patel *et al.*, 1989). It was provided by C. Goodman and was used at a dilution of 1:1 for whole mount embryo staining.

**anti-FasII (1D4):** a mouse monoclonal antibody for FasciclinII which is normally distributed on the three major longitudinal connective axons in later embryonic development (Seeger *et al.*,1993). It was generated by G. Helt from the Goodman lab. It was used at a dilution of 1:4 for whole mount embryo staining.

## 2.2 Digoxigenin-labeled RNA probes

***DER*:** anti-sense *DER* RNA probe was prepared from a bluescript vector containing the *DER* cDNA insert (provided by E. Bier).

***slit*:** anti-sense *slit* RNA probe was prepared from a bluescript vector containing the *slit* cDNA insert which was generated by M. Sonnenfeld.

***reaper*:** anti-sense *rpr* RNA probe was prepared from a bluescript vector containing the *rpr* cDNA insert (provided by H. Steller).

### **2.3 Embryo Collecting and Staging**

Adult *Drosophila melanogaster* were placed in plastic tri-pour beakers capped with 60 X 15 mm plastic petri dishes. Embryos were laid on these petri dishes which were filled with apple juice agar and supplemented with a dab of live yeast paste (Fleischmann's dry yeast). The yeast was a source of food and served to stimulate egg laying. For standard collections, the plates were changed twice daily, at approximately 9h00 and 17h00. The day collection was then placed at 18°C overnight and the embryos continued to develop at half their normal rate. If not ready to fix, the overnight collection and the previous day's collection from 18°C was placed at 4°C in order to arrest embryonic development. These embryo collecting procedures allowed for optimally staged embryos for these experiments (stage 12 - stage 17). Embryos were stored for a maximum of 72 hours if immediate fixing could not be performed.

Embryos were staged according to Campos-Ortega and Hartenstein (1985). A particular stage was identified using a combination of morphological features including the differentiation and subdivision of the presumptive gut (yolk) and mature gut, the degree of extension of the procencephalon, and the length of the germband and nerve cord.

### **2.4 Heat shock protocols**

0-4 hour collections of embryos which were subsequently aged to stage 12-stage 16 and standard embryo collections (see above) were subjected to a range of heat shock administrations based on the experiment. Please see results section for descriptive account of heat shock regimes (duration of heat shocks and recovery times). The embryos were collected on apple juice agar plates. Heat shocks were applied as these apple juice agar plates were floated in a 37°C water bath.

## **2.5 General Immunocytochemistry for *Drosophila* Embryo Whole Mounts (adapted from protocol by Nipam Patel).**

If embryos were at 4°C, they were left at room temperature for 1.5 hours prior to fixation. This allowed microtubules to repolymerize in order to restore axon morphology. While in the apple juice agar plates, the embryos are soaked with 50% commercial bleach for 5 minutes. The embryos were then rinsed with double distilled water onto a nitex sieve, as the bleach is removed into a waste container. The embryos were gently blotted with a kimwipe to absorb excess water and then transferred to a glass scintillation vial, containing a 1:1 fixative mixture of 3.7% formaldehyde in phosphate-buffered saline (1X PBS - pH 7.4): heptane. The embryos were fixed for 30 minutes.

After fixation, the bottom aqueous layer (PBS + fix) was removed with a Pasteur pipette. An equal volume of methanol was then added forcibly and the vials were shaken violently for 20 seconds. This procedure served to crack the embryos out of their vitelline membrane. While the vitelline remains at the heptane-methanol interface, the devitellinized embryos fall to the bottom of the vial, making for easy removal by a pipette. The embryos were transferred to 12 X 15 mm test tubes and washed 3X with methanol, each time transferring to a new test tube. The embryos were then washed 3X more with methanol in the same tube and finally transferred to a final new test tube. Methanol rinses served to remove any residual heptane and fix. For convenience, the embryos were sometimes stored in methanol at 4°C for a few days to several weeks.

The methanol was replaced with 1X PBS containing 0.2% triton-X (PBT). The embryos were then washed 3X more with PBT and then placed on a rotator for 20 minutes. After 20 minutes, the embryos were removed from the rotator. At this stage, the embryos tended to be 'sticky', adhering to the sides of the tube. Prior to removal of the PBT, a pipette was used to expel solution to dislodge the embryos so that they could

settle to the bottom of the tube. Care was always taken in ensuring the embryos were always submerged in solution. Because of the high surface tension of water, the liquid - air interface must never touch the tissue. The embryos were washed 1X more with PBT.

The PBT was removed to approximately 40 mL (ensuring that the tissue was completely submerged). The embryos were then blocked with normal goat serum (NGS) in PBT (1:15 dilution) for 30-45 minutes on an orbital shaker at room temperature. After blocking, the primary antibody was added to the NGS/PBT mixture (embryos containing a P[*lacZ*] element were incubated with rabbit anti  $\beta$ -galactosidase at 1:150 dilution). The embryos were incubated with primary antibody overnight at 4°C on an orbital shaker. (N.B. It was preferable to perform antibody staining with excess embryos, rather than too few embryos, as this decreased background problems. If using an orbital shaker, a tube 6 embryos deep does not pose a problem.)

On the following morning, the embryos were washed 5X with PBT and were then placed on the rotator for 4-8 hours. During this time, the embryos were washed with PBT every 0.5-1.0 hour. If washed overnight, the embryos were washed on the rotator at 4°C. The extensive washing process served to reduce background staining. The embryos were then re-blocked in NGS + PBT (1:10 dilution) for 30-45 minutes. The embryos were incubated in secondary antibody for 2 hours at room temperature. Donkey anti-rabbit coupled to horseradish peroxidase at 1:150 dilution was used if the primary antibody was rabbit anti- $\beta$ -galactosidase. After incubation, the embryos were washed 5X with PBT and then placed on a rotator for 2-4 hours with periodic PBT washes or overnight on the rotator at 4°C.

The peroxidase reaction is then performed in which the embryos were incubated in PBT containing 0.33 mg/mL diaminobenzidine (DAB). After a 2 minute incubation time, the reaction substrate hydrogen peroxide was added to 0.03%. The reaction was

monitored through a dissecting scope at 25X magnification. Care was taken to limit the reaction mixture's exposure to light. The reaction was stopped by diluting with PBT after the desired signal and depth of colour was attained. This occurred when the cell marker appeared sharp and unambiguous in contrast to the background and/or before the background reached a level which would render the tissue useless. The DAB reaction yields a golden brown precipitate. Alternatively, a blue/black precipitate was obtained by the addition of 8% cobalt chloride solution to the DAB reaction mixture to a final concentration of 4 mL/mL.

The embryos were then washed 1X more with PBT and then received a graded dehydration with ethanol: 50%, 70%, 90%, 95%, 3X 100%. The embryos were then cleared and preserved in methyl salicylate.

## **2.6 XGAL Immunocytochemistry (adapted from protocol by Christian Klämbt)**

X-gal staining was the final measure employed in screening mutants or transgenic fly lines for the stable incorporation of an enhancer trap or reporter fusion construct of interest. As above, embryos were dechorionated with 50% sodium hypochlorite (commercial bleach) for 5 minutes. They were then rinsed via distilled water into nitex-lined wells. The multi-welled unit containing the embryos was blotted dry and was then immersed in a 100X15 mm square style polystyrene petri dish containing heptane. After this initial equilibration, the pure heptane was exchanged for heptane saturated with fixative (2.5% gluteraldehyde in 1X PBS, pH 7.4). The embryos were fixed for 20 minutes while shaking on an orbital shaker. The fixative was then removed and recycled and the embryos were washed 4X with fresh heptane. After each heptane wash, the embryos were blotted dry. 1X PBS with 0.2% triton-X (PBT) was applied forcibly via a squirt bottle to the embryos in the wells. This procedure removes any residual heptane

and separates the embryos which tend to clump together following drying after the heptane washes. The embryos were then immersed in a fresh square petri dish containing PBT. Fresh PBT was exchanged in the tray every 15 minutes for 1 hour. The final PBT wash was exchanged for room temperature-equilibrated Xgal staining solution (10 mM phosphate buffer, pH 7.2; 150 mM sodium chloride; 1 mM magnesium chloride; 3.1 mM potassium ferrocyanide; 3.1 mM potassium ferricyanide; 3.1% Triton-X 100) and incubated for ten minutes. During this time, the reaction solution was initiated by heating Xgal staining solution over a bunsen burner until the solution just became cloudy (due to the triton), immediately followed by the addition of 25  $\mu\text{L}/\text{mL}$  of 8% Xgal in dimethylsulfoxide (DMSO). The reaction solution was then allowed to cool for a few minutes before being added to the embryos. The reaction occurred at room temperature overnight, or alternatively at 37°C for 2-6 hours. When satisfied with intensity of the label, the reaction mixture was removed and the embryos were washed several times with PBT.

### **2.70 Preparation of RNA Probe for *in situ* hybridization** (protocol adapted from Dervla Mellerick)

Labeled RNA probes were generated by *in vitro* transcription of linearized DNA with T3 or T7 RNA polymerases using digoxigenin-labeled uridine-triphosphate as a substrate. RNA probe was synthesized from the cDNA plasmid prep. prepared by Christian Smith. Based on the optical density, 10 mg of DNA was removed for restriction enzyme digestion and linearization of the DNA. It was added to a microfuge tube along with 10 mL 10X restriction enzyme buffer, sterile water to 95 mL and 5 mL of appropriate restriction enzyme (i.e. for sense or antisense). The DNA was digested for 3 hours in a 37°C water bath. After digestion, an equal volume of phenol chloroform was

added and the DNA was centrifuged at 4°C for 2 minutes. The top aqueous layer was then removed to a new microfuge tube and 10 mL of 3M NaOAc and 3 volumes of ethanol was added. The DNA was then precipitated overnight at -20°C. The DNA was then centrifuged for 5 minutes at maximum rpm at 4°C. The liquid was then aspirated off and the DNA pellet was resuspended in 200 mL of 70% cold ethanol and re-centrifuged for 2 minutes at 4°C. The liquid was again aspirated off and the pellet was air dried on ice for 30 minutes to 1 hour before being resuspended in DEPC treated (diethylpyrocarbonate - 0.1 %) double distilled water (ddH<sub>2</sub>O) to give approximately 0.5 mg/mL (i.e. assuming 10 mg cDNA was digested, it was resuspended in 20 mL water). The RNA transcription reaction was performed using reagents from the Boehringer Mannheim DIG RNA Labeling Kit (SP6/T7); catalogue number 1175025. For the reaction, 2 mL of the pure DNA template (i.e. 1 mg based on original optical density reading) was added to a microfuge tube along with 2 mL 10X NTP labeling mix, 2 mL 10X transcription buffer, 1 mL RNAase inhibitor, 11 mL DEPC treated ddH<sub>2</sub>O (i.e. up to 18 mL), followed by the final addition of T3 (cat.# 1031163 BMC) or T7 RNA polymerase (i.e. for sense or antisense or depending on orientation of insert) to get a total volume of 20 mL. The microfuge tubes were mixed gently, centrifuged briefly and incubated for two hours in a 37°C water bath. The transcription reaction was stopped with 2 mL 200 mM EDTA, pH 8.0. The RNA was precipitated with 0.1 volume of 4M lithium chloride and 3 volumes -20°C chilled ethanol. The contents were mixed well and the RNA was precipitated overnight at -20°C. The following morning, the microfuge tubes were centrifuged at maximum rpm for 15 minutes at 4°C. The contents were aspirated off and the pellet was washed with 100 mL -20°C-chilled 70% ethanol:30% DEPC-treated ddH<sub>2</sub>O. The tubes were again centrifuged for 5 minutes at 4°C, followed



by the aspiration of the supernatant. The pellet was dried on ice for 30-45 minutes, followed by resuspension in 100 mL DEPC-treated ddH<sub>2</sub>O and storage at -20°C.

### **2.71 Antibody and RNA *in situ* Double Labeling** (protocol adapted from Dervla Mellerick)

RNAase precautions were taken throughout this protocol. Double distilled water, and buffer and salt stock reagents were incubated with 0.1% diethylpyrocarbonate (DEPC) overnight, followed by autoclaving to inactivate DEPC. Similarly, all autoclave sensitive stock reagents were aliquoted and prepared in DEPC treated double distilled water. All glass culture tubes, pasteur pipettes, microfuge tubes, tips and caps used were RNAase free.

### **2.72 Antibody staining prior to *in situ* hybridization**

Embryos were dechorionated with bleach and collected as per general antibody staining, however they were rinsed with embryo wash (7% NaCl and 0.05% Triton X-100) into the nitex sieves. The embryos were gently blotted with a kimwipe to absorb excess water and transferred to a glass scintillation vial, containing the fixative mixture. The fix mix comprised of 2mL embryo wash, 2mL 10% formaldehyde, 1mL 5X fixation buffer and 5mL heptane. The 5X fixation buffer consisted of 800mM KCl, 200mM NaCl, 20mM EGTA (pH 8.0), 5mM spermadine, 2mM spermine-HCl, and 150mM Pipes (pH 7.4). The vials were shaken vigorously for a few seconds and then placed on the rotator, where the embryos were fixed for 25 minutes. Periodically, the vials were removed from the rotator and shaken vigorously. After 25 minutes, the vials were removed and allowed to sit for 5-10 minutes or until the layers separated adequately. (Detergent will permeate the heptane phase and cloud the mixture.)

After fixation, the bottom aqueous layer was removed and the embryos were devitellinated with methanol as per general antibody staining. The embryos were transferred to 12 X 15 mm test tubes and further washed 4X with methanol, followed by 5X ethanol washes. New glassware accommodated the early washes to reduce probability of any residual heptane and fix.

After the final alcohol rinse, half of the ethanol was removed and replaced with PBTH. PBTH consists of 1X phosphate buffered saline (PBS, pH 7.4) containing 0.1% Tween 20 and 0.05 mg/mL heparin sodium salt (Gibco BRL, cat# 15077-019). The tube was inverted and the embryos were banged down to the bottom. After settling, the ethanol/PBTH mixture was removed and the embryos were washed 5X with PBTH, followed by the placing of the tubes on the rotator for 20 minutes. The embryos were then rinsed 1X in PBTH containing 0.25 mg/mL tRNA (BMC, cat# 109525), followed by incubation in 100  $\mu$ L of blocking buffer for 40 minutes on an orbital shaker. The blocking buffer contained 2 mg/mL BSA (Sigma, cat# 9048-46-8) and 1 mL/mL of 20 units/mL RNAase inhibitor (BMC) in PBTH containing tRNA. The primary antibody was diluted in the blocking buffer (see section Antibodies re: dilution specifics for designated primary antibodies) and the embryos were incubated overnight at 4°C on the orbital shaker.

The following morning, the embryos were washed 5X with PBTH and were then placed on the rotator for 4-8 hours. During this time, the embryos were washed with fresh chilled PBTH every 0.5-1.0 hour. If washed overnight, the embryos were washed on the rotator at 4°C.

For secondary antibody incubation, the embryos were rinsed in PBTH with tRNA as above and re-blocked in blocking buffer for 40 minutes. The secondary antibody (either goat anti-mouse IgG or donkey anti-rabbit IgG conjugated to horse-radish

peroxidase) was then diluted at 1:100 to 1:150 in the blocking buffer and the embryos were incubated for two hours at room temperature on the orbital shaker. After incubation, the embryos were washed 5X with PBTH and placed on the rotator for 2-4 hours with periodic PBTH washes.

When ready, the peroxidase reaction is then performed in which the embryos were incubated in PBTH containing 0.5 mg/mL diaminobenzidine (DAB), 0.06% hydrogen peroxide and 1 mL/mL of 20 units/mL RNAase inhibitor. 300 mL of this reaction mix was added per tube and the reaction was monitored through a dissection scope at 25X magnification using a black background. The reaction was stopped by diluting with PBTH after the intensity of the signal relative to background was attained. Care was taken to minimize background levels as high background before *in situ* hybridization will render the tissue useless.

The embryos were washed 2X more in PBTH and then post-fixed for ten minutes in a 4:1 ratio of PBTH to 10% formaldehyde. After removal of the post-fix, they were then rinsed 3X with PBTH. Following the final PBTH rinse, half of the PBTH was removed and replaced with ethanol. The tubes were capped and the embryos were banged to the bottom. They were then rinsed 3X with ethanol. If desired, the embryos could be stored at -20°C at this stage before proceeding to *in situ* hybridization.

### **2.73 RNA *in situ* hybridization label**

The embryos were rinsed in 50% ethanol/50% xylenes and then cleared in 100% xylenes for 2.5 hours while shaking on the orbital shaker. They were then re-rinsed in fresh 50% ethanol/50% xylenes, followed by 5X 100% ethanol washes. Glassware was changed for each wash to remove all traces of xylenes. Embryos were then rinsed in 50%

methanol/50% PBT (1X PBS with 0.1% Tween-20). While in 50% methanol/50% PBT, the embryos were transferred to RNAase free 1.5 mL microtubes.

The embryos were then post-fixed in a 4:1 ratio of PBT to 10% formaldehyde for 10 minutes. The microtubes were placed on their sides on the orbital shaker platform. The embryos were then rinsed 3X with PBT for 2 minutes each. The embryos were then treated with 50 mg/mL of proteinase K in PBT for exactly 3 minutes. During the digestion, the tubes were shaken gently, but this agitation was arrested 25 seconds before the 3 minutes was over, in order to allow the embryos to settle. At 3 minutes, the liquid was removed and the reaction was stopped by rinsing the embryos 2X with 2 mg/mL glycine in PBT. The embryos were rinsed 2X more with PBT, followed by a 10 minute post-fix as previously described. The embryos were then washed 4X with PBT for 2 minutes each.

The embryos were then washed for 3X 2 minutes in 50% PBT/50% prehybridization (prehyb) solution at room temperature. Prehyb solution consists of 50% formamide, 300mM NaCl, 10mM Tris-HCl (pH 6.8), 10 mM Na Phosphate, 1X Denhardt's solution (Sigma, cat# D-9905 - contains 1% solution of Bovine Serum Albumin, Ficoll, and Polyvinylpyrrolidone), 5mM EDTA (pH 8.0), and 1 mg/mL tRNA. The embryos were then washed in room temperature 100% prehyb solution for 5 minutes, followed by a 1 hour incubation in prehyb solution that had been pre-equilibrated to 53°C, while shaking gently in the hybridization oven (set at 53°C).

During prehybridization, a 1:10 dilution of RNA probe was prepared and heated for 20 minutes on 60°C dry heating block. It was then immediately placed on ice until ready for hybridization. When ready for hybridization, the prehyb solution was removed and replaced with 53°C equilibrated hybridization solution and probe. The hybridization solution is the same as prehyb, except there is no tRNA and it includes 10% w/v dextran

sulfate. Depending on the probe, final dilutions in hybridization solution varied from 1:500 to 1:2500 (please see probe and antibodies section). RNA hybridization took place overnight in the hybridization oven without shaking.

The following morning, the embryos were washed through a graded series of posthybridization (posthyb) solution to PBT at 53°C. Posthyb is the same as prehyb, however there is no tRNA. While shaking, the embryos were washed for 20 minutes each in 4:1, 3:2, 2:3 and 1:4 parts posthyb to PBT, followed by 2X 20 minutes washes in 100% PBT. They then received one final wash in room temperature PBT.

The embryos were then treated with 20 mg/mL RNAaseA in the 37°C water bath for 25 minutes, followed by 3X PBT washes at room temperature. They were then incubated with a 1:2000 dilution of anti-DIG antibody in PBT for 1 hour while shaking on the orbital shaker. After antibody incubation, the embryos received 4X20 minute washes with PBT. For convenience, the embryos were sometimes stored in PBT overnight and the alkaline phosphatase reaction was performed the following day.

The embryos then received 3X5 minute washes in detection solution. Detection solution consists of 100mM NaCl, 50mM MgCl<sub>2</sub>, 100mM Tris-HCl (pH 9.5), and 0.1% Tween 20. The final detection solution wash was then removed and replaced with the reaction solution. Reaction solution consisted of 4.5 mL 4-Nitro blue tetrazolium chloride (NBT) and 3.5 mL X-phosphate/ 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) each per mL of detection buffer. To facilitate the monitoring of the reaction, the reaction mix and embryos were then transferred from the microtubes to 12 x 75 mm test tubes and the reaction was allowed to proceed in the dark. Reaction times varied from 1-10 hours, depending on the concentration of probe. Care was taken to minimize the reaction mix's exposure to light, when assessing the level of signal.

The reaction was stopped by removing the reaction mix and washing 7X with PBT, followed by 3X washes in 1X PBS. The embryos then received a graded series of glycerol washes, from 30% glycerol/70%PBS to 50% glycerol/50% PBS to final storage in 70% glycerol/30% PBS at 4°C.

For viewing and mounting, the embryos were washed back down the glycerol gradient to 100% PBS and then received a graded dehydration with ethanol as per general antibody labeling protocol, followed by clearing in methyl salicylate. The tissue was assessed immediately, as the reaction product is somewhat soluble in methyl salicylate.

## **2.8 Dissection of Embryonic Nerve Cords and Subsequent Processing for TEM** (protocol adapted from J.R. Jacobs)

A thermanox slide was affixed to a glass slide with a dab of silicone cement. Thermanox serves as a sticky substrate for the tissue to adhere to. A 2 cm<sup>2</sup> well was created around the thermanox slide, using the silicone sealant. The well was deep enough to hold low surface tension solvents. A large patch of double sided tape was placed inside the well. With a razor blade, a cut was made 4 mm from the edge of the tape. With forceps, clumps of appropriately aged embryos were placed on the larger patch of tape. The chorion was rolled off using forceps and each embryo was then placed dorsal surface up, on the 4 mm strip of tape. Once a satisfactory number of embryos had been transferred, the large patch of sticky tape was removed. Schneider's medium (Life Technologies, cat#: 11720-034) was used as a dissecting medium to preserve tissue integrity. The embryos were dissected with an electrolytically sharpened tungsten needle. These are made by placing tungsten wire down the shank of a 25 gauge needle on a 1 mL syringe. The needle was sharpened to a rapidly tapering tip in 10% NaNO<sub>2</sub> at 10 V.

The embryos were poked at one end with the needle tip and buffer is allowed to enter the embryos for 10 seconds. The embryos is re-entered with the needle and the needle is run along the embryos as close to the dorsal surface as possible, essentially cutting the embryos loose from the vitelline membrane. As the needle was lifted, the embryos were removed from its vitelline package. The embryos were gently placed ventral surface down on the slide and the head and tail are ensured to be firm against the thermanox. With the needle, the gut was rolled out and the epidermis was peeled back to adhere to the thermanox. The ventral nerve cord was revealed.

All processing took place within the silicone well, as the nerve cords remained adhered to the thermanox. The Schneider's media was extensively washed out and replaced with fresh 2% FLUKA gluteraldehyde in 0.1 sodium cacodylate buffer (pH 7.4) and the nerve cords were fixed for 10 minutes. This was followed by extensive washes in cacodylate buffer. Nerve cords from embryos containing a P[*lacZ*] element were incubated in X-gal staining solution (10 mM phosphate buffer, pH 7.2; 150 mM sodium chloride; 1 mM magnesium chloride; 3.1 mM potassium ferrocyanide; 3.1 mM potassium ferricyanide) for 5 minutes. This solution was then removed and replaced with the same staining solution to which 25 mL/mL of 8% Bluo-gal in dimethylsulfoxide (DMSO) was added. The reaction solution was prepared by first heating the X-gal staining solution to 65°C, followed by the addition of the Bluo-gal substrate. The nerve cords were incubated overnight at room temperature.

The reaction solution was removed and the nerve cords were fixed for 30 minutes in primary EM fix (2% paraformaldehyde and 2.5% gluteraldehyde in 0.1 M sodium cacodylate, pH 7.4). The tissue was washed extensively with cacodylate buffer, followed by fixation in 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 minutes. The nerve cords were then washed in cacodylate and then equilibrated with distilled water, before

staining with 5% uranyl acetate for 30 minutes. The nerve cords were then washed with distilled water.

The nerve cords were then dehydrated with a series of washes in 50%, 70% and 90% ethanol for 10 minutes each. This was followed by 2X washes each in 95% and 100% ethanol for 10 minutes. Finally, they were washed in 100% ethanol for 30 minutes. The embedding procedure started with the samples being first incubated in a 1:1, followed by a 1:3 ratio mixture of absolute ethanol to plastic each for 1 hour. They were then incubated in 100% plastic overnight in the dessicator. The plastic consisted of 4.4 g Araldite, 6.2 g Epon, 12 g DDSA and 0.3 mL DMP-30. On the following morning, fresh plastic was exchanged in the well, and the plastic was polymerized for 48 hours in the oven at 80°C, while under vacuum.

After plastic polymerization, the plastic was removed with the thermanox from the glass slide by sliding a razor blade between the thermanox and glass slide. Individual embedded nerve cords were cut out of the block, trimmed and mounted on a blank block in sagittal orientations using commercial crazy glue. A few drops of plastic was applied over the embedded dissection and the whole block was placed in the oven for 12 hours to polymerize. The mounted dissection was then trimmed using a razor blade to prepare for sectioning. 60-90 nm ultrathin sections were obtained and collected onto slot grids which were coated with 0.2% formvar. The sections were post-stained with lead citrate for three minutes followed by extensive washing in boiled water. They were viewed with a transmission electron microscope.



## **2.9 Photography and Film Processing**

Whole mount embryos which were cleared and stored in methyl salicylate were mounted with permount under a cover slip on glass slides. They were viewed and photographed through a Zeiss Axiophot microscope. Kodak technical pan 35 mm film at a speed of 25 ASA was used for black and white pictures. The film was developed with Kodak technidol liquid developer. Fujichrome 64 Professional T "RTP" 35 mm film at a speed of 64 ASA was used for colour photographs.

## RESULTS

### 3.0 *argos* is a negative regulator of midline glia survival

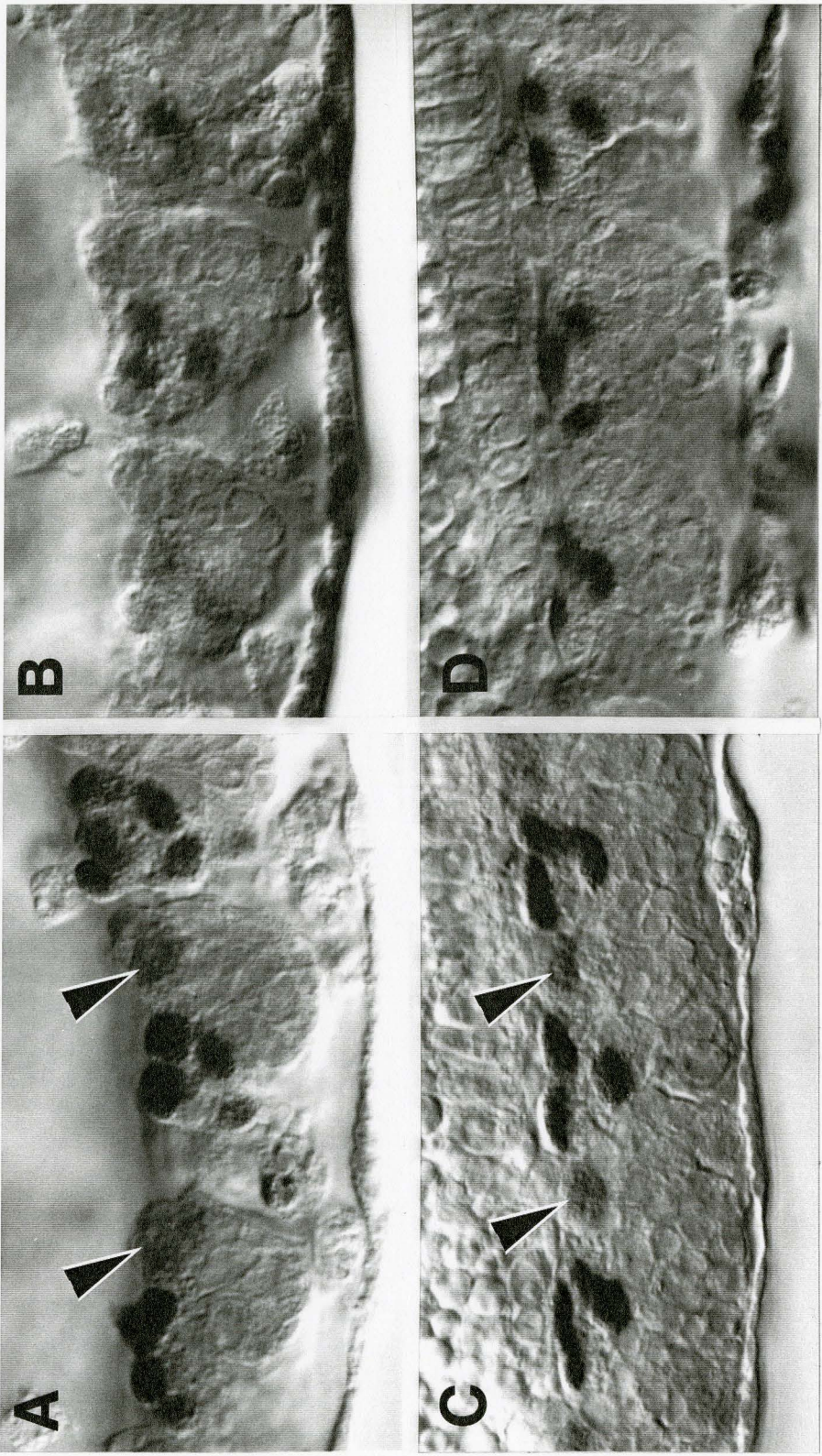
Different numbers of MG and MG in different positions within in each segment survive through embryogenesis. Thus, the pattern of MG survival is stochastic (Sonnenfeld and Jacobs, 1995a). This suggests that MG survival is regulated by non cell autonomous means. Furthermore, genes whose hypomorphic or hypermorphic mutations impart an increase or decrease in MG number respectively, might have a wildtype function which accounts for a developmental decrease in MG number. Such a gene might encode a negative regulator of MG survival. Experiments described below suggest that *argos* might function in this manner.

*argos* expression is coincident with the temporal pattern of MG apoptosis, following morphological establishment of the commissural axons which cross the midline of the VNC. As monitored with the *argos*<sup>w11</sup> enhancer trap, wildtype *argos* expression was first detected at late stage 12, but it becomes more pronounced and resolved to 2.61 +/- 0.13 MG/segment (n=10) at stage 13 (Fig. 1B). It should be noted that the above mean and all subsequent expressed means are presented with standard error. The means were calculated by averaging the MG cell counts from the abdominal segments A1 to A7 for each embryo which were in turn averaged for n number of embryos. The spatial distribution of *argos* expression in the midline remains relatively static, but does increase

slightly to  $2.92 \pm 0.07$  MG/segment ( $n=10$ ) by stage 15 (Fig. 1D) and to  $3.14 \pm 0.10$  MG/segment ( $n=10$ ) by stage 17 (Fig. 2A). The discrete expression pattern of *argos* is unique because most genes whose expression is restricted to the MG like *slit* and *DER* are expressed throughout the MG lineage in an initial population of 8-12 MG/segment (Fig. 6A,B; Dong and Jacobs, in preparation). One exception is *pointed*, a gene encoding an ETS domain containing transcription factor, necessary for terminal MG differentiation and survival (Klänbdt, 1993) which like *argos*, is only expressed in a subset of the MG lineage (Dong and Jacobs, in preparation). *argos* expression was contrasted with the expression of AA142, a MG specific enhancer trap which has yet to be molecularly characterised. The pattern of AA142 expression provides a good representation of cell reduction within the MG lineage through apoptosis (Sonnenfeld and Jacobs, 1995a). AA142 is first expressed in the midline of early stage 12 embryos, and is clearly expressed in 5-6 MG per segment at stage 13 (Fig. 1A). AA142 is expressed weakly in the MGP which occupy a posterior position in each neuromere. AA142 expression is reduced to  $3.83 \pm 0.16$  MG/segment ( $n=11$ ) by stage 15 (Fig. 1C) and further to about 3.2 MG/segment by stage 16 (Fig. 5A) as the MG are lost through apoptosis. It is interesting to contrast the expression pattern of AA142 to that of *argos*. *argos* is the only characterised MG marker to date whose expression in the MG lineage increases through embryogenesis. Furthermore, the unique expression pattern of *argos* at the onset of MG apoptosis is suggestive of it functioning in such a process.

**Figure 1. *argos* expression is restricted to the subset of MG which survive.**

Embryos containing one copy of the AA142 (A,C) and *argos*<sup>w11</sup> (B,D) enhancer traps were stained with antibodies against  $\beta$ -galactosidase to compare their wildtype expression patterns. Each panel represents sagittal views of three ventral nerve cord (VNC) segments, with the anterior of the embryo at left and dorsal at top. Unless stated otherwise, all subsequent figures have panels with embryos oriented in this manner. Immediately after commissure separation in stage 13 embryos, there are 5-6 AA142 expressing MG (A) and only 3 *argos*<sup>w11</sup> expressing MG (B). The MGP which occupy a posterior position in each segment are denoted by arrowheads in A and C. By stage 15, apoptosis has reduced the number of AA142 expressing MG (C), but not the number of *argos*<sup>w11</sup> expressing MG (D).



*argos* is believed to act as a negative regulator in determination and differentiation pathways in the developing eye disc, wing vein primordia, chordotonal organ precursors, and the embryonic ventral ectoderm (Freeman *et al.*, 1992a; Kretzschmar *et al.*, 1992; Okano *et al.*, 1992; Freeman, 1994a; Sawamoto *et al.*, 1994; Brunner *et al.*, 1994; Golembo *et al.*, 1996a; Okabe *et al.*, 1996; Freeman, 1996). We demonstrate here a similar role for *argos* in the midline, acting as a negative regulator of MG survival.

*argos<sup>w11</sup>*, a hypomorphic allele, is a disruptive P-element insertion in the regulatory region upstream of the *argos* open reading frame (Freeman *et al.*, 1992a). At the end of embryogenesis when the MG apoptotic event is normally complete, stage 17 *argos<sup>w11</sup>* homozygotes have extra surviving *argos* expressing MG (5.05 +/- 0.15 MG/segment, n=10; Fig. 2B). Similarly, stage 17 *argos<sup>A7</sup>* homozygotes, an amorphic allele created by the imprecise excision of the *argos<sup>w11</sup>* P-element, have a slightly more severe phenotype with 5.30 +/- 0.15 MG/segment (n=10) surviving, identified by the *slit1.0lacZ* reporter fusion construct (Fig. 2C). These extra surviving cells represent only the MGA and the MGM, as there are no *slit1.0lacZ* marked MGP which are normally located posterior to the posterior commissure in the dorsal midline (see arrowhead). However, from an initial population of 10-12 general MG, Dong and Jacobs, (in prep.) discovered that there are 4 MGPs. One can infer that there are 6-8 MGA/MGM. Therefore, it appears that not all MGA and MGM survive in *argos* loss of function mutants. Indeed, cell death profiles were observed in some *argos<sup>A7</sup>* homozygote embryos (data not shown). Apoptotic bodies were characterised at the light level as small condensed silhouettes labelled by immunoreactivity to perdurant lacZ (Sonnenfeld and

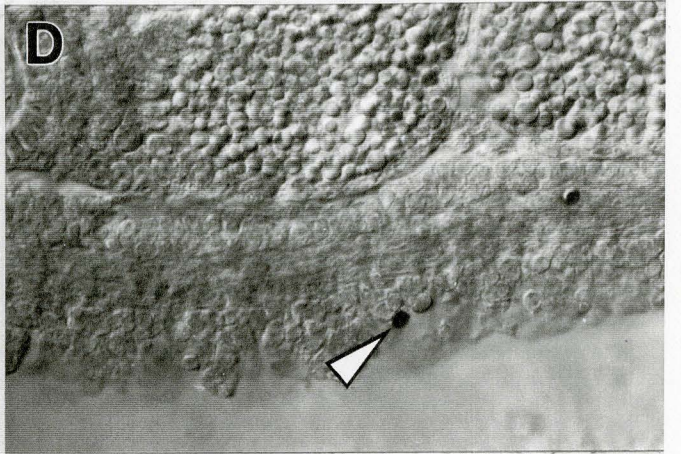
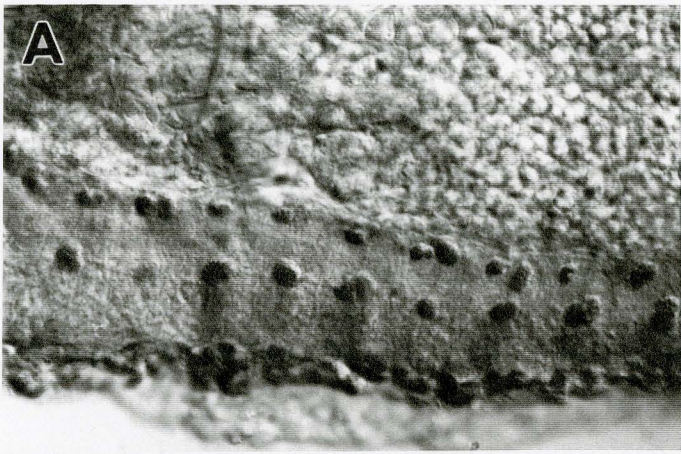


Jacobs, 1995a). These condensed profiles are also identifiable by their displacement and expulsion from the midline (Sonnenfeld and Jacobs, 1995b). To further confirm the absence of any surviving MGP in the *argos* null mutant, embryos were incorporated with the enhancer trap X55 which is strongly expressed in the MGP among other MEC neuronal lineages. By stage 16 in wildtype embryos, most if not all the MGP have entered apoptosis (Fig. 3A). This conclusion follows that in the normal reduction of the MG through apoptosis, there is a virtual 0% survival rate for the MGP (Sonnenfeld and Jacobs, 1995). Similarly in *argos* null embryos, there are no surviving MGP (Fig. 3B).

The effect of ubiquitous over-expression of *argos* through the *hsp70* promoter (Sawamoto *et al.*, 1994) was also assessed. To identify the MG, the *slit1.OlacZ* reporter was incorporated into a stable line of four copies of *HSargos*. Embryos were administered heat shocks beginning at stage 13 when apoptosis is first observed within the MG lineage and processed at stage 16 when the apoptotic event is normally complete. Three 45 minute heat shocks, each separated by 45 minute recoveries was sufficient to direct all the MG into apoptosis, as evidenced by numerous cell death profiles (Fig. 2D). Two 45 minute heat shocks resulted in the survival of only 0.36 +/- 0.04 MG/segment. Most if not all of these surviving glia were in the position of the MGM, situated between the anterior and posterior commissure. Even a single 45 minute heat shock with two copies of *HSargos* was sufficient to reduce MG numbers through apoptosis. While wildtype *argos* expressing MG have not been observed to undergo apoptosis (see below), they can be forced to cell death through ubiquitous over-expression of *argos*, as assayed

**Figure 2. Variations in the number of surviving MG through alterations in Argos function.** Embryos containing the *argos*<sup>w11</sup> enhancer trap (A,B) and *slit1.0lacZ* reporter construct (C,D) were labeled with antibodies to  $\beta$ -galactosidase. All panels show embryos at stage 17, a stage by which the MG apoptotic event is complete. In embryos containing one copy of the *argos*<sup>w11</sup> enhancer trap, there are wildtype numbers of surviving MG expressing *argos* (A). In embryos homozygous for the *argos*<sup>w11</sup> hypomorphic mutation, there are increased numbers of surviving MG expressing *argos* (B). Similarly, embryos homozygous for the amorphic allele *argos*<sup>Δ7</sup>, have increased numbers of surviving MG (C). There are no *slit1.0lacZ* marked MGP which are normally located posterior to the posterior commissure in the dorsal midline (see arrow); see Fig. 3A,B. Ectopic expression of *argos* directed by four copies of P[*HSargos*] dramatically reduces the number of MG (D). Cell death profiles are observed (see arrowheads).





through the *argos*<sup>w11</sup> enhancer trap in a background of two copies of *HSargos* (data not shown).

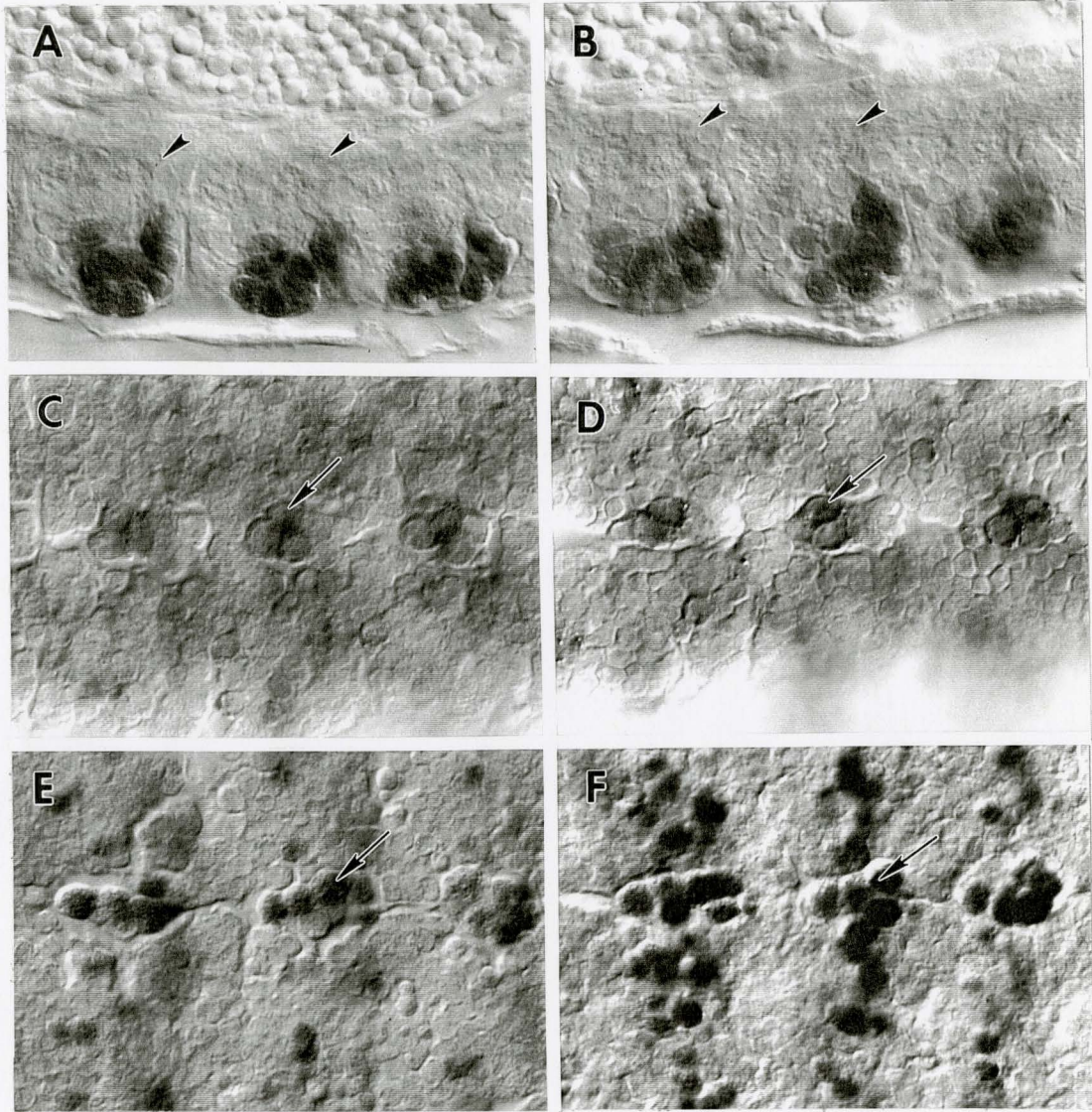
The MG were most susceptible to ubiquitous over-expression of *argos* during the normal period of apoptosis mediated MG reduction, stages 12 through 16. Embryos processed at stage 12 and which received a series of heat shocks leading up to and including this stage did not display as severe a reduction in MG relative to stage 16 embryos receiving the same heat shock regime. Similarly, heat shocks administered later than stage 16 and/or during larval development had a very minor, if any effect on MG number.

An alternative hypothesis to explain why supernumerary MG are detected in *argos* loss of function mutants, is that they are derived at the expense of the other mesectodermal lineages. Wildtype *argos* might function as a negative regulator of MG determination. Because of their relatively high cell numbers in the MEC, two such lineage candidates are the six ventral unpaired median neurons (VUMs) and the six neurons and glia progeny of the median neuroblast (MNB) lineage (Klämbt *et al.*, 1991; Patel *et al.*, 1989). The VUMs and MNB progeny occupy a ventral position in the posterior region of each segment in the CNS midline. These cells were visualised through the incorporation of the X55 enhancer trap and the VUMs and MNB progeny were also individually labeled through the monoclonal antibodies 22C10 and 4D9, respectively (see methods). Both wildtype and *argos*<sup>A7</sup> mutant embryos have 10-12 X55 expressing cells in each segment of the ventral midline, as viewed in the sagittal plane (Fig. 3A,B). As assessed by 22C10, both wildtype and *argos*<sup>A7</sup> embryos have 4-5 clearly

**Figure 3. The VUMs and MNB develop independently of Argos function.**

Embryos containing the X55 enhancer trap (A,B) which is expressed in the MGP, VUMs, and MNB with its support cells were labeled with antibodies to  $\beta$ -galactosidase. The VUMs (C,D) and the MNB and its progeny (E,F) were further analysed by using the monoclonal antibodies 22C10 and 4D9, respectively. *argos*<sup>A7</sup> homozygotes (B,D,F) were identified through absence of the balancer chromosome specific *P[actin-lacZ]* staining as assayed through antibodies to  $\beta$ -galactosidase. Embryos in A and B are in sagittal view, while C-F represent VNC dissections in frontal view. All panels show stage 16 embryos. In both wildtype (A) and *argos*<sup>A7</sup> (B) embryos, there are no surviving MGP, as assayed by the absence of X55 expressing cells in the posterior part of each segment in the dorsal midline (see arrowheads). In both wildtype (A) and *argos*<sup>A7</sup> mutants (B), there are 10-12 X55 expressing cells, which are the VUM neurons and MNB and its progeny. Individually, there are 4-5 VUM neurons observable at the chosen plane of focus in both wildtype (C) and *argos*<sup>A7</sup> mutants (D) and 5-6 MNB and support cells in both wildtype (E) and *argos*<sup>A7</sup> (F) embryos. Clusters of these cells are denoted by arrows.





visible VUM neurons arranged in grape-like clusters (Fig. 3C,D). Finally, both wildtype and *argos*<sup>A7</sup> embryos have 5-6 clearly visible Engrailed labeling MNB progeny cells in each segment of the ventral midline (Fig. 3E,F). Characteristically, these cells can be observed extending in a linear array.

The studies outlined above suggest that *argos* mediates the reduction of MG number and acts independently of other MEC lineages.

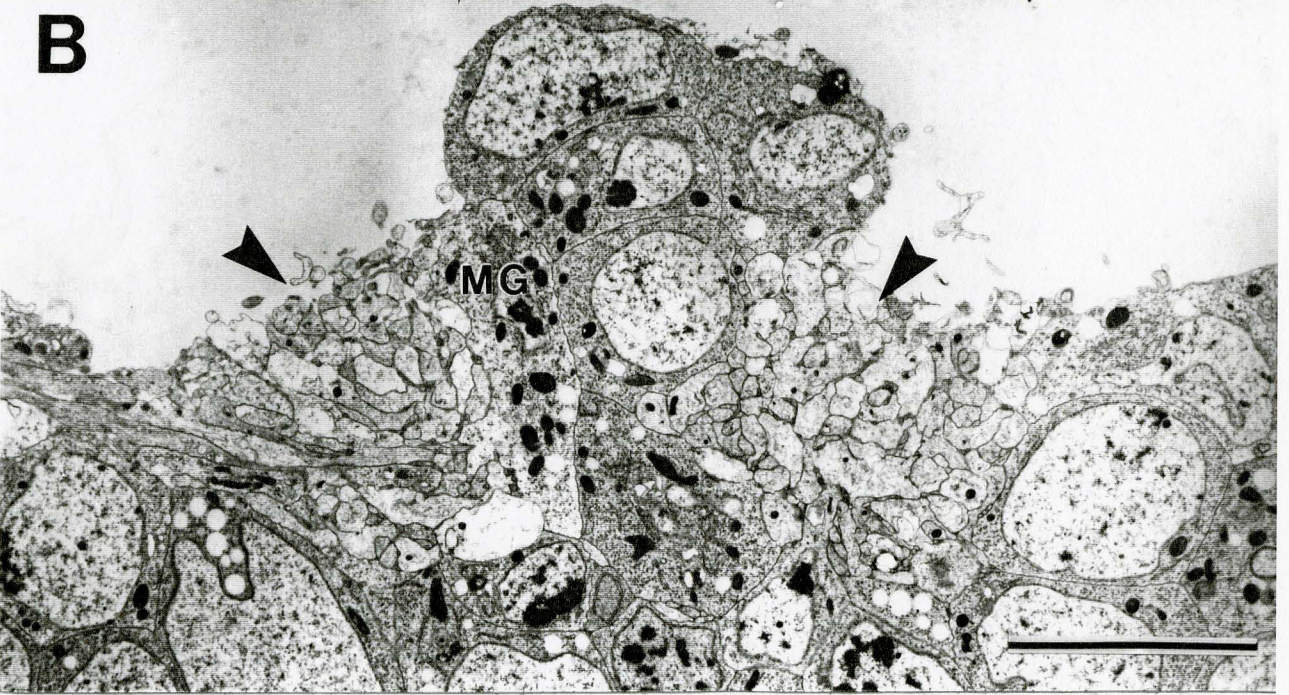
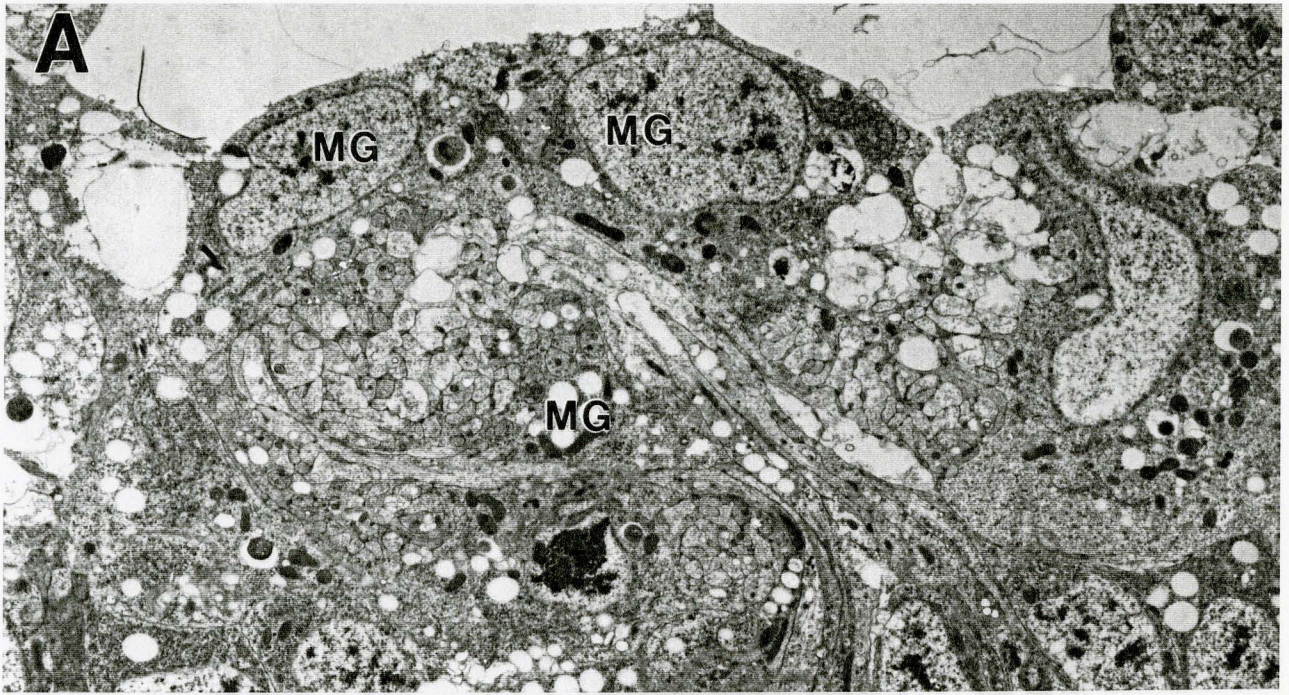
### **3.1 Ectopic loss of MG compromises axon ensheathment and affects central nervous system morphology**

The MG are intimately involved in commissural axon tract pioneering, morphogenesis, and maintenance in the establishment of the CNS (Jacobs and Goodman, 1989; Rothberg *et al.*, 1990; Klämbt *et al.*, 1991; Sonnenfeld and Jacobs, 1994; Sonnenfeld and Jacobs, 1995a; Harris *et al.*, 1996; Tear *et al.*, 1996). Therefore, the removal of MG through ubiquitous over-expression of *argos* would be expected to have a profound effects on CNS development.

Ultrastructural analysis of the consequences of *HSargos* elicited MG loss in stage 14 embryos shows that the commissural axons are not ensheathed as compared to the wildtype heat shock control of the same age (Fig. 4). In the wildtype embryo, the MG lamellipodia enwrap the commissures, while this characteristic is much reduced in the *HSargos* embryo. The one remaining MG does display some ensheathment. Furthermore, there is evidence of axon membrane damage on the dorsal surface of the commissures in the *HSargos* embryo. As in wildtype, the single surviving MG in this *HSargos* embryo

**Figure 4. MG loss through *argos* misexpression compromises commissural axon ensheathment.** Wildtype embryos receiving heat shock treatment have normal numbers of MG ensheathing the commissural axons (A). Ectopic expression of *argos* in *HSargos* embryos results in reduced numbers of MG and considerable desheathment of the commissural axons is observed (B, see arrowheads). Scale bar: 4  $\mu\text{m}$ .







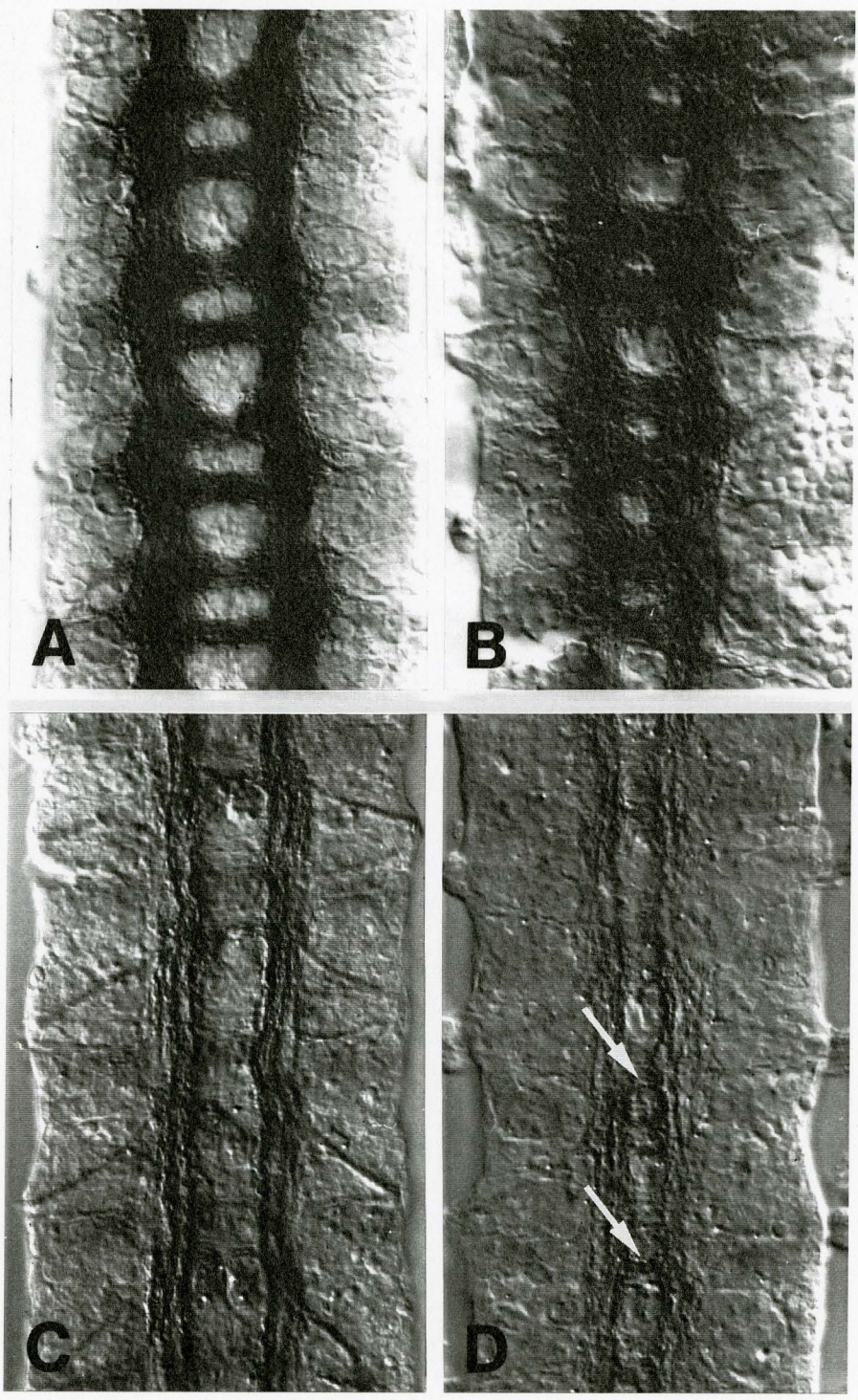
shows typical characteristics of differentiated glia, including extensive endoplasmic reticulum and electron lucent cytoplasm (Jacobs and Goodman, 1989a; Sonnenfeld and Jacobs, 1994).

Perturbations in the CNS axon tract architecture were analysed. Wildtype and *HSargos* embryos received two 45 minute heat shocks, separated by a 45 minute recovery and followed by a long final recovery of 4.25 hours. Therefore, these stage 16 embryos received heat shock treatments beginning at stage 12 when the commissural axon growth cones make their initial trajectories to the midline. This is followed by the modeling and separation of the commissures by the MG. The embryos were processed and labeled with the monoclonal antibody, BP102 which recognises an uncharacterised carbohydrate moiety on axons of the CNS. In the heat shocked wildtype control, the pioneering and subsequent separation of the anterior and posterior commissures appears to have proceeded typically, and by this stage, the commissures and the longitudinal connectives form a ladder-like structure (Fig. 5A). However, the commissures of the *HSargos* embryo appear to be incompletely separated and are broader than those of the wildtype (Fig. 5B). Furthermore, the CNS appears slightly narrower than that of wildtype.

The broader commissures of *HSargos* embryos was of interest because it resembles the phenotype of the *roundabout* loss of function phenotype (Seeger *et al.*, 1993; Goodman, pers. communication). In *roundabout* mutants, axons that are normally ipsilaterally directed along the longitudinal connectives, cross the midline. To more accurately characterise this phenotype, the embryos were labeled with a MAb to Fasciclin II. Fasciclin II distribution is specific to a subset of axon fascicles of the longitudinal



**Figure 5. MG loss through *argos* misexpression affects commissural axon tract morphology.** Each panel shows VNC dissections in frontal view with anterior at top. All embryos received heat shocks beginning at stage 12, followed by aging to stage 16 before processing, (for specifics, see text). The CNS axon tracts were stained with BP102 MAb (A,B) and FasII MAb (C,D) through HRP immunocytochemistry. Heat shocked wildtype controls have normal CNS axon tract morphology (A,C). The ladder-like structure formed by both the anterior and posterior commissures and the longitudinal axon tracts is apparent (A). Bilaterally represented longitudinal tracts with three labeled fascicles are observed (C). Ectopic expression of *argos* in *HSargos* VNCs alters commissural axon regularity (B,D). The commissures appear more fuzzy and broader (B) and there is misexpressed FasII immunolabeling crossing the midline (D, see arrows). The CNS of *HSargos* VNCs are also narrower than those of wildtype.



connectives and it is normally absent from segments of commissural axons crossing the midline. To ensure complete cell death in the MG lineage, the embryos were subjected to three 45 minute heat shocks each separated by 45 minute recoveries and followed by a final 2.75 hour recovery prior to processing. Therefore, the time of application and the overall duration of the heat shock and recovery series remained the same as that described for the MAb102 experiment. In the stage 16 wildtype heat shock control, three clearly defined fascicles are observed in the longitudinal connectives, while there is no ectopic FasII deposition crossing the midline (Fig. 5C). In *HSargos*, the longitudinal connective fascicles appear more irregular and are less clearly defined (Fig. 5D). Furthermore, there is ectopic FasII expression that appears to circumvolute the midline. Certain segments are affected more severely (see arrows). Again, the CNS is more narrow in *HSargos* embryos.

Interestingly, aspects of the MG, 102 and FasII phenotypes in *HSargos* embryos resemble phenotypes of hypomorphic and null mutations in the *spitz* group and *DER* pathway genes (Klämbt *et al.*, 1991; Raz and Shilo, 1992; Sonnenfeld and Jacobs, 1994; Dong and Jacobs, unpublished data). In mutants for these classes of genes, the MG die, resulting in detrimental developmental consequences for the CNS cytoarchitecture.

### **3.2 The *spitz* group and *DER* pathway are required for MG survival and are epistatic to *argos***

The Spitz group/*DER* signaling cassette is required for numerous developmental decisions in a variety of tissues during *Drosophila* embryogenesis (Bier *et al.*, 1990; Raz

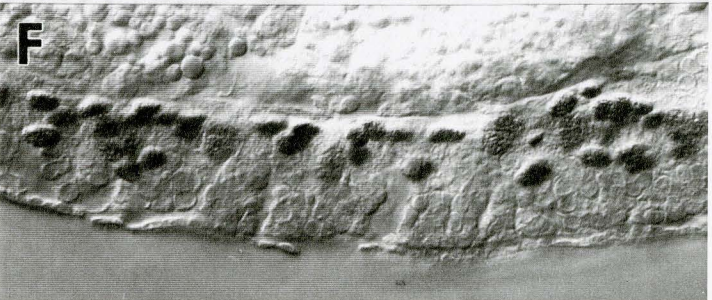
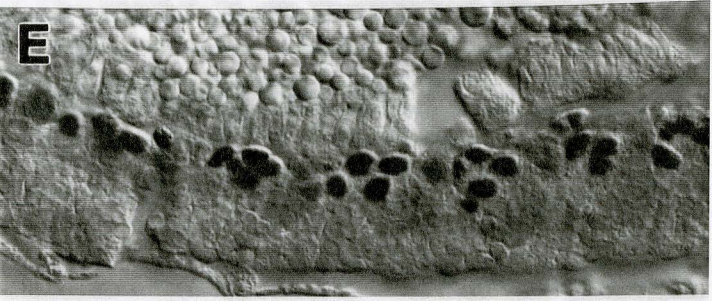
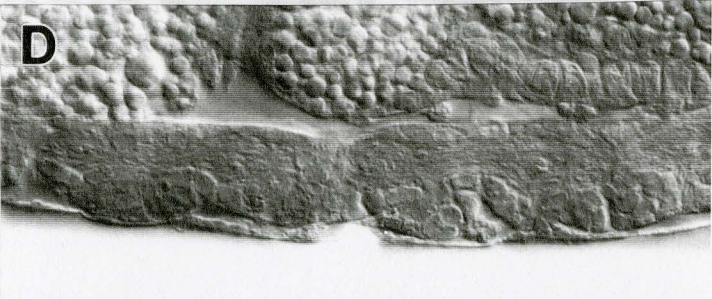
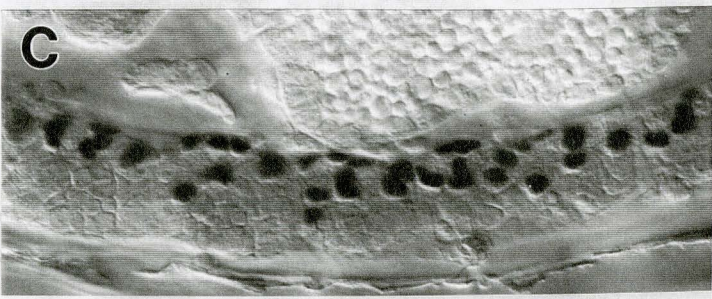
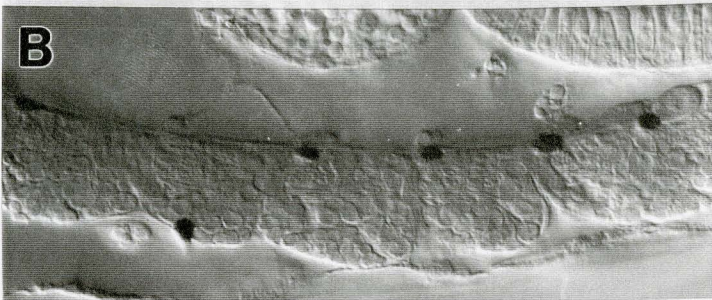
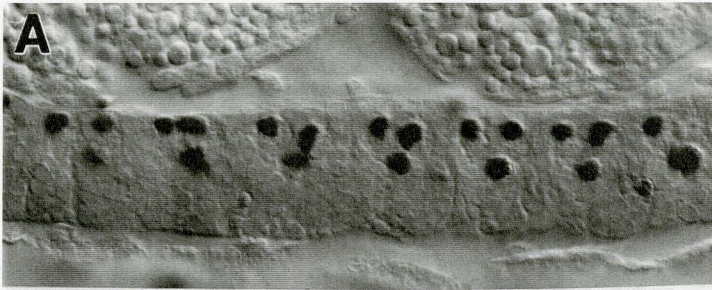
and Shilo, 1992; Rutledge *et al.*, 1992; Clifford and Schüpbach, 1992; Baumann and Skaer, 1993; Sturtevant *et al.*, 1993; Kolodkin *et al.*, 1994; Klämbt, 1993; Schweitzer *et al.*, a,b; Golembo *et al.*, 1996a,b; Freeman, 1996). In the midline, *spitz* group loss of function mutants like *Star*, *rhomboid*, *spitz*, and *pointed* and various allelic mutants of *DER* result in MG death (Klämbt *et al.*, 1991; Sonnenfeld and Jacobs, 1994). Therefore, like *HSargos*, the *spitz* group and *DER* pathway loss of function mutants affect MG survival.

Mutants of the *spitz* group/*DER* pathway were incorporated with the enhancer trap AA142 to identify the MG. By stage 16 in a wildtype embryo, apoptosis has reduced the MG lineage to 3.2 MG/segment and the surviving MG assume an inverted ladder-like arrangement at the dorsal midline (Fig. 6A). In stage 16 *rho*<sup>438</sup> homozygotes, an amorphic allele created by the imprecise excision of the P-element in the *rhomboid* X81 enhancer trap (Freeman *et al.*, 1992b), there was fewer than one surviving MG per segment and of those, many were displaced dorsally (Fig. 6B). However, ubiquitous over-expression of *rhomboid* through the *hsp70* promoter resulted in supernumerary MG (Fig. 6C). These embryos received five 20 minute heat shocks from stage 12 through to stage 16. The *faint little ball* (*flb*) locus encodes DER, and *flb*<sup>2E07</sup> is a hypomorphic lethal allele. In *flb*<sup>2E07</sup> homozygotes, there were no AA142 positive cells remaining by stage 16 and it appears that most if not all MG succumb to apoptosis by this stage (Fig. 6D). Prior to stage 16 however, there were still some surviving MG per embryo as labeled by AA142 (data not shown). With *Ellipse*<sup>Bl</sup>, a gain of function mutation in DER, embryos displayed a delay in the reduction of MG numbers (Fig. 6E). Stage 15 *Ellipse*

**Figure 6. The *spitz* group/*DER* pathway is required for MG survival.**

Embryos containing the AA142 enhancer trap were stained with antibodies to  $\beta$ -galactosidase. All panels represent stage 16 embryos, except for E which represents a stage 15 embryo. In wildtype embryos, apoptosis has reduced the number of MG to approximately 3 MG/segment by stage 16 and they assume an inverted L-shape arrangement (A). In embryos homozygous for the amorphic *rho*<sup>438</sup> allele (B) and the hypomorphic *flb*<sup>2E07</sup> allele (D), there were significantly reduced MG numbers. In D, there were no AA142 expressing cells remaining. Embryos homozygous for the gain of function *DER* allele *Elp*<sup>B1</sup>, displayed a delay in the reduction of MG numbers, so that there were slightly increased numbers of AA142 expressing cells at stage 15 (E). Ectopic expression of *rhomboid* in *HSrho* embryos results in supernumerary MG per segment (C). Similarly, in C38-Gal4; *UAS-Draf* embryos in which an activated form of *Draf* is expressed in a subset of the mesectoderm, there are supernumerary MG up to stage 16 (F).





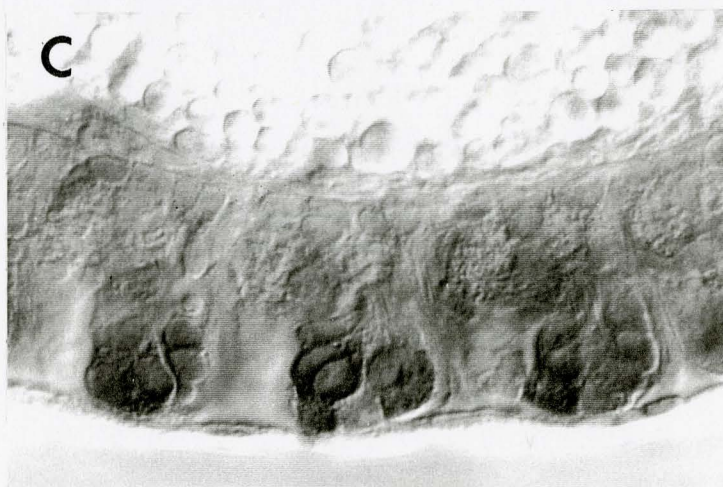
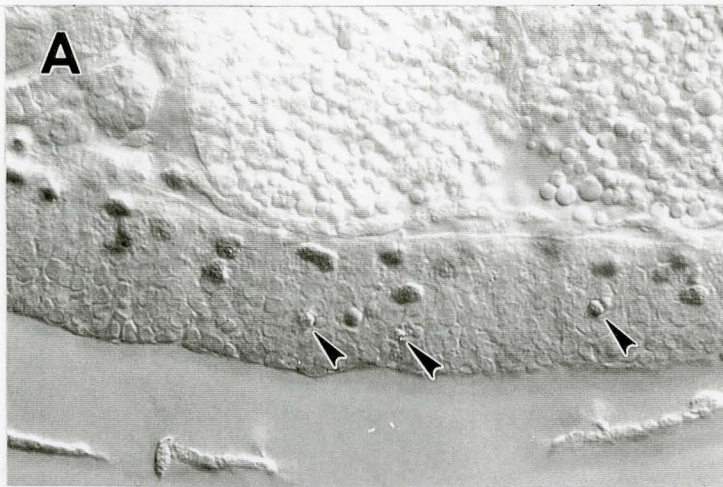
homozygotes had  $4.02 \pm 0.08$  MG/segment ( $n=12$ ) while similarly staged wildtypes had  $3.82 \pm 0.09$  MG/segment ( $n=10$ ). Stage 16 *Ellipse* homozygotes had  $3.46 \pm 0.10$  MG/segment ( $n=18$ ). Finally, over-expression of *D-raf* (*UAS-Draf*) in the MG lineage through the C38 Gal 4 enhancer trap (Yeh *et al.*, 1995) resulted in supernumerary MG (Fig. 6F) in embryos up to early stage 16. By late stage 16 however, there was ectopic cell death in the MG lineage of C38;*UAS-Draf* embryos (Fig. 7A). Some insights into this surprising result follows from observations of the *lacZ* expression pattern of the C38 Gal4 enhancer trap. As assessed in C38;*UAS-lacZ* wildtype embryos, C38 is expressed at stage 13 in dorsally located cells in the midline, whose morphology and position relative to the commissures is suggestive of the MG (Fig. 7B). C38 is also expressed in a tight cluster of cells located more ventrally, whose appearance and position is suggestive of the VUMs. By later stages, C38 is no longer expressed in the MG, but is exclusive to the VUMs and perhaps some cells of the MNB progeny (Fig. 7C).

Loss of function alleles of the various components of the *spitz* group/*DER* pathway result in ectopic MG death, as in the case of *HSargos*. Conversely, both loss of function alleles of *argos* and over-expression constructs of *rhomboid* and *D-raf* lead to increased numbers of surviving MG. Therefore, these data suggest that *argos* and the *spitz* group/*DER* signaling cassette have opposing functions in the regulation of MG survival. Furthermore, *in vivo* studies in the embryonic ventral ectoderm and *in vitro* cell culture studies show that *argos* expression is induced by the DER pathway, and that *argos* can in turn inhibit this pathway through DER receptor inactivation (Golembo *et al.*,

**Figure 7. Late stage C38-Gal4; *UAS-Draf* embryos exhibit increased MG apoptosis.**

By late stage 16, there is ectopic MG cell death in C38-Gal4; *UAS-Draf* embryos, as observed with the enhancer trap AA142 (A). Numerous apoptotic AA142 expressing cell bodies can be seen (see arrowheads). The expression pattern of C38 in the mesectoderm was elucidated through C38-Gal4; *UAS-lacZ* embryos which were stained with antibodies to  $\beta$ -galactosidase. At stage 13, C38 is expressed in dorsally located cells which appear to be the MG (arrows). C38 is also expressed in a more ventrally located cluster of cells in the mesectoderm which is outside the focal plane in this photomicrograph (B). By stage 16, C38 is only expressed by the ventral mesectodermal cells, whose morphology is suggestive of the VUMs and perhaps some cells of the MNB group (C).





1996a; Schweitzer *et al.*, 1995b;). What is the epistatic hierarchy between the *spitz* group/*DER* pathway and *argos* in the context of the regulation of MG survival?

A number of homozygous double mutant combinations between genes at various levels of the *spitz* group/*DER* pathway and the hypomorphic allele *argos<sup>w11</sup>* were analysed. In the *rho<sup>38</sup>-argos<sup>w11</sup>* double mutant, there is less than one surviving MG per segment by stage 16, but *argos* expression is still quite strong (Fig. 8A). However, in *Star<sup>HN23</sup>; argos<sup>w11</sup>* embryos, the few surviving MG express *argos* very weakly (Fig. 8B). Similarly, a double mutant of *argos<sup>w11</sup>* with the *E55* deficiency which uncovers *spitz*, resulted in very few surviving MG (Fig. 8C). Some of these surviving MG expressed *argos* strongly, while others expressed *argos* considerably less. In *flb<sup>2E07</sup>; argos<sup>w11</sup>* double mutants, there were a few *argos<sup>w11</sup>* positive cells remaining, but it was unclear whether these were surviving MG, or displaced cells from other *argos* positive tissues (Fig. 8D). Finally, in *pnt<sup>9J31</sup>-argos<sup>w11</sup>* double mutants, there was virtually no midline expression of *argos* (Fig. 8E). Therefore, the *spitz* group/*DER* loss of function phenotype was observed in all cases (compare to Fig. 7B,D), suggesting that this signaling cassette functions epistatically and upstream to *argos*. Furthermore, normal *argos<sup>w11</sup>* enhancer trap expression requires *spitz* group/*DER* pathway function.

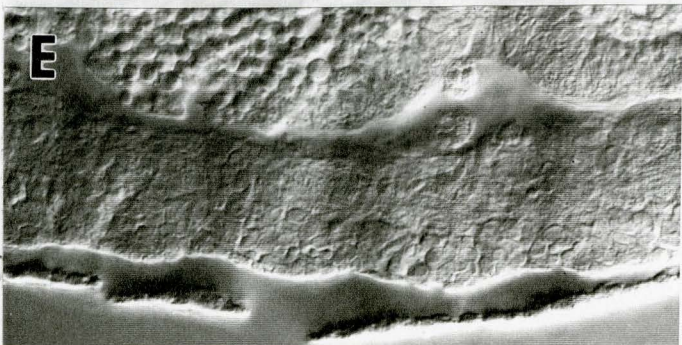
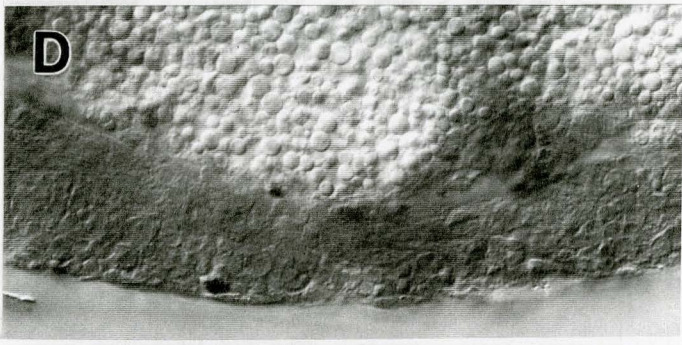
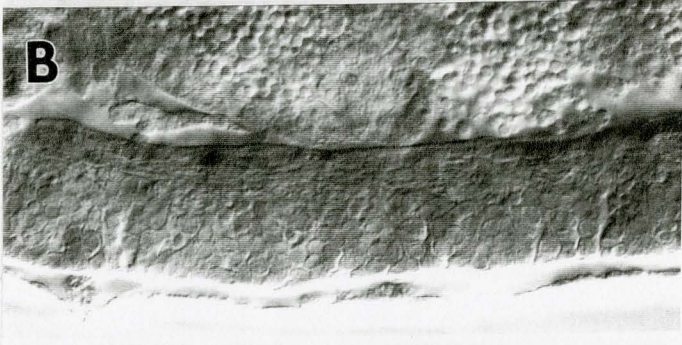
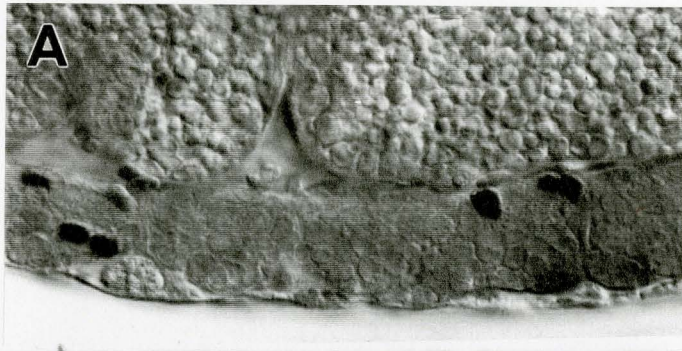
### 3.3 Argos mediates the down-regulation of DER mRNA levels

Argos has been shown to inhibit *spitz* mediated EGF receptor transphosphorylation, when assayed by amount of tyrosine phosphorylation of immunoprecipitated DER from S2 cell extracts (Schweitzer *et al.*, 1995b). The

**Figure 8. The *spitz* group/*DER* pathway is epistatic to *argos*.**

Double homozygotes of hypomorphic or null alleles in the *spitz* group/*DER* pathway with the hypomorphic *argos*<sup>w11</sup> allele are shown. *argos*<sup>w11</sup> is an enhancer trap and therefore embryos were stained with antibodies against  $\beta$ -galactosidase. The reduced MG number phenotype typical of single *spitz* group/*DER* pathway loss of function mutants is exhibited in all cases. In all double mutants, except for *rho*<sup>38</sup>-*argos*<sup>w11</sup> (A), *argos*<sup>w11</sup> enhancer trap expression is considerably reduced. *rhomboid*: *rho*<sup>38</sup>-*argos*<sup>w11</sup> (A). *Star*: *S*<sup>IN23</sup>; *argos*<sup>w11</sup> (B). *spitz*: *DfE55*; *argos*<sup>w11</sup> (C). *DER*: *flb*<sup>2E07</sup>; *argos*<sup>w11</sup> (D). *pointed*: *pnt*<sup>9J31</sup>-*argos*<sup>w11</sup> (E).





immediacy of this inhibition suggests a physical interaction of the Argos antagonist and the EGF receptor, followed by the internalization of the ligand-receptor complex. A potentially longer lasting mechanism of negative regulation would be the reduction of *DER* transcript levels (Sturtevant *et al.*, 1994). Secreted Argos might ultimately direct MG death through the attenuation of *DER* transcription.

To address this hypothesis, the effect of ubiquitous expression of *argos* on *DER* mRNA levels was analyzed. Wildtype and *HSargos* embryos carrying the *slit1.0lacZ* MG marker were subjected to a one hour heat shock, followed by a 45 minute recovery prior to processing. Wildtype heat shock controls received heat shocks at early stage 12 and stage 14 and aged to late stage 12 and stage 15 respectively, have normal numbers of MG and all are expressing *DER* mRNA (Fig. 9A,C). *HSargos* embryos which received similarly timed heat shock applications show a marked decrease in the number of surviving MG and the level of *DER* mRNA of surviving MG is significantly reduced (Fig. 9B,D). In *HSargos* embryos, the MG are displaced from their wildtype position. MG lacking contact with other MG expressed lower levels of *DER* mRNA than those MG which appear to be contiguous with other MG (data not shown).

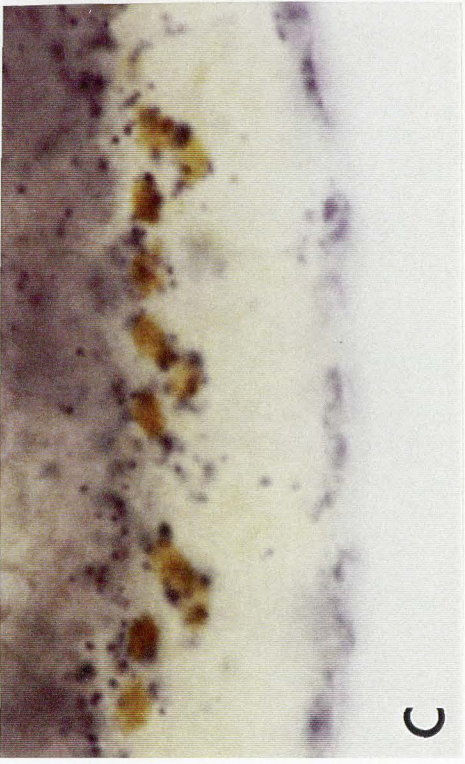
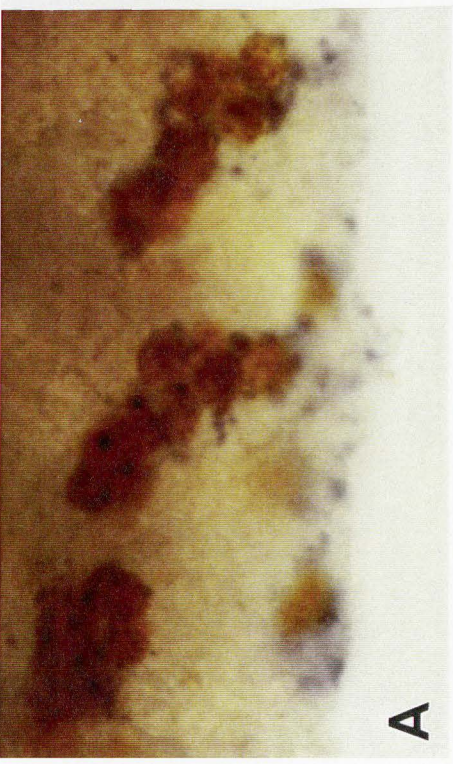
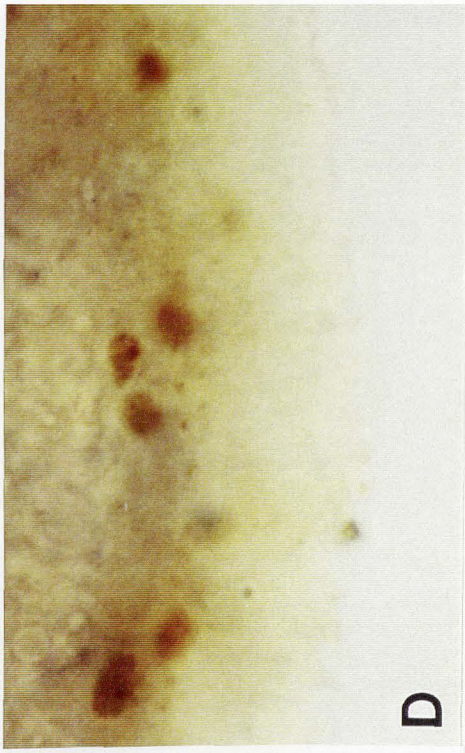
### **3.4 *argos* is only expressed in a subset of the MG**

As previously described, *argos* is unique in that it is expressed in a subset of cells of the MG lineage. Furthermore, the number of *argos* expressing MG does not decline during the natural reduction in the MG lineage due to apoptosis. Moreover, it appears that these *argos* expressing MG are the ones that survive beyond embryogenesis, as *argos*

**Figure 9. *DER* mRNA down-regulation following *argos* misexpression.**

Wildtype (A,C) and *HSargos* embryos (B,D) were subjected to a one hour heat shock beginning at stage 12 (A,B) and stage 14 (C,D) followed by a 45 minute chase. Embryos are doubly labeled for *slitlac1.0* expression as detected by antibodies against  $\beta$ -galactosidase (brown) and for *DER* mRNA (blue) as detected by *in situ* hybridization with a digoxigenin-labeled *DER* anti-sense RNA probe and visualised through alkaline phosphatase cytochemistry. The stage 12 (A) and stage 15 (C) wildtype embryos have *slit1.0lacZ* expressing MG which express significant levels of *DER* mRNA. The similarly staged *HSargos* embryos (B,D) have *slit1.0lacZ* expressing MG with much reduced levels of *DER* mRNA.





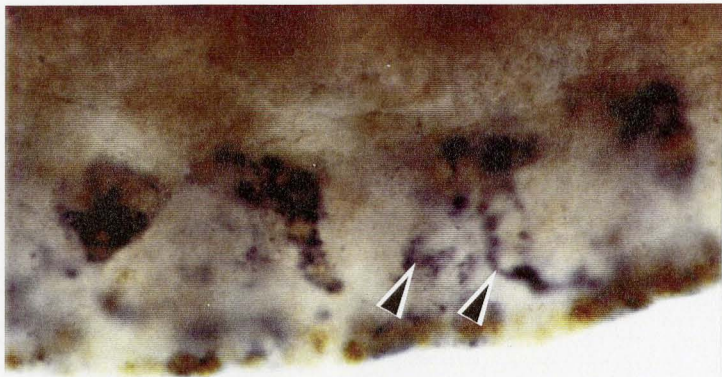
9-9

expressing MG in *argos*<sup>w11/+</sup> wildtype embryos have not been observed to express *reaper*, as assayed through RNA *in situ* hybridization (n=30), (data not shown). Figure 8 shows the enhancer trap expression patterns of *argos* relative to the temporal and spatial mRNA transcript patterns of two other MG specific markers, *slit* and *DER* in *argos*<sup>w11/+</sup> embryos. *slit* and *DER* expression first appear in early stage 12 embryos at the time of germ band retraction. This precedes *argos* expression, but by late stage 12 *argos* was first observed in one to two cells of the 8-10 *slit* and *DER* expressing cells, which were observed in a dorsal to ventral pattern in each segment (Fig. 10A,B). In stage 14 embryos, approximately three MG per segment are expressing *argos* and these same cells are also expressing *slit* and *DER* (Fig. 10C,D). At the posterior of each segment are *slit* and *DER* expressing cells which lack *argos* enhancer trap expression. It is also interesting to note the ventrally displaced *slit* expressing MG (Fig. 10C, arrowhead), which typically lacks for *argos* enhancer trap expression. MG become dislocated from the area of the commissural axons while undergoing apoptosis (Sonnenfeld and Jacobs, 1995a). By stage 16, apoptosis has dramatically reduced the number of *slit* and *DER* expressing MG, so that the only surviving MG are those that express both *argos* and *slit* and *DER* (Fig. 10E,F).



**Figure 10. The subset of MG which survive express *argos*.**

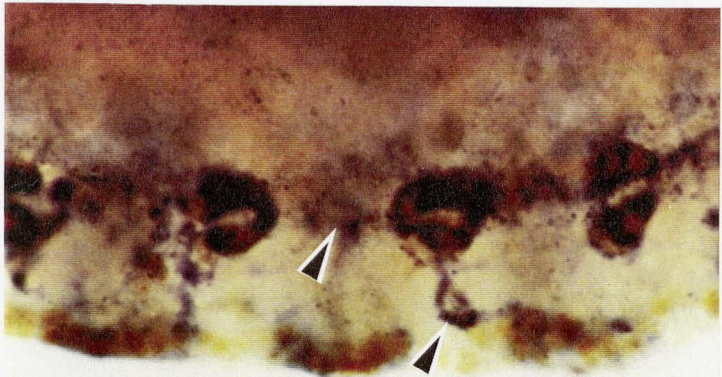
The wildtype expression pattern of the *argos*<sup>w11</sup> enhancer trap, *argos*<sup>w11/+</sup> (brown staining) is contrasted with the expression patterns of *slit* mRNA (blue staining; A,C,E) and *DER* mRNA (blue staining; B,D,F) in the MG. At stage 12 (A,B), *slit* and *DER* expression is observed throughout the MG lineage while *argos* expression is sparse. At stage 14 (C,D) *argos* is expressed in only a subset of the *slit* and *DER* expressing cells. Note the *slit* and *DER* expressing MG in the posterior of each segment which lack *argos* expression (arrowheads). Also note the ventrally displaced *slit* expressing cell typically absent for *argos* expression (arrowhead, C). By stage 16 (E,F), the only surviving MG are those that express both *argos* and *slit* and *DER*.



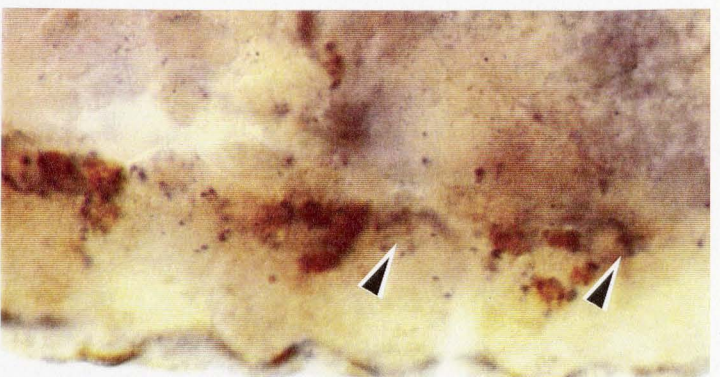
**A**



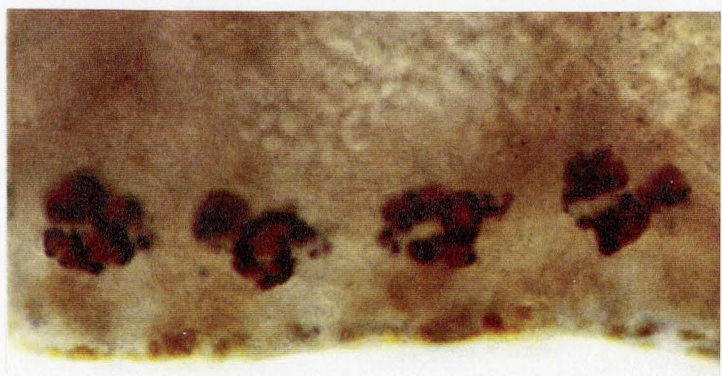
**B**



**C**



**D**



**E**



**F**

## SUMMARY AND DISCUSSION

### 4.0 *argos* and the *spitz* group/*DER* signaling cassette function to regulate MG cell numbers

From the mesectoderm, a surplus of MG are specified. However, the MG is a transient cell type. After serving guidance and morphogenetic roles in the establishment of commissural axons, most undergo apoptosis to leave an average of 3.2 MG per segment by the end of embryogenesis. The surviving MG display intersegment variability in their number and position relative to the commissures (Sonnenfeld and Jacobs, 1995a). Therefore, the regulation of the number and position of surviving MG is expected to be mediated by non cell autonomous means. Previous studies have identified the *spitz* group/*DER* signaling cassette of genes as critical for MG survival because loss of function mutations in *rhomboid*, *Star*, *spitz*, *DER*, and *pointed* result in apoptosis of virtually all the MG (Klämbt *et al.*, 1991; Klämbt, 1993; Raz and Shilo, 1992; Sonnenfeld and Jacobs, 1994). We provide evidence to further support the hypothesis that the *spitz* group/*DER* pathway is a positive regulator of MG survival. Gain of function alleles of *DER* (*Ellipse<sup>BI</sup>*) result in a delay in the reduction of MG numbers. Furthermore, misexpression of *rhomboid* and *D-raf* (*UASD-raf*) result in supernumerary surviving MG.

Our data suggest that Argos serves as a non-autonomous factor imparting the negative regulation of MG survival. Misexpression and loss of function perturbations of the *argos* gene have opposite MG phenotypes to the respective misexpression and loss of function disruptions of genes of the *spitz* group/*DER* pathway. In embryos lacking *argos* function, there are extra surviving MG in each segment while ectopic over-expression of *argos* results in increased apoptosis among the MG. Furthermore, the MG are most sensitive to *argos* misexpression during the normal course of MG number reduction. The initiation of wildtype *argos* expression is coincident with the onset of the apoptotic program in the MG lineage. *argos* enhancer trap expression increases gradually in the MG lineage up to a point where it is only expressed in that subset of MG which survive past embryogenesis. *argos* enhancer trap expression in the midline begins after the mesectodermal cells have been determined, precluding a determinative role for *argos*. Moreover, the other MEC lineages examined, the VUMs and MNB, develop independently of *argos* function.

Loss of function alleles of the various elements of the *spitz* group/*DER* pathway and *HSargos* constructs result in similar phenotypes not only in the midline, but in other tissues as well. In the developing eye, mutations in *spitz*, *Star*, *DER* and *pointed* and ectopic expression of *argos* perturb proper photoreceptor determination and differentiation (Freeman, 1994b; Tio *et al.*, 1994; Koldkin *et al.*, 1994; Freeman, 1996; O'Neill *et al.*, 1994; Freeman, 1994a; Sawamoto *et al.*, 1994; Brunner *et al.*, 1994). Similarly, *spitz/DER* group nulls and hypomorphs and ectopic *argos* result in a lack of determination of ventral ectodermal tissue, wing veins, and chordotonal organs (Golembo

*et al.*, 1996a; Sturtevant *et al.*, 1993; Sawamoto *et al.*, 1994; Bier *et al.*, 1990; Okabe *et al.*, 1996). Conversely, embryos lacking *argos* function have an increased number of cells in each of these tissues (Freeman *et al.*, 1992; Okano *et al.*, 1992; Kretzshmar *et al.*, 1992; Sawamoto *et al.*, 1994; Okabe *et al.*, 1996; Golembo *et al.*, 1996). These previous studies suggest a highly regulated balance of opposing positive and negative signals in time and space which ultimately imparts the specification of cell fate (for review, see Freeman, 1997). We propose a similar mechanism for the *spitz* group/*DER* pathway and *argos* in regulating the number and position of surviving MG.

#### 4.1 *argos* requires *spitz* group/*DER* pathway function

How does *argos* function relate to *spitz/DER* group action? *argos* transcription is induced by the *spitz* group/*DER* pathway (Golembo *et al.*, 1996a). Similarly, from double mutant analysis, we have shown that the *spitz* group/*DER* pathway is epistatic and upstream of *argos* and *argos* expression in the MG depends on the *spitz* group/*DER* pathway signal. It should be noted that the *argos*<sup>w11</sup> mutation was used in the double mutant analysis because it is an enhancer trap and therefore it provides the added benefit of monitoring *argos* expression in *spitz/DER* pathway mutant backgrounds. While the *argos*<sup>w11</sup> mutation is not a null, it does result in a MG phenotype virtually indistinguishable from that of *argos*<sup>A7</sup>.

In these double mutants, the level of *argos* enhancer trap expression was correlated with the severity of the *spitz* group/*DER* pathway mutations and their corresponding position in the signaling cascade. While the *spitz* group/*DER* mutant



phenotype of MG death was manifested in each case, the double mutant involving *rhomboid* displayed significant *argos* expression as compared to the other *argos* and *spitz/DER* pathway double mutants. It is believed *rhomboid* encodes an integral membrane protein (Bier *et al.*, 1990). It is postulated to be associated with cell adhesions, as Rhomboid was discovered to be concentrated in plaque-like structures and at adherens junctions at the apical surface of ventral midline cells (Sturtevant *et al.*, 1996). Rhomboid co-localises with Armadillo, the *Drosophila*  $\beta$ -catenin homolog. Furthermore, *rho* mutants result in a loss of cell to cell contiguity in various mesectodermal lineages (Sonnenfeld and Jacobs, 1994). However, while a *rho* null mutant might result in a decrease in the efficacy of adhesion between two cells, the signaling mechanism might remain essentially intact. Therefore, even in a *rho* mutant background, a threshold might eventually be reached where enough *spitz* signal is harboured to activate *argos* transcription.

It is important to contrast the *rho*<sup>38</sup>-*argos*<sup>w11</sup> phenotype with that of the *Star*<sup>JIN23</sup>;*argos*<sup>w11</sup> double mutant. *Star* is another putative integral membrane protein with similar patterns of regulation and expression to Rhomboid (Kolodkin *et al.*, 1994; Bier *et al.*, 1990). *rho* and *Star* exhibit strong genetic interactions as assayed during wing vein development (Sturtevant *et al.*, 1993). Therefore, *Star* might operate directly with Rhomboid through a cell adhesion mechanism. They might act in concert as necessary accessory proteins. Through the apposition of cell surfaces, Rhomboid and *Star* might facilitate Spitz processing and release (Schweitzer *et al.*, 1995a; Golembo *et al.*, 1996b) and specify the Spitz mediated DER pathway signal (Sturtevant *et al.*, 1993). The more

severe phenotype in terms of very low *argos* enhancer trap expression in *Star<sup>IIN23</sup>;argos<sup>w11</sup>* double mutants relative to *rho<sup>38</sup>-argos<sup>w11</sup>* suggests that Star is the more limiting component in this relationship.

While it is necessary to first determine the subcellular distribution of Spitz and Star, it is interesting to speculate that along with Rhomboid, they too might be concentrated at adherens junction complexes (Sturtevant *et al.*, 1996). The adherens junction is a cell-cell signaling centre and serves as an intracellular bridge between adhesive and growth factor receptors, like members of the cadherin family and DER, respectively (for review, see Sastry *et al.*, 1996; Kirkpatrick and Peifer, 1995). *shotgun* encodes *Drosophila* E-cadherin, the only classic cadherin isolated so far from invertebrates (Oda *et al.*, 1994; Tadashi *et al.*, 1996; Tepass *et al.*, 1996). While E-cadherin is predominantly expressed in the embryonic epidermis, there is evidence that it is also expressed in the midline.

Mutations of the *spitz* gene which encodes the *Drosophila* TGF- $\alpha$  homolog (Rutledge *et al.*, 1992) do not completely abolish *argos* enhancer trap expression in the MG. A number of possibilities exist to explain the *spitz* phenotype. While the *E55* deficiency results in a null for *spitz* function, there is a maternal contribution of *spitz* (Mayer and Nüsslein-Volhard, 1988; Rutledge *et al.*, 1992). Furthermore, there is likely some basal auto-phosphorylation activity of DER in the absence of Spitz induction (Scweitzer *et al.*, 1995a,b). It is also likely that DER is regulated by further tissue specific ligands (Clifford and Schupbach, 1994). In addition to *spitz* and *argos*,

molecular and genetic evidence has shown that *vein* encodes a candidate EGF-R ligand and its expression is specific to a subset of ventral midline cells (Schnepp *et al.*, 1996).

In the case of double mutants involving *DER* and *argos*, the low but observable levels of *argos* enhancer trap expression is likely the result of the *flb*<sup>2E07</sup> allele being a relatively weak hypomorphic mutation compared to other *DER/flb* alleles (Raz *et al.*, 1991). However, *flb*<sup>2E07</sup> homozygotes are embryonic lethal and while its mutation has not been molecularly identified, there is likely some biological activity, enough to propagate a signal to activate some *argos* transcription. There is also low but detectable levels of *argos* enhancer trap expression in some pre-apoptotic MG in *pnt*<sup>9J31</sup>-*argos*<sup>w11</sup> double mutants. *pnt* encodes an ETS-related transcription factor and is believed to be a downstream target of DER signaling (Klämbt, 1993; Klaes *et al.*, 1994). Indeed, PntP2 can be phosphorylated by MAP kinase *in vitro* (Brunner *et al.*, 1994). There are likely more nuclear protein targets of DER signaling which in turn regulate the expression of MG specific genes like *argos*.

Another possible mechanism of MG survival regulation and one that might involve a *spitz* group/*DER* independent pathway, may be mediated by axon contact (Sonnenfeld and Jacobs, 1995a). There's a vertebrate precedent for such a model in the rat optic nerve. Differentiating oligodendrocytes are dependent on putative axon-derived signals for survival (Raff *et al.*, 1993). Through analysis of mutant and transgenic embryos of *Drosophila*, it was discovered that the degree of MG survival was positively correlated with the amount of axon contact (Sonnenfeld and Jacobs, 1995a). A mutation in *commissureless*, a gene which encodes an axon guidance molecule presented by the



MG to the commissural axons, results in an absence of commissural axons crossing the midline (Seeger *et al.*, 1993; Tear *et al.*, 1996). The resulting decrease in axon-MG contact was correlated with an advanced time course of apoptosis and a decrease in MG survival (Sonnenfeld and Jacobs, 1995a). Furthermore, upon the application of a relatively mild heat shock regime to *HSargos* embryos, the surviving MG are always in the position of the MGM. Surviving MG express *argos*. The MGM receives the most axon contact, as it resides between the anterior and posterior commissures. The close apposition of axons might contribute some buffering capacity, rendering the MGM more resistant to Argos. It would be interesting to probe for a possible *spitz* group/*DER* pathway independent activation of *argos* transcription by analysing *argos* enhancer trap expression in *commissureless* mutants. Alternatively, a commissural axon dependent mechanism might partially regulate Spitz group mediated DER signaling. MG like the MGM might be more predisposed for survival because the increased axonal contact it receives leads to more intense and localised DER signaling. Such a hypothesis can be tested by probing for a suppression of the HS-*rho* elicited supernumerary MG phenotype in a *commissureless* mutant background.

#### **4.2 Argos opposes the Spitz group/*DER* pathway**

*in vivo* and *in vitro* analysis suggests that Argos inhibits the activation of the EGF receptor (Schweitzer *et al.*, 1995b). My examination of the *spitz* group/*DER* pathway and *argos* function in the MG substantiates this conclusion. Further insights were obtained into *argos* function through the study of gain of function mutations and transgenics in the

embryonic ventral midline. Targeted overexpression of *D-raf* in a subset of mesectodermal cells results in an increase in MG numbers up to early stage 16. However apoptosis reduces the MG to below wildtype numbers by later embryogenesis.

Expression of the C38 Gal4 enhancer trap shifts from the MG to exclusive expression in the VUMs by these later stages. As *argos* expression is induced by the *spitz* group/*DER* pathway, it follows that overexpressed *D-raf* in the MG would lead to higher levels of secreted Argos protein. The half-life of Argos is greater than 4 hours (Freeman, 1994a) and therefore augmented levels of Argos would remain at later embryonic stages even after the hyperactivated raf pathway in the MG has since been shut off. Without this positive inducer of MG survival, elevated levels of Argos may lead to ectopic MG death.

A single induction of ectopic expression of *rho* by the hsp-70 promoter at stage 9-10, results in increased MG number at stage 12 (Sonnenfeld and Jacobs, 1995a). However, without continued ubiquitous overexpression of *rho*, MG number declined towards wildtype numbers and there was evidence of apoptosis earlier than normal. This study demonstrated that continued induction of ectopic *rho* leads to supernumerary MG surviving to the end of embryogenesis. To date, it is unknown whether these increased MG numbers persist into larval stages in the absence of continued ectopic *rho* expression.

With *Ellipse*, a gain of function mutation of *DER*, there was a slight increase in MG numbers until stage 16 or alternatively stated, there was a delay in the reduction of MG numbers. *Ellipse* mutants do not lead to increased or misexpressed levels of DER transcript or gene product in the eye disc (Zak and Shilo, 1992). Indeed the mutation resides in the coding region and not the regulatory regions of the gene (N. Baker, personal

communication). Therefore, the *Ellipse* mutation likely imparts a structural disruption to the DER protein. Because it does not result in a deregulation of autophosphorylation activity, *Ellipse* might lead to a disruption in the efficacy of receptor-ligand interaction. This leads to one hypothesis to explain the delay of MG reduction in *Ellipse* mutants. Argos-DER but not Spitz-DER binding capacity might be lowered. Therefore, the negative regulation of the DER pathway is slowed. However, if this putative *Ellipse* structural perturbation is relatively mild, perhaps the excess Argos that might have since pooled in the system can overcome this handicap.

#### **4.3 Spitz group/DER pathway and Argos function in other *Drosophila* tissues**

A very elegant study by Freeman, (1996) gave strong evidence that reiterative signaling through the DER pathway leads to the development of all cell types of the ommatidium. The model works as a sequential series of inductions beginning with the central R2, R5, and R8 cells differentiating as photoreceptors. Ommatidial development progresses with the successive recruitment via concentric rings with the next ring containing the remaining photoreceptors, followed by the ring of cone cells, primary pigment cells and secondary/tertiary pigment cells. Spitz is produced by and specifies all these cell types through DER activation. However, upon specification, the DER pathway inhibitor Argos is also induced. Because Argos has a greater range of diffusion than Spitz, it blocks the induction of the more distal concentric rings of cells yet to be determined, so that inappropriate recruitment does not take place. As the wave of

induction moves peripherally, a specific threshold of Spitz mediated DER signaling will overcome this local Argos inhibition.

A similar mechanism is proposed for the patterning of the ventral ectoderm (Golembo *et al.*, 1996b). After Star and Rhomboid mediated processing of secreted Spitz, the ligand diffuses and creates a gradient of DER activation. Proximal ectodermal cells receive the highest levels of DER signaling and in turn induce *argos* expression. Argos is secreted and downregulates the DER pathway in the more distal cells, thereby preserving the DER signaling gradient.

The *spitz* group and *argos* also function in the development of chordotonal organs (Okabe *et al.*, 1996). This context is unique because *argos* is expressed in yet to be identified cells surrounding the chordotonal organ precursor. The embryonic ectoderm and developing eye disc are tissues in which the DER pathway and Argos signaling are inherent as these populations of cells both produce and are affected by Argos. Similarly, the MG offers a more discrete population of cells, and provides further insights into the Spitz group/DER pathway and Argos function.

#### **4.4 Model of Spitz group/DER pathway and Argos function in the MG**

The maintenance of high levels of DER signaling imparts survival to the MG, activating a positive terminal differentiative program. Indeed hyperactivated DER signaling is believed to activate the ETS-related transcription factor Pointed, a gene whose expression exemplifies a terminal differentiated state (Klämbt, 1993; Klaes *et al.*, 1994). Interestingly, like *argos*, *pointed* is only expressed in a subset of MG, the subset

of MG which survive to hatching (Dong and Jacobs, in prep.). It appears that these MG are being primed to serve as commissure ensheathing glia at post-embryogenesis.

Another downstream effect of the Spitz group/DER signaling pathway is the initiation of MG migration. Migration of the MG is required for the morphogenesis of the commissural axon tracts (Klämbt *et al.*, 1991). In *spitz* group mutants, early MG migration does not occur and the commissures remain fused (Klämbt *et al.*, 1991). It remains to be seen whether *drifter* is a downstream target of the DER pathway. *drifter* encodes a POU domain containing transcription factor, another element necessary for proper MG migration (Anderson *et al.*, 1995). There likely remains multiple downstream nuclear proteins which are targets of the DER signaling pathway, and which direct MG terminal differentiation.

I propose that unrestricted diffusion of inductive factors among the MG is unlikely. The MG are a discrete group of cells in which a subset of 3 out of 12 MG progenitors survive. Only this subset expresses *argos*, presumably in response to highly localised Spitz group mediated induction. Between the MG, juxtacrine signaling (cell contact-dependent signaling) involving a membrane associated Spitz molecule would impose a finer tuned level of specification over secreted Spitz mediated paracrine signaling (Fagotto and Gumbiner, 1996). However, through cell culture and *in vivo* analysis, only secreted Spitz was demonstrated to induce DER autophosphorylation and is sufficient to account for the ventralisation of the embryonic ectoderm (Schweitzer *et al.*, 1995a). Nonetheless, there exists the possibility of tissue specific variation in the use of secreted versus membrane bound Spitz, and a role for membrane bound Spitz between the

MG should not be dismissed. Schweitzer *et al.*, (1995a) used S2 cells which lacked Rhomboid and Star and possibly other necessary accessory proteins, when assaying the biological activity of membrane bound Spitz. Spitz's vertebrate homolog, TGF- $\alpha$  is active both as a membrane bound and secreted form (Wong *et al.*, 1989). While spitz is ubiquitous, there are enhanced patterns of expression in certain tissues. Furthermore, the overlapping enhanced expression patterns of *spitz* and *DER* are consistent with a biological activity imparted by a membrane bound Spitz (Rutledge *et al.*, 1992; Zak *et al.*, 1990). It would be interesting to examine a transgenic involving MG targeted UAS-membrane bound Spitz to see if it reproduces the supernumerary surviving MG phenotype. This hypothesis of a membrane anchored Spitz initiating the mechanism of the regulation of MG survival doesn't rule out an earlier role for secreted Spitz. Spitz is postulated to emanate from the mesectodermal precursor cells to pattern the ventral ectoderm during stages 10-11 (Golembo *et al.*, 1996b). However, such a model would require a Spitz diffusion range to far exceed its proposed signaling domain of 3-4 cell diameters (Freeman, 1994b; for review, see Freeman, 1997).

The adherens junction along with possible accessory proteins like Star and Rhomboid, might mediate the close apposition of MG apical cell surfaces and promote Spitz localisation. Therefore, Spitz is concentrated to this specialised area between adhering MG where there is likely a local enrichment of its receptor, DER (Zak and Shilo, 1992). The MG with the most concentrated coupling of this adhesion and signaling pathway network achieve the highest levels of DER signaling. In turn, those same MG will be the first to express *argos*, an indicator that they have achieved survival

status. Argos is then secreted and binds to DER on both *argos* and non *argos* expressing MG. *argos* expressing MG might be refractory to normal wildtype levels of Argos in the surrounding ECM. Furthermore, continued induction of overexpressed *rho* or *D-raf* results in supernumerary surviving MG which appear to be refractory to Argos.

There is an alternative hypothesis to explain the resistance of *argos* expressing MG to Argos mediated apoptosis. Rhomboid forms plaque-like densities and co-localises with adherens junction proteins (Sturtevant *et al.*, 1996). Star might be another constituent of these structures and together they may be members of an adhesion complex. While these plaques concentrate high levels of Spitz mediated DER signaling, they might exclude normal wildtype levels of Argos (Stemerding and Jacobs, submitted). Therefore, in wildtype, *argos* expressing MG would escape autocrine inhibition. However, flooding the MG with ectopic *argos* overcomes this hindrance, and Argos directs even *argos* expressing MG to apoptosis.

MG not expressing *argos* might have reduced levels of adhesion contacts and hence lower DER signaling and are therefore amenable to *argos* mediated apoptosis. During the normal time course of MG reduction through apoptosis (stage 13-16), all MG express comparable levels of *DER* transcript (Figure 10) up to the point where cell death is believed to be imminent (Figure 9). Although MG destined to die lack the necessary elevated levels of DER signaling, they likely express sufficient levels of DER protein to efficiently receive Argos. In turn, Argos binding promptly impedes Spitz mediated DER signaling as assayed through the inhibition of DER transphosphorylation (Schweitzer *et al.*, 1995b) and later attenuates *DER* transcription (this study). Argos might also lead to

the down-regulation of other *spitz/DER* pathway products or conversely, up-regulate the expression of differentiation inhibitors like Yan (Lai and Rubin, 1992) and Ras pathway negative regulators like Gap1 (Gaul *et al.*, 1992).

In *argos<sup>w11</sup>* homozygotes, DER inhibition is likely reduced. *argos<sup>w11</sup>* represents a P-element insertion upstream of the coding region and is not a structural mutation (Freeman *et al.*, 1992a). The insert is downstream of the putative transcriptional start site and might therefore correspond to a mutation in the regulatory domain of translation. As a result, reduced levels of functional protein might be translated. In turn, increased numbers of MG would achieve sufficient DER signaling, leading to activation of *argos* and survival. With complete removal of Argos function through the *argos<sup>47</sup>* null allele, all MG escape Argos inhibition. However, not all MG survive. It's probable that survival also requires a certain threshold level of DER signaling which is unattainable without the requisite number of cell-cell contacts. This model is substantiated by evidence that the MGP have a 0% survival rate in both wildtype (Sonnenfeld and Jacobs, 1995a) and in *argos<sup>47</sup>* mutants. The MGP occupy a position relatively distant from the rest of the MG population (see Figure 1A) and it's implausible that they experience intense DER signaling. However, while the MGP express DER, it is not known whether a complete Spitz group mediated DER pathway is intact in these cells. Alternatively or in conjunction with the *spitz* group/*DER* and *argos* pathway, the CNS axons may play a role in regulating the number of MG that survive to hatching (Sonnenfeld and Jacobs, 1995a).



#### 4.5 Loss of MG affects CNS cytoarchitecture

Another facet of this thesis was to examine the effect of the lack of MG on CNS morphology. Subsequent to *HSargos* elicited MG loss, embryonic ventral nerve cords were examined at the light and electron microscopic level. Ultrastructural analysis showed that commissure ensheathment was severely compromised. The removal of MG also leads to fuzzy and thicker commissures, a phenotype reminiscent of the *roundabout* mutation (Seeger *et al.*, 1993). Indeed, the commissures appear to contain more axons than normal. Further confirmation of a semblance to the *robo* phenotype came from analysis of FasII distribution. Normally, the MP1 CNS interneuron, a medial longitudinal connective interneuron, projects ipsilaterally along the longitudinal connectives. However, in *robo* mutant embryos, the MP1 growth cones turn medially and projects across the midline where it fasciculates with its contralateral homolog, forming typical circular MP1 pathways (Seeger *et al.*, 1993). We were unable to identify the neuronal identity of the FasII expressing fascicle traversing the midline in *HSargos* embryos. The *roundabout* gene encodes a transmembrane receptor on growth cones and axons (C. Goodman, pers. comm.) and is thought to respond to a negative repulsive cue, emanating from the midline. Perhaps, *HSargos* elicited MG loss removes this putative ligand for Robo, resulting in a phenotype which is mimicked by a removal of Robo receptor function. In *HSargos* embryos, this phenotype is exhibited only after a long chase to stage 16, following the final heat shock administration. *HSargos* embryos processed shortly after the last heat shock at stage 14, exhibited a phenotype very similar to wildtype (data not shown). Complete MG death following ectopic overexpression of *argos* is not

immediate. Therefore, unlike *robo* mutants, the growth cones and axons of *HSargos* embryos have functional Robo receptor to respond to the negative repulsive factors, which likely remain relatively abundant at stage 12 or 13. If embryo processing takes place at stage 14, there is not enough time for a *robo*-like phenotype to be manifested. However, if MG death is assured following a longer recovery, a *robo*-like phenotype is observed. Therefore, ipsilaterally projecting growth cones and axons might remain sensitive to the Robo-mediated repulsive signaling mechanism beyond the normal time course that their axon trajectories are established. A more plausible hypothesis is that the MG continue to play a role in the repulsion of axonal growth cones from the midline which continue to appear after the initial axon tracts have been established.

It is also important to note that the MG also express a putative attractive cue for commissural axons (Seeger *et al.*, 1993; Tear *et al.*, 1996). *commissureless* is expressed by the MG and in *commissureless* mutants, commissural axons fail to cross the midline. Why doesn't *HSargos* exhibit a *commissureless* phenotype? The *robo* phenotype in *HSargos* embryos is explained by *robo* being epistatic to *commissureless*, as revealed through double mutant analysis. One possibility to explain the double mutant phenotype of *robo* and *comm* is that Comm inhibits the Robo-mediated repulsion of commissural growth cones, thereby allowing these growth cones to cross the midline (Tear *et al.*, 1996).

It would be interesting to search for MG loss related consequences to the CNS during larval development. *argos* continues to be expressed in the MG. However, preliminary experiments of ubiquitous overexpression of *argos* in larvae does not yield

MG death. This experiment needs to be repeated, employing a more robust HS regime.

An alternative approach is to induce MG death during embryogenesis and age the animals to hatching. While viability is substantially reduced in *HSargos* embryos, some do hatch.

These larvae exhibit lethargic behaviour and it would be interesting to probe for further repercussions of MG loss and other CNS defects.

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