MODULATORS OF SPITZ GROUP/DER SIGNALING DIFFERENTIALLY AFFECT MIDLINE GLIA SURVIVAL AND DIFFERENTIATION

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By

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ABSTRACT

In *Drosophila melanogaster*, the genes of the *spitz* group and of the DER signaling pathway function together to communicate localized developmental signals to the cells of many tissues. The embryonic midline glia (MG), a mesectodermal lineage essential to proper morphogenesis of the axon tracts of the ventral nerve cord (VNC), depend on *spitz* group signaling for survival and differentiation. Loss of function of any of the *spitz* group genes or of *DER* results in a decrease in the number of MG and subsequent defects in the formation of the axon tracts. These defects include a medial collapse of the longitudinal axons and fusion of the posterior and anterior commissures. Ectopic expression of Rhomboid, a putative seven pass transmembrane protein which is a member of the *spitz* group, generates supernumerary glia. Directed expression of DER^{A887T}, an activated form of the Drosophila EGF receptor, sSpi, a diffusible ligand, or Ras^{V12}, a constitutively activated variant of a monomeric G-protein, have the same effect on MG number. It is proposed that the *spitz* group/DER signaling pathway act to promote survival of MG precursor cells.

In addition, expression of *Draf* and *pntP1* are found to increase the number of surviving supernumerary MG, however these signaling molecules are determined to be less effective at promoting the survival of the MG, based on their decreased ability to generate supernumerary MG. Furthermore, a subset of the supernumerary MG created in embryos in which *Draf* has been misexpressed appear to be incompletely differentiated or insulated from normal programmed cell death (PCD) by expression of this transgene. The effect of

multiple cytoplasmic signaling pathways, activated by *spitz* group/DER signaling, on MG survival and differentiation is examined and discussed.

Ectopic expression of *rhomboid* generates supernumerary MG. This process is suppressed by mutation of genes of the *spitz* group or of *DER*. Evidence that Rho functions upstream of Spi, Star and DER is presented. The implications of these data and their relevance to previously published models which propose the molecular actions of the *spitz* group/DER pathway genes are discussed.

Finally, the role of *spitz* group/DER signaling in the activation of downstream target genes is explored. Overexpression of these genes results in increased expression of *pointed*, *argos*, and *rhomboid*. A model for *spitz* group function in signal amplification is proposed.

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CONTRIBUTIONS

Dr. J. R. Jacobs performed the fixed and stained embryos for *sli*1.0-Gal4/UAS-DER^{A887T}. Mythili Nadella fixed and stained embryos for *sim*-Gal4/UAS-ras^{V12S35}; AA142, *sim*-Gal4/UAS-ras^{V12G37}; AA142 and *sim*-Gal4/UAS-ras^{V12C40}; AA142. Dr. J.R. Jacobs and R. Battye provided technical assistance in the preparation of the composite images.

INTRODUCTION

The central nervous system of the *Drosophila* embryo is a complex structure which requires a sophisticated program for its development. The coordinated action of many cell types and genes is responsible for the generation of the various components of this system. This introduction will focus primarily on the determination and differentiation of the Midline Glia (MG) and the role of these cells in the organization and construction of the Ventral Nerve Cord (VNC).

1.0 The midline glia and their role in VNC Development

The embryonic ventral nerve cord is divided into segmental units which develop as a serially repeated structure. Each individual unit can be further divided into two subunits, the left and right neuromeres. Each contains an identical set of cell types which contribute to the proper development of the VNC within each segment (reviewed in Goodman and Doe, 1993). Within each pair of neuromeres, the longitudinal axon tracts and the anterior and posterior commissures, which run perpendicular to the longitudinals and project across the midline, develop in a consistent pattern (reviewed in Goodman and Doe, 1993). The axons tracts of the mature VNC appear as a ladder-like structure which may be revealed immunohistochemically with the monoclonal antibody BP102 (Klämbt *et al.*, 1991). The midline glia (MG) and several other mesectodermal lineages are involved in the correct formation of the axon tracts (Klämbt *et al.*, 1991).

It is thought that the MG play an important role initially in commissural axon pathfinding and later in the separation of the anterior and posterior commissures (Klämbt *et al.*, 1991). The exact role of the MG in this process is not certain, however it is

believed that the MG direct commissure formation by generating localized signals through the expression of several midline and MG specific genes.

D-netrins are expressed and secreted by the MG and function, like highly similar proteins in vertebrates and C. *elegans*, as attractants during the process of axon guidance (Harris *et al.*, 1996). Loss of function of these key signaling molecules results in a failure of the commissural axons in crossing the midline (Harris *et al.*, 1996).

commissureless (*comm*) codes for a gene product which is made by the MG and transferred to axons as they cross the midline (Tear *et al.*, 1996). The exact function of this novel membrane protein is uncertain, although loss of function results in the loss of both commissures (Seeger *et al.*, 1993; Tear *et al.*, 1996). Thus it appears that Commissureless, like the D-Netrins, functions as an attractant in growth cone guidance.

In contrast, *roundabout* (*robo*) (Seeger *et al.*, 1993) appears to function as a receptor for a ligand which acts to repulse axons (Seeger *et al.*, 1993; C.S. Goodman, pers. comm.). It is suggested that Slit, which is manufactured and secreted by the MG (Rothberg *et al.*, 1990), may function as the ligand for Robo (C.S. Goodman, pers. comm.). Loss of function of *robo* does not affect the initial crossing of the commissural growth cones over the midline but these axons inappropriately cross-back over the midline, instead of projecting ipsilaterally. This gives rise to a thickened commissures phenotype, similar that which is observed when the MG undergo premature apoptosis after the initial formation of the commissures has been allowed to occur (Stemerdink and Jacobs, 1997). Loss of function of *slit* generates a different phenotype (Rothberg *et al.*, 1990; Klämbt *et al.*, 1991; Sonnenfeld and Jacobs, 1994). In *slit* null mutant embryos the longitudinal axons tracts are collapsed about the midline, demonstrating that loss of

function at this locus results in a more severe phenotype than the loss of its putative receptor, *robo* (C.S. Goodman, pers. comm.). This suggests that *slit* has additional functions in this system or that other receptors exist which can interact with this ligand. It is possible that these receptors are expressed on the longitudinal axons and prevent them from collapsing on the midline in wildtype embryos.

A large pool of MG progenitors function initially in the guidance of the commissural axon tracts, however only approximately 6 of these cells persist to stage 13 and function in the separation of the anterior and posterior commissures (Sonnenfeld and Jacobs, 1995). These 6 surviving cells can be further subdivided into 3 pair, the anterior, middle and posterior midline glia MGA, MGM and MGP respectively (Jacobs and Goodman, 1989). During commissure separation, the MGM migrate posteriorly over the MGA and come to lie between the anterior and posterior commissural axon tracts (Klämbt *et al.*, 1991). In addition the MGP move anteriorly into the adjacent segment where they become positioned posterior to the posterior commissure (Klämbt *et al.*, 1991). The MGA remain anterior to the anterior commissure (see fig. 2, Klämbt *et al.*, 1991). Proper migration appears essential, as glia in embryos mutant for the transcription factors *drifter* or *tramtrack* are observed to wander, unable to reach their normal positions, which results in fusion of the commissures (Anderson *et al.*, 1995, Geisen *et al.* 1997). Additionally, embryos mutant for genes of the *spitz* group, which effect MG survival and differentiation have fused commissures (Klämbt and Jacobs , 1991; Sonnenfeld and Jacobs, 1994; Scholz *et al.*, 1997; Stemerdink and Jacobs, 1997).

After separation of the commissures, approximately half of the MG undergo apoptosis (Sonnenfeld and Jacobs, 1995). This process appears to involve programmed

cell death (PCD) (Dong and Jacobs, 1997; Stemerdink and Jacobs, 1997), and survival may correlate with axon contact (Sonnenfeld and Jacobs, 1995). These remaining cells persist to the end of embryogenesis and act to ensheath the commissures (Jacobs and Goodman ,1989).

1.1 Apoptosis reduces the number of MG

Up to 12 mesectodermal cells (MEC) may express glial specific genes such as *slit* or the enhancer trap AA142 (Dong and Jacobs, 1997). It seems likely that these cells participate in the initial guidance of the commissural axon growth cones (Jacobs and Goodman, 1989; Klämbt *et al.*, 1991; Tear *et al.*, 1996, Harris *et al.*, 1996). At stage 13, this MG precursor pool is reduced to about 5.3 cells per segment, which subsequently function in the separation of the commissures (Sonnenfeld and Jacobs, 1995). Of these, only an average of 3.2 MG per segment remain to function in the separation of the commissures (Sonnenfeld and Jacobs, 1995). In each of these cases, it is believed that programmed cell death (PCD) is involved in reducing the number of MG which survive. The coordinated actions of the genes *reaper* (*rpr*), *head involution defective* (*hid*) and *grim*, which are uncovered in the deficiency *H99*, appear to control this process under wildtype conditions (Dong and Jacobs, 1997; Zhou *et al.*, 1997). It should however be noted that misexpression of *rpr* alone may be capable of inducing the MG to undergo apoptosis (J.R. Jacobs, unpublished data). The 3 genes uncovered by *H99* do not appear to carry out the cell death program, but likely function to regulate or initiate this process, perhaps through the upregulation of caspase function (White *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996; reviewed in McCall and Steller, 1997; Dong and Jacobs, 1997; Zhou *et al.*, 1997).

The number of MG that survive these two apoptotic events is consistent and appears to be regulated by two distinct processes. A loss of function of the cell death genes *rpr*, *grim*, and *hid* caused by the deficiency *H99* appears to block cell death in the MG lineage, resulting in an increase in the number MG observed at both stage 13 and stage 17 (Dong and Jacobs, 1997; Zhou *et al.*, 1997). Induction of the *spitz* group/*DER* pathway genes or of the *Draf/MAPK* pathway has a similar effect (Sonnenfeld and Jacobs, 1995; Stemerdink and Jacobs, 1997), suggesting that *spitz* group signaling acts to perpetuate the survival of the MG by blocking the action of the cell death initiators, *rpr*, *grim*, and *hid* (see below). Furthermore, it has been demonstrated that loss of function of the *Drosophila* homologue of the EGF receptor (*DER*) or of genes in the MAPK pathway, such as *ras* or the *Drosophila* homologue of MAPK (*rolled*), enhances the ability of *hid* overexpression to mediate apoptosis in the MG (Kurada, Bae and White, pers. comm.). Loss of function of *rolled* or of *Draf*, known as MAPKKK in mammalian systems, also improves the ability of *rpr* overexpression to induce cell death (Kurada, Bae and White, pers. comm.).

Misexpression of *ras* or of *Draf* down-regulates *hid* mRNA levels in the MG (Kurada, Bae and White, pers. comm.). Conversely, overexpression of *spitz* group genes does not affect *rpr* mRNA transcription in the midline. However, it has been demonstrated that the mammalian cell death inhibitor Bcl-2, and it's *C. elegans* homologue ced-9 can partially block Rpr from inducing cell death in a *Drosophila* cell culture systems (Hisahara *et al.*, 1998). This is an important fact, since it may indicate a possible mechanism for the normal inhibition of the function of this gene product. It is possible that Rpr function is down-regulated by the action of a hypothetical *Drosophila*

Bcl-2/ced-9 homologue, which may itself be activated through a phosphorylation cascade that may involve interaction with DRaf. A similar mechanism exists to control apoptosis in vertebrates (reviewed in Farrow and Brown, 1996; reviewed in Hunter, 1997).

Thus it appears that signaling events involving the *spitz* group genes and the DER and MAPK pathways, act to assure the survival of a subset of the MG. In addition the survival of the final complement of MG, those that perpetuate until the end of embryogenesis, has been correlated with axon contact (Sonnenfeld and Jacobs, 1995). It has been observed that MG which establish contact with the commissural axons are more likely to survive the second phase of apoptosis, which begins at stage 13 and reduces the number of MG from 5.3 to 3.2, than are glia that do not initiate this contact. In *comm* mutant embryos, which have no commissural axon tracts, the number of surviving glia is decreased suggesting that the commissures are important to MG survival. The MG which do not undergo apoptosis migrate laterally and position themselves in contact with the longitudinal axon tracts (fig. 5A and B, Sonnenfeld and Jacobs, 1995). At present it is not known whether misexpression of *spitz* group genes is sufficient to mediate MG survival in the absence of commissural axon contact.

1.2 The *spitz* group/DER genes function in MG development

The *spitz* group/DER pathway is essential for the proper morphological development of many tissues in both embryonic and adult *Drosophila melanogaster*. Loss of function of the *spitz* group genes *rhomboid* (*rho*), *Star* (*S*), *spitz* (*spi*) and *pointed* (*pnt*) give rise to similar mutant phenotypes in many adult tissues, including the wing veins and photoreceptors (Sturtevant *et al.* 1993; reviewed in Freeman, 1997). Embryonic tissues including the malpighian tubules, the chordotonal organs, the ventral ectoderm and the CNS also require the *spitz* group genes for development (Mayer and Nüsslein-Volhard 1988; Klämbt *et al.* 1991; Kim and Crews, 1993; Okabe *et al.*, 1996). They are proposed to be components of a signal transduction pathway widely employed in the development of *Drosophila*.

As mentioned, the *spitz* group plays a major role in the development of the embryonic CNS (Klämbt *et al.*, 1991). Loss of function of any of the genes in this pathway results in characteristic defects in the development of the axons tracts. In particular, commissure fusion and a collapse of the longitudinal tracts upon the midline, which results in a narrowing of the VNC, are observed. It appears that the premature death and incomplete differentiation of the MG result in this phenotype, since these cells are then no longer able to function in commissure formation and in the separation of the anterior and posterior tracts (Klämbt *et al.*, 1991; Sonnenfeld and Jacobs, 1994). The individual components of this complex signaling mechanism will be examined in greater detail below, in order to fully explain their molecular characteristics and expression patterns within the developing embryos.

1.21 *spitz*

spitz encodes a signaling molecule that contains an EGF (epidermal growth factor) domain, which functions is protein-protein interactions (Rutledge *et al.*, 1992; Rao *et al.*, 1995). Spitz is synthesized as a membrane bound protein structurally similar to Tumor Growth Factor-α (TGF-α) which in mammalian systems binds to and activates ErbB-1, a mammalian EGF receptor subtype (Rutledge *et al.*, 1992; reviewed in Massagué, 1990). Similarly, Spitz appears to act through DER, a *Drosophila* receptor tyrosine kinase with a high degree of structural similarity to the vertebrate EGF receptor (Rutledge *et al.*, 1992; Schweitzer *et al.*, 1995).

spitz is expressed ubiquitously during embryonic development, but appears to require further processing in order to participate in the determination of discrete groups of cells or tissues such as the ventral ectoderm and the MG (Mayer and Nüsslein-Volhard, 1988; Rutledge *et al.*, 1992; Schweitzer *et al.*, 1995; Golembo *et al.*, 1996). Additionally, *spitz* is expressed in the developing eye disc, where it participates in photoreceptor determination (Freeman, 1994b). It is thought that the membrane-bound form of Spitz (mSpi) is non-functional and that cleavage forms an active, diffusible secreted molecule which is required for activation of the receptor (Schweitzer *et al.*, 1995). TGF-α is also synthesized as a membrane bound precursor, which functions as a secreted ligand (reviewed in Massagué, 1990). Temporal and spatial regulation of Spitz release from the membrane is believed to specify regions of the embryo in which *spitz* signaling is active (Schweitzer *et al.*, 1995; Golembo et. al., 1996). This control is achieved through regulation other of genes, such as *rhomboid* (*rho*) and *Star* (*S*) (discussed below), which are thought to facilitate or perform this cleavage event (Bier *et al.*, 1990; Schweitzer *et*

al., 1995). Additionally, sSpi may only diffuse a short distance, exerting its effects over 3-4 cell diameters (Freeman, 1994b), which would ensure a localized response.

1.22 *faint little ball (flb)*

The *faint little ball* (*flb*) locus encodes the *Drosophila* homologue of the Epidermal Growth Factor Receptor (*DER*) (Price *et al.*, 1989; Schejter and Shilo, 1989). DER is a member of the receptor tyrosine kinase (RTK) superfamily and is structurally related to the mammalian Erb family of EGF receptors (Ullrich *et al.*, 1984; Livneh *et al.*, 1985; Schejter *et al.*, 1986; as cited in Raz *et al.*, 1991). *DER* is expressed ubiquitously during embryogenesis and appears to function in the development of many tissues including the ventral ectoderm, the chordotonal organs and the MG (Raz *et al.*, 1990; Raz *et al.* 1991; Raz and Shilo, 1992; Raz and Shilo, 1993). Like the members of the *spitz* group, *DER* is also implicated in the development of multiple cell types in the ommatidia (Freeman, 1996) and in the wing veins (Sturtevant *et al.*, 1993). Due to its ubiquitous expression, *flb* mutant embryos suffer from a variety of defects associated with the role of the receptor in the proper development and survival of multiple cell and tissue types (Schejter and Shilo, 1989).

Ligand binding induces receptor dimerization. This in turn activates signaling through both auto-phosphorylation and trans-phosphorylation of the dimerized receptors (reviewed in Ullrich and Schlessinger, 1990; Schlessinger and Ulrich, 1992; as cited in Clifford and Schüpback, 1994). This activated ligand-receptor complex is then able to induce activation of multiple downstream signal transduction pathways, which in turn communicate the signal to the cell nucleus (Clifford and Schüpback, 1994).

1.23 *rhomboid* (*rho*)

rhomboid (*rho*) encodes a seven-pass trans-membrane protein with little homology to other known proteins (Bier *et al.*, 1990). Rho does however contain a PEST sequence, which is found in many proteins that are rapidly turned-over (Rogers *et al.*, 1986; as cited in Bier *et al.*, 1990) suggesting that the activity of this protein can be temporally controlled with some precision. *rho* is expressed during embryogenesis in many tissues including the chordotonal organs, the hindgut, the ventral ectoderm and the mesectoderm (Bier *et al.*, 1990). In addition *rho* is also expressed in a spatially restricted pattern within the wing and eye discs. *rho* is essential for the proper development of the wing veins and the photoreceptors (Sturtevant *et al.*, 1993; Freeman *et al.*, 1992).

Embryonic expression in the presumptive mesectodermal precursors begins early in embryogenesis at the cellular blastoderm stage (see Bier *et al.*, fig.5). After ventral closure, *rho* expression is observed in a strip two cells wide along the midline of the embryo (Bier *et al.*, 1990; Lanoue, Gordon and Jacobs, unpublished observation). These same cells also express *single-minded* (*sim*), a gene required for determination of mesectodermal identity (Crews *et al*, 1988; Nambu *et al.*, 1991). As development proceeds, *rho* expression gradually becomes restricted to a sub-population of the midline cells, including the MG. Rho appears to be concentrated in plaques in the plasma membrane and is often co-localised with other proteins, such as Armadillo, which are associated with adherens junctions (Sturtevant *et al.*, 1996). It is postulated that an unknown mechanism is involved in targeting the protein to these patches on the cell surface (Sturtevant *et al.*, 1996).

Rho function appears to be essential for *spitz* group signaling (Klämbt *et al.*, 1991; Freeman *et al.*, 1992; Sturtevant *et al.*, 1993). In adult flies, *rho* mutant clones have missing wing veins and a reduced number of the photoreceptor cells in the ommatidia (Freeman *et al.*, 1992; Sturtevant *et al.*, 1993). In embryonic CNS development, loss of function at the *rho* locus results in premature apoptosis and improper differentiation of the MG leading to commissure fusion and narrowing of the VNC (Klämbt *et al.*, 1991; Sonnenfeld and Jacobs, 1994; Dong and Jacobs, 1997; Stemerdink and Jacobs, 1997). Overexpression of *rho* using a heat-shock inducible promoter (*hsp70*), results in an increase in MG number and photoreceptor number, and in a thickening of the wing veins (Sonnenfeld and Jacobs, 1995; Stemerdink and Jacobs 1997; Freeman *et al.*, 1992; Sturtevant *et al.*, 1993).

It has been proposed that Rho somehow acts to cleave or facilitate cleavage of the membrane bound precursor of Spitz (Schweitzer *et al.*, 1995). Due to its spatially and temporally restricted expression pattern, relative to *DER* and *spi*, *rho* could act in this manner to specify the domain of *spitz* group signaling (Bier *et al.*, 1990; Schweitzer *et al.*, 1995). In support of this hypothesis, genetic evidence exists which demonstrates that *spi* is epistatic to *rho* (Schweitzer et. al., 1995).

1.24 *Star* (*S*)

Star is a novel, single-pass transmembrane protein which has no sequence similarity to any other known proteins (Kolodkin *et al.*, 1994). Like Rho, Star contains two PEST sequences, and as such may be subjected to rapid degradation. During embryonic development *Star* expression is observed at stage 9-10 in a row of mesectodermal cells 1-2 cells wide located at the midline (Kolodkin *et al.*, 1994). Later,

during germ band retraction, *Star* expression becomes restricted to clusters of cells that are probably the MG (Kolodkin *et al.*, 1994). It has been proposed that this spatially and temporally restricted expression pattern coupled with Star's potential for rapid degradation enable it to act in the restriction of *spitz* group signaling. It is hypothesized that Star acts with Rho in facilitating the release of sSpi (Schweitzer *et al.*, 1995).

1.25 Cytoplasmic signal transduction pathways

As mentioned, activation of DER initiates the activation of multiple cytoplasmic signaling cascades. Phosphorylation of the receptor allows binding of Drk, the *Drosophila* homologue of the mammalian adapter protein Grb-2 (Clifford and Schüpbach, 1994). Drk mediates activation of the *Drosophila* homologue of Ras1, Ras, a small non-receptor G-protein which can activate several cytoplasmic targets (reviewed in Marshall, 1995; and Katz and McCormick, 1997). In particular, Ras is known to interact with Raf, Phosphoinositol-3'- kinase (PI3'K) and Jun-Kinase (Jnk), transducing signals via these messengers (reviewed in Katz and McCormick, 1997).

Induction of the DRaf signaling cascade results in phosphorylation of Rolled, the *Drosophila* homologue of ERK/MAPK (mitogen activated protein kinase) (Schweitzer *et al.* 1995, Gabay *et al.*, 1997). This in turn results in the activation of several transcription factors (reviewed in Su and Karin, 1996). In *Drosophila*, the actions of the ets family transcription factors PointedP2 and Yan appear to be regulated in this manner (O'Neill *et al.*, 1994; Rebay *et al.*, 1995). Recent data supports the conclusion that DER signaling in the midline works through the MAPK pathway (Scholz *et al.*, 1997; Stemerdink and Jacobs,1997) (See figure 1). Furthermore, overexpression of *pointedP1*, a constitutively

active form of *pointed*, which is a target of the MAPK pathway, also increases the number of MG (Scholz *et al.*, 1997).

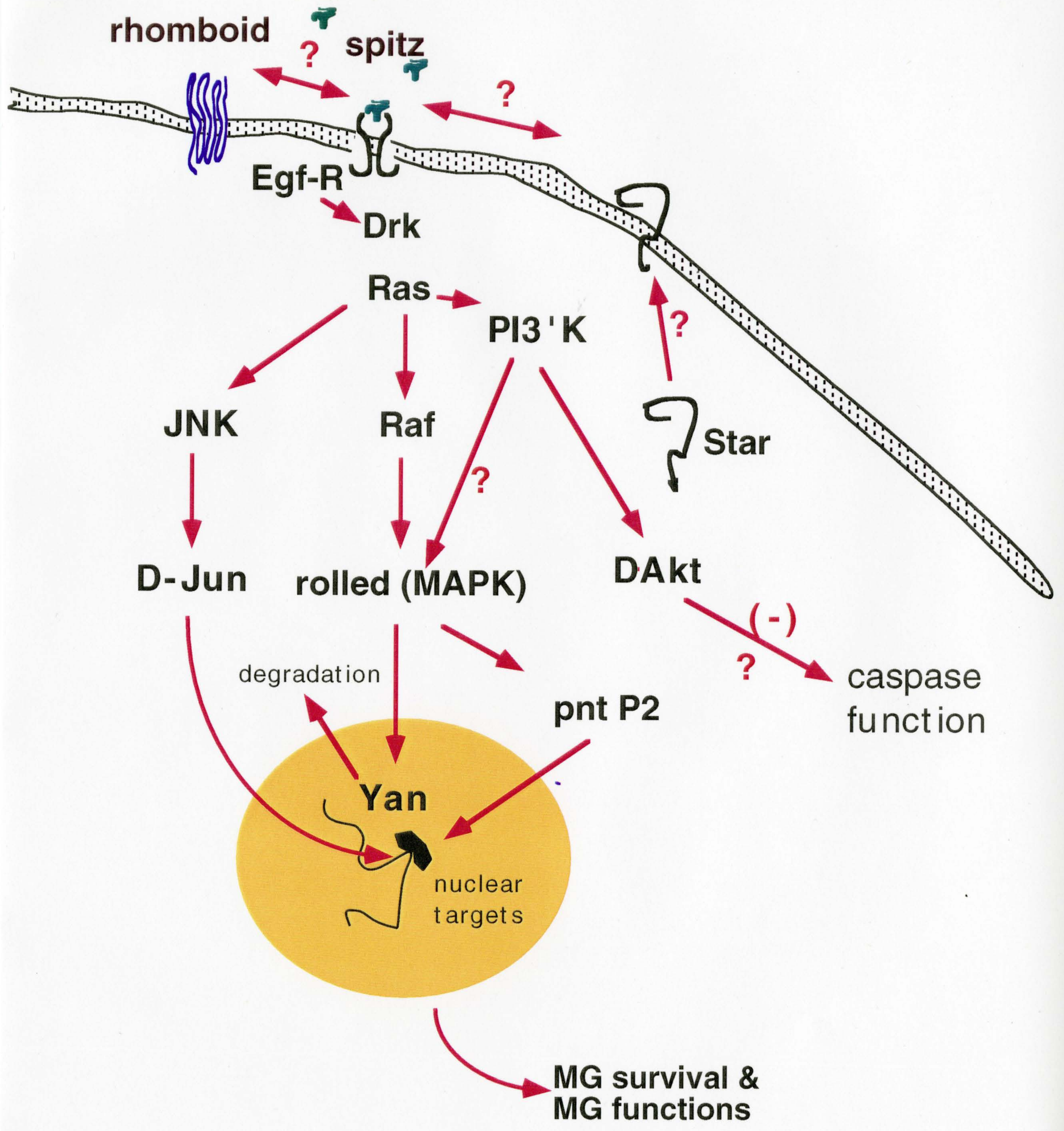
PI3'K, which is also activated by Ras, has multiple effectors. Primarily, PI3'K acts by generating phosphoinositide second messengers which in turn activate downstream targets (reviewed in Carpenter and Cantley, 1996). Non-calcium dependent isoforms of Protein kinase C (PKC) are a major target of these second messengers. It has been demonstrated that these signaling molecules can in turn upregulate MAPK function (reviewed in Carpenter and Cantley, 1996). In addition, PI3'K can activate Protein Kinase B (PKB or Akt) which, in mammalian systems, appears to function to block apoptosis (Kennedy *et al.*, 1997). It is believed that Akt performs this function, at least in part, by downregulating the function of a subset of the caspases. Genetic analysis of *Dakt1*, the *Drosophila* homologue of this gene, confirms that it negatively regulates apoptosis in some tissues (Staveley *et al.*, 1998). Directed misexpression of *p35*, a viral caspase inhibitor, was shown to block apoptosis in *Dakt1* mutant embryos, while loss of function of *rpr*, *hid* and *grim* did not suppress the mutation. This suggests that Dakt acts independently of these cell death genes, perhaps by blocking caspase function, as observed in mammals.

When activated by RTK signaling, Ras is also capable of activating Jun, a transcription factor, through the upregulation of Jun kinase (JNK) (reviewed in Katz and McCormick, 1997).

Figure 1. Summary of extracellular and intracellular signal transduction pathways associated with DER

Summary of *spitz* group /DER pathway in *Drosophila*. The current model suggests that Rho and S act to cleave Spitz from a membrane bound form (mSpi) to a secreted, activated ligand (sSpi). sSpitz then acts through DER to transduce a signal in the receiving cells. Activation of DER is proposed to result in the subsequent activation of multiple cytoplasmic signaling cascades. These in turn cause changes in gene expression and other cellular processes. See text for further details.

Figure 1



In vertebrate neurons, activation of Jun kinase or overexpression of Jun results in apoptosis of these cells (Ham *et al.*, 1995; Xia *et al.*, 1995; as cited in Scholz *et al.*, 1997). Contrary to this fact, overexpression of activated D-Jun, the *Drosophila* homologue of Jun, appears to prevent cell death of the MG while promoting apoptosis in other midline neuronal cell types (Scholz *et al.*, 1997).

It is noteworthy that activation of PI3'K may indirectly activate JNK in mammals (reviewed in Carpenter and Cantley, 1996).

1.26 *pointed* (*pnt*) and *yan*

Pointed (Pnt) and Yan are transcription factors which contain DNA binding domains similar to those found in members of the mammalian ets family (Karim *et al.*, 1990; Lai and Rubin, 1992). The *pointed* locus codes for two isoforms, PointedP1 which is constitutively active and PointedP2 which must be phosphorylated in order to be translocated to the nucleus. Only the P2-type transcript is localized to the MG, whereas the P1 transcript is produced in the lateral glia (Klämbt, 1993). Loss of function of *pointed* results in fusion of the commissurral axon tracts, as described for other members of the *spitz* group. This phenotype appears to result from improper differentiation rather than premature death of the MG, which normally act to separate the commissures (Klämbt, 1993). Overexpression of *pointedP1*, but not *pointedP2*, in the midline results in a moderate increase in MG number (Scholz *et al.*, 1997). PointedP2 activity is positively regulated by MAPK phosphorylation, which causes nuclear localization from the cytoplasm, and is believed to affect MG differentiation through the activation of transcription of MG specific target genes (Klämbt, 1993; Klaes *et al.*, 1994).

yan is expressed in the embryonic midline and is a negative regulator of differentiation whose function appears to oppose that of *pointed* (Rebay and Rubin, 1994; Scholz *et al.*, 1997). MAPK phosphorylation of Yan inactivates the transcription factor inducing localization to the cytoplasm where it may be rapidly degraded (Lai and Rubin, 1992; Bruner *et al.*, 1994). Yan and Pointed are each capable of binding to Ets responsive sequences, thus it seems likely that they compete for these binding sites in the promoter regions of target genes (Klämbt, 1993; O'Neill *et al.*, 1994; Rebay and Rubin, 1995; Albagli *et al.*, 1996). Activation of these targets then depends upon the relative phosphorylation states Pnt and Yan, which is determined by the level of MAPK pathway activity (Rebay and Rubin, 1995). Yan activation results in the upregulation of *hid* mRNA levels in the MG (Kurada, Bae and White, pers. comm.). Therefore it is possible that Pointed could function to block *hid* expression in cells that have had MAPK activity increased in response to activation of DER, thereby promoting survival of the MG.

1.27 *argos* (*aos*)

Argos (Aos) is a secreted signaling molecule which functions non-autonomously to negatively regulate cell fate decisions, including survival (reviewed in Freeman, 1997, Stemerdink and Jacobs, 1997). In the embryonic CNS, loss function at the *aos* locus results in supernumerary MG , while ectopic expression induces apoptosis in these cells (Stemerdink and Jacobs, 1997). *spitz* group/DER pathway signaling is required the expression of *aos*. *pointed* is required for this function (Scholz *et al.*, 1997; Stemerdink and Jacobs, 1997).

Aos appears to negatively regulate signaling through DER. Ectopic expression of *aos* leads to a significant decrease in the level of *DER* mRNA expression (Stemerdink and

Jacobs, 1997). Aos may also interfere with signaling by binding to the receptor, blocking access to ligands such as sSpi (Schweitzer *et al.*, 1995; Golembo *et al.*, 1996; reviewed in Freeman, 1997). Mosaic analysis in the eye demonstrates that Aos functions non-autonomously, and may diffuse up to 10-12 cell diameters (Freeman *et al.*, 1992)

It is hypothesized that *spitz* group/DER signaling is able to reach higher levels in some cells than in others. These cells are therefore induced to turn on *aos* earlier than are others. Aos then diffuses to neighbouring cells and blocks DER signaling, which results in apoptosis or changes in the differentiation program of these cells (Golembo *et al.*, 1996, Stemerdink and Jacobs, 1997, Reviewed in Freeman, 1997). Therefore it appears that the DER pathway forms an inhibitory feedback loop through the activation of *aos* expression.

1.3 Objective and rationale for research and investigation

The embryonic midline glia make an excellent model system for the examination of *spitz* group function, primarily due to the fact that development of these cells is so well characterized (Jacobs and Goodman, 1989; Klämbt *et al.*, 1991). Since we are only dealing with the effects of a particular experimental protocol on one cell type, it is relatively easy to analyze data and compare between trials in this system. In addition, the phenotype generated by loss of function in *spitz* group genes is unambiguous, characterized by a decrease in MG number or axon tract collapse and commissure fusion. This makes it possible to detect subtle changes in phenotype more easily than in other systems (Klämbt *et al.*, 1991; Sonnenfeld and Jacobs, 1994). As well, the phenotype associated with gain of function of *spitz* group/DER pathway genes is equally unambiguous. These embryos have supernumerary MG, which is also easily measured,

further increasing the attractiveness of this system as a model (Sonnenfeld and Jacobs, 1995; Dong and Jacobs, 1997; Zhou *et al.*, 1997; Stemerdink and Jacobs, 1997).

Recent publications from the Jacobs lab have demonstrated that both loss of function of the cell death genes uncovered by the deficiency *H99*, and overexpression of *spitz* group/DER pathway genes generate supernumerary glia (Sonnenfeld and Jacobs, 1995; Dong and Jacobs, 1997; Stemerdink and Jacobs, 1997). I seek to determine the relationship between these two sets of genes, by determining if they regulate the same process and by addressing the mechanism by which this is accomplished.

In this thesis, I examine two processes. First, I address the question of how the *spitz* group genes interact to generate a localized signaling mechanism which acts to promote survival and differentiation of the MG. This is accomplished through epistatic analysis of *spitz* group function, using overexpression of *rho* or *sspi* in conjunction with other *spitz* group/DER pathway loss of function mutations. In addition, the effects of *spitz* group signaling on regulation of *rho* expression are observed.

Next, I explore the downstream signal transduction machinery associated with DER. A thorough experimental program is undertaken to explore the relative contributions of various cytoplasmic signaling cascades to the processes of survival and differentiation that are controlled by *spitz* group signaling. This is done by targeting the expression of these genes to the MG using the Gal4-UAS system (Brand and Perrimon, 1993). The outcome of these experiments is compared to results observed in embryos deficient for cell death. Parallels are established and key differences are discussed. Markers such as cell position and morphology as well as regulation of downstream target gene

expression are used to assess the effect of changing expression of different genes on MG differentiation and survival.

MATERIALS AND METHODS

2.0 *Drosophila melanogaster* strains

Drosophila melanogaster strains were obtained from the Bloomington Stock Centre unless otherwise noted. All flies were raised and maintained on sucrose-salts-yeast-agar medium at room temperature (22-25 °C). The wildtype strain used was Canton-S p-element free (CS-P) in all cases. All lines carrying mutant chromosomes were balanced over balancer chromosomes containing a P[*actin*-LacZ] reporter transposon. Mutant homozygotes were detected embryonically by a lack of *actin*-LacZ labelling. A double mutant recombinant line for HS*rho*1B; rho^{38}, AA142/TM3 as well as a recombinant line for rho^{38}, AA142/TM3 were kindly provided by C. Stemerdink (see Stemerdink and Jacobs, 1997). The enhancer trap lines 1B; X55 and 1B; P223 lines were also provided by C. Stemerdink. 1B; AA142 was obtained from the lab stocks. All other lines were made myself, unless otherwise noted.

2.01 Mutants and Transgenics

rhomboid $^{\Delta 38}$: a null allele created by imprecise p-element excision of the transposon present in *rhomboid* ^{X81} which has a cytological position at 62A1-2 (Freeman *et al.*, 1992).

spitz ^{1}: also referred to as spi^{IIA}. This is an EMS induced allele of the *spitz* gene, which is located at position 37E2-38E. (Nüsslein-Volhard *et al.*, 1984).

Star ^{IIN3}: an EMS-induced allele of *Star*, which has a cytological position at 21E1-2 (Nüsslein-Volhard *et al.*, 1984).

faint little ball ^{2E03}: a second chromosome weak hypomorphic allele of the *Drosophila* EGF Receptor (DER), cytological location at 57F1 (Nüsslein-Volhard *et al.*, 1984).

pointed ^{9J31}: a third chromosome EMS-induces loss of function allele, located at cytological position 94F (Jürgens *et al.*, 1984; Klämbt, 1993). Kindly provided by S. Crews.

HS*rho*1B: full length *rho* cDNA fused to the *hsp70* heat-shock inducible promoter. Ectopic expression construct located on the X chromosome (Sturtevant *et al.*, 1993). Kindly provided by E. Bier.

2.02 UAS constructs

All UAS constructs contain coding DNA for a gene of interest sub-cloned into the pUAST vector (Brand and Perrimon, 1993). This vector contains the yeast UAS (upstream activating sequences) promoter. Binding of Gal4, a yeast transcription factor, which is provided *in trans* by crossing the UAS line to a line carrying the Gal4 coding sequence fused to an enhancer element, activates expression of the downstream coding region in a Gal4 dependent manner (Brand and Perrimon, 1993).

UAS-*sspi*: Third chromosome viable insertion of a transposon carrying the target gene *secreted-spi* A full length *spitz* cDNA was modified by the addition of a stop codon, by PCR, at the putative Spitz cleavage site at lysine-129.(Schweitzer *et al.*, 1995). Obtained from B. Shilo.

UAS-*rho*: Third chromosome viable insertion of transposon carrying the target gene *rho*. A cDNA encompassing the full coding region was used. (Xiao *et al.,* 1996). Obtained from J. Nambu.

UAS-*DER*^{A887T}: third chromosome viable insertion of transposon carrying the target gene activated *DER*. This is a constituatively active mutant, which has a single amino acid substitution. Threonine is substituted for alanine at position 887 (N. Baker??, submitted to Developmental Biology). Kindly provided by N. Baker

UAS-*RAS*^{V12} : second chromosome viable insertion of transposon containing the target gene *Ras1*. A single amino acid substitution, valine for glycine at position 12, makes this form of Ras1 constitutively active.(Karim and Rubin, 1998). Obtained from F. Karim.

UAS-*RAS*^{V12S35} : second chromosome viable insertion of transposon containing the target gene *Ras1*. Two amino acid substitutions were made, valine for glycine at position 12 and serine for threonine at position 35. This is an activated variant of Ras1 that cannot interact with or activate PI3'K. (Karim and Rubin, 1998). Obtained from F. Karim.

UAS-*RAS*^{V12G37} : X chromosome viable insertion of transposon containing the target gene *Ras1*. Two amino acid substitutions were made, valine for glycine at position 12 and glycine for glutamine at

position 37. This is an activated variant of Ras1 that cannot interact with or activate DRaf. (Karim and Rubin, 1998). Obtained from F. Karim.

UAS-*RAS*^{V12C40} : X chromosome viable insertion of transposon containing the target gene *Ras1*. Two amino acid substitutions were made, valine for glycine at position 12 and cystine for tyrosine at position 40. This is an activated variant of Ras1 that cannot interact with or activate DRaf. (Karim and Rubin, 1998). Obtained from F. Karim.

UAS-*RAS*^{WT} : second chromosome viable insertion of transposon containing the target gene *Ras1*. A full length *ras1* cDNA was used (Karim and Rubin, 1998). Obtained from F. Karim.

UAS-*Draf* : third chromosome viable insertion of transposon containing the target gene *Draf* (Brand and Perrimon, 1994)

UAS-*pntP1*: third chromosome viable insertion of transposon containing the target gene *pntP1* (Klämbt, 1993; Scholz *et al.*, 1997). Kindly provided by C. Klämbt.

UAS-*pntP2* : third chromosome viable insertion of transposon containing the target gene *pntP2* (Klämbt, 1993; Scholz *et al.*, 1997). Kindly provided by C. Klämbt.

UAS-*Dakt* : second chromosome lethal insertion of transposon containing the target gene *Dakt* (Staveley *et al.*, 1998). Kindly provided by A. Manoukian.

UAS-*Dakt (CAA142X)* : second chromosome viable insertion of transposon containing a dominant negative form of the target gene *Dakt* (Staveley *et al.*, 1998). DAkt has been altered by the fusion in frame of a PH domain. This is hypothesised to make this variant of DAkt dominant negative. Kindly provided by A. Manoukian.

2.03 Enhancer traps and reporter gene fusion constructs

P element enhancer traps are instrumental in elucidating temporal and spatial patterns of gene expression during *Drosophila* development (Bier *et al.*, 1989, Bellen *et al.*, 1989). In general, P element vector contain the *E. coli* gene *ß-galactosidase* under the control of a weak promoter which cannot initiate expression of the transgene on its own. Random insertion of the transposon into the regulatory region of a gene, places this construct under the transcriptional control of the enhancer element associated with the target gene. *ß-galactosidase* is then transcribed in a pattern identical to that of the flanking gene (O'Kane and Gehring, 1987). The following enhancer traps and reporter constructs were used.

AA142: an enhancer trap containing a P element insertion at the cytological position 66D. The gene in which the transposon is inserted has not yet been molecularly characterised (Klämbt *et al.*, 1991).

AA142142 is strongly expressed in the MGA and MGM and weakly in the MGP, beginning at stage 12 (Klämbt *et al.*, 1991; Sonnenfeld and Jacobs, 1994)

P223: an enhancer trap containing a P element insertion at the cytological position 98. The gene in which the transposon is inserted has not yet been molecularly characterised. P223 is expressed in the MP1 and MP2 neurones from stage 12.

X55: an enhancer trap containing a P element insertion at the cytological position 56F. The gene in which the transposon is inserted has not yet been molecularly characterised. X55 labels the MG, the VUMs and the MNB from stage 12 (Klämbt *et al.*, 1991).

X81: an enhancer trap containing a P element insertion into the regulatory region of the *rhomboid* gene. Expression of this transgene mimics that of *rhomboid* (Bier *et al.*, 1990).

pointed^{1227}: a P element insertion into the middle of P2 exonI (Scholz *et al.*, 1993). This results in a hypomorphic allele of the *pointed* gene. *pointed*^{1277} is expressed in a subset of the MG, beginning at stage 13.

argos^{w11}: an enhancer trap containing a viable P element insertion at the cytological position 73A3,4. This insertion creates a mildly hypomorphic allele of *argos* (Freeman *et al.*, 1992). In heterozygotes,

argos^{W11} is expressed in a subset of MG which do not undergo apoptosis (Stemerdink and Jacobs, 1997).

***sim*-Gal4**: enhancer trap containing the yeast transcription factor Gal4 under the transcriptional control of the *sim* enhancer. Expression of this transgene is initiated at stage 9 in the entire mesectoderm, but eventually becomes restricted to the MG by stage 17 (see appendix 1). Kindly provided by S. Crews.

***sli*1.0-Gal4**: enhancer trap containing the yeast transcription factor Gal4 under the transcriptional control of the *slit* enhancer. Expression of this transgene is initiated at stage11 in the presumptive MG and in the MP1 neurones and continues past the end of embryogenesis (Scholz *et al.*, 1997)(see appendix 1). Kindly provided by C. Klämbt.

2.1 Antibodies

anti-ß-galactosidase: a commercial rabbit polyclonal antibody (Cappel) used to identify gene expression patterns of lines carrying a P[*lacZ*] element. A dilution of 1:150 was used for whole mount embryo staining.

BP102: a mouse monoclonal antibody which recognises an uncharacterised carbohydrate moiety which is found on the surface of the longitudinal and commissural axon tracts (Klämbt *et al.*, 1991). BP102 was obtained from C. Goodman. A dilution of 1:5 was used for whole mount embryo staining.

22C10: a mouse monoclonal antibody which recognises an antigen located on the VUM cell bodies and on many CNS and PNS axons and cells, including the chordotonal organs (Fujita *et al.*, 1982). A dilution of 1:10 was used for whole mount embryo staining. Kindly provided by G. Boulianne.

anti-engrailed (4D9): a mouse monoclonal antibody which recognises Engrailed, a nuclear protein expressed in the MNB and its progeny (Patel *et al.*, 1989). anti-engrailed was obtained from C. Goodman. A dilution of 1:5 was used for whole mount embryo staining.

2.2 Digoxigenin-labeled RNA probes

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

rhomboid : anti-sense *rho* probe was prepared by *in vitro* transcription from a bluescript vector containing a PCR generated fragment of the *rho* cDNA (prepared by R. Battye and M.Gordon).

2.3 Embryo Collecting and Staging

Adult *Drosophila melanogaster* were placed in perforated plastic beakers capped with 60 X 15 mm plastic petri dishes (Fisher Scientific). Embryos were laid and collected on these plates which had been filled with an apple juice-agar mix a supplemented with a small dab of live yeast paste (Fleishman's dry yeast), which stimulated egg laying and acted as a source of food. For standard collections were changed twice daily, once in the morning and once in the evening. Plates collected in the morning (overnight collection) were placed at 4 °C in order to arrest development of the embryos. Plates collected in the early evening were placed at 18 °C overnight so that the rate of embryonic development was slowed to one half its normal rate. Plates were moved from18 °C to 4 °C the next morning to halt development. This allowed for optimally staged embryos (stage 12- 17) for most experiments. Embryos were stored at 4 °C for a maximum of 72 hours if fixation was not performed immediately. For some heat-shock experiments 4hr embryos were required (see protocol below). Plates were collected at 1or 2 hr intervals and aged to 4hrs at 25 °C.

Embryos were staged according to Campos-Ortega and Hartenstein (1985). Individual stages were identified through the examination of the degree of extension of the germband and nerve cord, and by the differentiation and subdivision of the developing gut. A combination of these traits was used to make the final determination.

2.4 Heat shock protocol

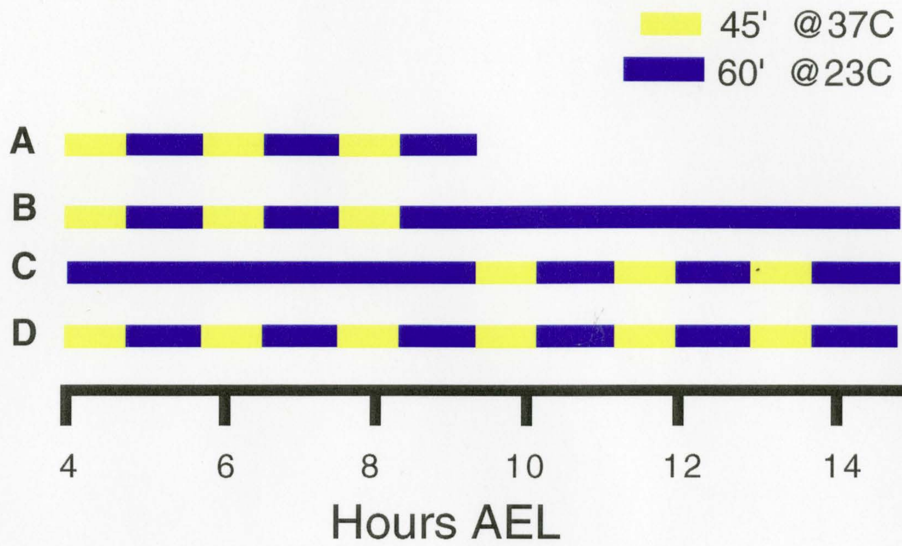
1-2 hour collections of embryos were subjected to a variety of heat-shock protocols depending on the experiment. All heat shock protocols consist of administration of 45 min heat-shocks, followed by 1 hour recoveries. Heat-shocks are carried out by floating the apple juice-agar filled petri plates containing the embryos in a 37 °C water bath. Recovery occurs at room temperature (22 °C-25 °C). Several trials were carried out to determine the duration of heat-shock that was effective at generating supernumerary glia in HS*rho*1B embryos, while damaging the embryos as little as possible. Based on Rho localisation and expression data published in (Sturtevant *et al.*, 1996) it was determined that 45 minute heat-shocks are optimal. Under normal circumstances, Rho is localised to plasma membrane plaques associated with cell junctions (Sturtevant *et al.*, 1996). It appears that a lengthy induction of the HS*rho*1B construct is required to ensure that Rho protein is distributed homogeneously in the plasma membrane and not restricted to plaques. Rho, has a short half-life, possibly because of the presence of a PEST sequence (Bier *et al.*, 1990). It is reported in (Sturtevant *et al.*, 1996) that after a one hour recovery, the level of Rho protein at the plasma membrane in heat-shocked embryos declines rapidly. Based on this data as well as experimental trials, it was determined that 45 min. heat-shocks followed by one hour recoveries are optimal, perhaps due to the fact that high levels of Rho can be maintained ubiquitously on the cell membranes.

Several protocols were used, based on the experiment being conducted, these are summarised in Figure 2. HS*rho*1B embryos were heat-shocked 3 or 6 times (Fig. 2A and 2D) from

Figure 2: Heat-shock protocols

Several heat-shock (HS) protocols were employed to induce ectopic expression of *rhomboid* during various periods of embryonic development. (A) Early HS protocol. 3 X 45 minute HS are administered, interspersed with 1 hour recoveries at room temperature. The first HS is given at stage 9 (4hrs. after egg laying (AEL)). Embryos are fixed and stained at stage 12-13. (B) Early HS protocol with long recovery. Modified from (A), embryos are collected and fixed at stage 16-17. (C) Late HS protocol, 3X45 min. HS are administered. These are started at stage 12-13 and the embryos are collected at stage 16-17 for fixation. (D) Long HS protocol. Essentially a combination of (A) and (C). Embryos are given 6X45 min. HS, starting at stage 9 and collected and fixed at stage 16-17.

Figure 2: Heat-shock protocols



stage 9 (4hrs after egg laying(AEL)) in order to examine the effects of sustained ectopic *rho* expression on stage 12-13 and 16-17 embryos respectively.

To examine the effects of late induction of ectopic *rho* expression on stage 16-17 embryos, 3 heat shocks were administered, starting at stage 12 (9-10hrs AEL) (Fig. 2B). Finally, embryos were heat-shocked 3 times, beginning at stage 9 (4hrs. AEL) and then left to recover until stage 16-17, after the final heat-shock (Fig. 2C). This was used to identify the requirement for sustained ectopic *rho* until the end of development (see results).

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

Embryos were dechorionated in bleach and then placed in a scintillation vial with 5ml heptane, 4.5ml phosphate buffered saline (PBS, pH 7.4) and 0.5ml 37% formaldehyde. Embryos were fixed for approximately 20 minutes, and then the lower aqueous fixative layer was removed with a Pasteur pipette. Embryos were devitellinized by violently adding methanol. Devitellinized embryos sank to the bottom and were removed with a Pasteur pipette to a glass test tube, and washed 3 times in methanol. Embryos were then transferred to a fresh test tube and washed twice more with methanol. The embryos were subsequently bathed in PBS with 0.2% Triton-X (PBT). 3 quick washes followed by a 20 minute wash on the rotator were performed. Embryos were washed twice more in PBT and then transferred, using a plastic pipette to a fresh test tube where they were washed twice more in PBT.

After washing, embryos were blocked for up to 40 minutes in 100ml PBT and 10ml of normal goat serum (NGS). Primary antibody was then added in the appropriate concentration and left overnight on an orbital shaker at 4°C. To wash off excess primary antibody, embryos were washed several times in PBT and left on the rotator for 6 hrs at room temperature or overnight at 4 ° C. Embryos were washed twice in PBT and blocked later the same day or the next day with NGS and PBT as previously described, secondary horseradish peroxidase (HRP) conjugated antibody was added, and allowed to react for 1.5-2 hours. Donkey or Goat anti-rabbit (Jackson Immunological)was used as a secondary for anti-ß-galactosidase, while Goat anti-mouse secondary was used for all other primary antibodies. Embryos were then washed 5 times in PBT and then placed on the rotator for about 30 min. in PBT. After additional washing in PBT, the embryos were re-suspended in a 0.33mg/ml 3,3'-Diaminobenzidine (DAB) solution for the peroxidase reaction. After a short incubation period, a 3% solution of hydrogen peroxide was added to the DAB solution. The reaction was stopped through the addition of PBT when desired colouration was obtained. The HRP staining yields a brown colouration. In double labelling, CoCl was added to the DAB to give a black reaction product. The embryos were then thoroughly washed to remove excess DAB and then dehydrated using an ethanol gradient (50%, 70%, 90%, 95%, 100%). Final storage was in methyl salicylate.

2.6 BrdU incorporation (adapted from Bodmer *et al.*, 1989)

Incorporation of Bromo-deoxyuridine was used to detect cells undergoing apoptosis. Embryos were dechorionated as previously described, collected on Nitex sieves and blotted dry with Kimwipes. These embryos were then immersed in a test tube containing octane equilibrated with 1X *Drosophila* Schneider's medium for 4 min. on the rotator. Embryos were then transferred with a Pasteur pipette, in a minimum volume of octane, to a scintillation vial containing 1mg/ml BrdU in 1X Schneider's medium. The remaining octane was removed by blowing air over the sample in order to evaporate it. Embryos were incubated in this mixture for 30 min., washed 5 times in distilled water and then collected onto a Nitex sieve. Embryos were hydrolysed with 2N HCl in PBT for 30 min. on the shaker and then washed with PBT. Embryos were fixed and processed for antibody staining as described above. A 1:50 dilution of anti-BrdU (Boehringer Mannheim) was used.

2.70 Preparation of RNA probes for *in situ* hybridization (adapted from protocol by Christian Smith)

Labelled RNA probes were generated from linearized template by *in vitro* transcription. A ribonucleotide mix containing digoxigenin-labelled uridine-triphosphate was used to create the labelled probes. cDNA plasmid preps were prepared from overnight bacterial cultures using a Quiagen Maxi Prep kit.

10µg of plasmid DNA was digested in 10µl 10X restriction buffer, sterile water to 95µl and 5µl of an appropriate restriction enzyme (depending on the construct used). DNA was digested for 3

hours at 38 °C. After digestion, the product was cleaned by adding an equal volume of phenol-chloroform mixture and was centrifuged for 2 min. The top aqueous layer was removed to a fresh microfuge tube and the DNA was precipitated overnight at -20 °C using 10µl of 3M NaOAc and 3 volumes of ethanol. The DNA was collected by first centrifuging for 5 min. at maximum rpm and then removing the removing the liquid with a micropipette. The pellet was resuspended in 200 µl of 70% ice-cold ethanol and re-centrifuged for 2 min. The liquid was then removed by aspiration and the pellet was air dried for 1 hour and then resuspended in DEPC treated (diethylpolycarbonate - 0.1%) double distilled water (ddH_2O) to give approximately 0.5 mg/ml (assume that 10µg cDNA digested, therefore 20µl ddH_2O).

In vitro transcription was performed using reagents from the Boehringer Mannheim DIG RNA Labelling kit, catalogue number 1175025. 2µl of DNA template mix was mixed with 2µl 10X NTP labelling mix, 2µl 10X transcription buffer, 1µl RNAse inhibitor, 11µl DEPC treated ddH_2O, followed by 2µl of T3 or T7 RNA polymerase (depending on the orientation of the insert). Transcription was allowed to proceed for 2 hours at 37 °C. The reaction was stopped by the addition of 2µl of 200mM EDTA, pH 8. The RNA probe was precipitated using 0.1 volume of 4M LiCl and 3 volumes of chilled ethanol. After mixing, the RNA was allowed to precipitate overnight at -20 °C. In the morning, the microfuge tube was centrifuged for 15min. The liquid was removed by aspiration and the pellet was washed in 100µl of chilled 70% ethanol/30% DEPC treated ddH_2O. The tubes were again centrifuged, for 5 min., and the liquid removed. The pellet was dried on ice, resuspended in 100µl DEPC treated ddH_2O and stored at -20 °C.

2.71 RNA *in situ* hybridization label (adapted from protocol by Dervla Mellerick)

Embryos were dechorionated in bleach and collected, as described above, however embryo wash (7% NaCl, 0.05% Triton X-100) was employed instead of water for rinsing. Embryos were then fixed in glass scintillation vials containing fixative mixture. The fix mix comprised 2ml embryo wash, 2ml 10% formaldehyde, 1ml 5X fixation buffer (800mM KCl, 200mM NaCl, 20mM EGTA (pH 8.0), 5mM spermadine, 2mM spermine-HCl and 150mM Pipes (pH 7.4))) and 5ml heptane. The vials were given a vigorous shake and then placed on a rotator for 20 min. After this period of rotation the contents of the vials were allowed to settle for 5-10 min., so that the layers could settle. The embryos were removed and washed in methanol as described for general antibody staining. After methanol washing, the embryos were washed 5X in ethanol. Embryos could be stored at this point at -20°C.

Embryos were rinsed in 50% ethanol/50% xylenes and then bathed for 2 hours in 100% xylenes, on an orbital shaker, in order to clear them. They were subsequently rinsed in 50%ethanol/50% xylenes and then 5X in 100% ethanol. Frequent glassware changes were required to ensure that no xylenes were left to contaminate the samples. Embryos were rinsed in 50% methanol/50% PBT (1X PBS with 0.1% Tween-20) and transferred to microfuge tubes (RNAse free).

The embryos were post-fixed for 10 minutes in a 4:1 mixture of PBT and 10% formaldehyde. The tubes were placed on their sides on an orbital shaker. 3X 2 min. rinses with PBT were then performed. Embryos were then treated with 50 mg/ml Proteinase K for exactly 3 minutes with gentle shaking. Great care was taken to ensure that the treatment did not exceed 3 minutes. This reaction

was stopped by removing the liquid and rinsing the embryos 2X with 2mg/ml glycine in PBT. Embryos were washed a further 2X in PBT, which was followed by another 10 min. post-fix and 4X 2 min. PBT washes.

The embryos were then washed 3X 2 min. in 50% PBT/50% prehybridization solution (prehyb) (50% formamide, 300mM NaCl, 10mM Tris-HCl (pH 6.8), 10mM Na Phosphate, 1X Denhardt's solution (1% solution of Bovine Serum Albumin (BSA), Ficoll and Polyvinylpyrrolidone), 5mM EDTA (pH 8.0), and 1mg/ml tRNA) at room temperature. Embryos were washed for 5 min. at room temperature in prehyb and then for 1 hour in prehyb that had been warmed to 53 °C, in the hybridization oven which had been set to 53 °C.

While the embryos were in prehyb solution, a 1:10 dilution of RNA probe solution was prepared and heated to 60 °C for 20 min. in a heating block. The melted probe was then placed on ice. After 1 hour in prehyb, this solution was removed and replaced with pre-warmed (53 °C) hybridization solution (hyb) (essentially the same as prehyb but without tRNA and with 10% w/v dextran sulphate). Depending on the concentration and effectiveness of the probe (as judged by previous experiments) a final dilution of between 1:500 and 1:2500 was used. RNA hybridization was allowed to take place overnight at 53 °C without shaking.

The next morning, the embryos were washed through a gradient of posthybridization solution (posthyb)/PBT mixtures at 53 °C. Posthyb is comparable to prehyd, however there is no tRNA. These washes were performed while shaking the tubes. Each lasted 20 min. and consisted of 4:1, 3:2,

2:3 and 1:4 parts posthyb to PBT followed by 2X 20 min. washes in 100% PBT. All tubes were then washed a final time in room temperature PBT.

Next, the embryos were treated with 20mg/ml of RNAseA at 37°C in a water bath to remove any unhybridized probe. They were then incubated with a 1:2000 dilution on anti-Dig antibody (Boehringer Mannheim) in PBT for 1 hour on the orbital shaker. Embryos were then washed 4X 20 in PBT and then stored overnight at 4°C.

The embryos were washed 3X 5 min. in detection solution (100mM NaCl, 50mM $MgCl_2$, 100mM Tris-HCl (pH 9.5) and 0.1% Tween 20). This was then replaced with reaction solution (4.5 µl 4-Nitro blue tetrazolium chloride (NBT) and 3.5 µl X-phosphate/ 5-Bromo-4-chloro-3-indoyl-phosphate (BCIP) each per ml of detection buffer). The embryos were then transferred back to glass tubes and the reaction was allowed to proceed in the dark.

The reaction was terminated by removing the reaction mix and washing 5X with PBT, followed by 2X with PBS. Embryos were then washed in a graded series of glycerol:PBS mixtures (30/70, 50/50) and finally stored in 70% glycerol/30% PBS at 4°C. Embryos were rehydrated and then dehydrated in an ethanol:water gradient (as described for general antibody work) before being placed in methyl salicylate for mounting and photography.

2.8 Light level photomicroscopy

Whole mount embryos which had been cleared in an ethanol gradient and stored in methyl salicylate were mounted with Permount on glass slides (Corning) and covered with cover slips

($18mm^2$,Corning). Slides were viewed and photographed through a Zeiss Axiophot microscope. Fujichrome 64 Professional T "RTP" 35mm film at a speed of 64 ASA was used for all colour photographs. Processed slides were scanned with a Nikon slide scanner and digitised using Adobe Photoshop. Composite images were assembled by Dr. J.R. Jacobs and R. Battye.

2.9 Cell counting

Cells counts were performed in most experiments presented in this thesis. In all cases, cells were counted and totalled for each segment of each embryo. 7 segments were counted per embryo, these are, the second most posterior segment and the next 6 segments directly anterior. In some embryos a segment may be omitted from counting if it was deemed damaged or too difficult to count accurately. Cell counts for each segment were totalled and added together for each embryo. This sum was then divided by the number of segments counted to give the mean value per embryo. The means of all embryos counted for a particular experiment were then added together and divided by the number of embryos in order to give the mean value per experiment. In all cases, (n=) refers to the number of embryos counted. Standard deviation of the mean was then calculated as described in Rosner, 1995. All values calculated for this thesis are given to 2 significant digits. All values incorporated form other sources are left in the format in which they were originally written.

Several transgenic lines are used. Values for controls are not explicitly represented. However, in all cases controls were examined to determine if the constructs alone were capable of generating an effect. All lines carrying HS*rho*1B were examined in the absence of heat-shock. No increase or

change in MG number was ever detected in these embryos. In addition, both *sli*1.0-Gal4 and *sim*-Gal4 were examined in the absence of any downstream UAS construct and no change in MG number was observed. The same observations were made for embryos carrying the UAS constructs in the absence of the Gal4 drivers. Furthermore, it has been reported that the Gal4-UAS system has very low levels of background expression (Brand and Perrimon, 1993).

RESULTS

Analysis of the *H99* deficiency has demonstrated that in the absence of programmed cell death, supernumerary Midline Glia (MG) survive. At stage 17, these embryos contain 11-12 cells per segment which express midline glial specific markers such as AA142 and *sli*1.0. Expression of *pointed* or *argos* is not upregulated in these extra glia (Dong and Jacobs, 1997).

3.1 Ectopic expression of *rhomboid* generates supernumerary glia

Earlier experiments suggested that overexpression of *rhomboid* was capable of producing supernumerary MG (Stemerdink and Jacobs, 1997; Sonnenfeld and Jacobs, 1995), a phenotype similar to that which is seen in embryos deficient for PCD. This observation is confirmed here. Published HS*rho*1B expression data suggests that Rhomboid is normally localized to plaques in the plasma membrane (Sturtevant *et al.* 1996). These plaques are normally associated with Armadillo and may be present at septate junctions between cells. Induction of *rhomboid* expression using the HS*rho*1B construct disturbs the normal process of localization and causes Rhomboid to be distributed throughout the plasma membrane (Sturtevant *et al.* 1996). Using this data as well as the results of several experimental trials, a heat-shock protocol was developed to give optimum *rhomboid* expression, determined as a maximum effect on MG number (see methods and materials for a complete description of this protocol). All heat-shocks are 45 minutes in duration followed by a 1 hour recovery.

In wildtype embryos 5.60 ±0.20 MG are visible at late stage 12 or early stage 13 (Sonnenfeld and Jacobs, Dong and Jacobs, 1997). This number is not greatly affected in embryos subjected to multiple heat-shocks. These embryos average 5.30 ±0.76 MG per segment (n=10), after the administration of 3 heat-shocks, beginning at stage 9 (Fig. 3A and Table1). Stage 17 WT embryos, having been subjected to 6 heat-shocks beginning at stage 9, are mildly affected, averaging 3.68 ±0.39 AA142 expressing cells per segment (n=12), relative to 3.2 ±0.1 for embryos that have not been subjected to heat-shocks (Sonnenfeld and Jacobs, 1995; Dong and Jacobs, 1997) (Fig. 3C and Table 1). This small discrepancy may be due to the fact heat-shocks appear to delay the development of these embryos (B. Lanoue and J. R. Jacobs, unpublished observation).

Induction of HS*rho*1B dramatically affects the survival of the MG. After 3 heat-shocks, stage 12/3 embryos average 7.90 ±1.34 cells per segment (n=9) (Fig. 3B and Table 1), compared to 5.60 ±0.20 for wildtype embryos. A further 3 heat-shocks maintains 6.84 ±0.82 cells per segment (n=9) to stage 16-17 (Fig. 3D and Table 1), while wildtype embryos have only 3.68 ±0.39 cells per segment. If 3 heat-shocks are induced later in development, beginning at stage 12, these embryos show a significant decrease in the number of MG rescued. Stage 16-17 embryos contain 4.72 ±0.36 cells per segment (n=10) (Fig. 3E and Table 1), suggesting that early ectopic expression of *rho* is required to block apoptosis of these cells. Maintenance of signaling appears essential as embryos which have received 3 heat-shocks beginning at stage 9 and then allowed to recover until stage 16-17 contain only 4.1 ±0.63 cells per segment (n=13). Directed misexpression of Rho using UAS-*rho* and either *sli*1.0-Gal4 or *sim*-Gal4 (described below) fail to produce an increase in MG number. It is possible that UAS-*rho* is incapable of producing

sufficient protein to overload the targeting mechanism and saturate the plasma membrane or that directed misexpression using these drivers is too spatially restricted to produce an effect.

Extra glia in HS*rho*1B embryos are distributed regularly throughout the VNC, in contrast to the supernumerary glia described in *H99* embryos, which appear clustered in the dorsal region of the nerve cord (Dong and Jacobs 1997; Zhou *et al.*, 1997). Ectopic expression of Rho in *H99* embryos, using HS*rho*1B, appears to correct this abnormality (B.R. Lanoue and J.R. Jacobs, unpublished observation).

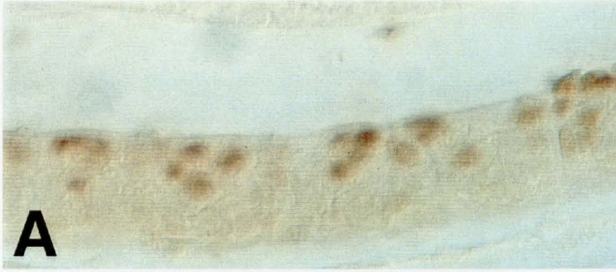
As in *H99* embryos, it is thought that these supernumerary MG arise from mesectodermal progenitors that have been prevented from undergoing PCD and not from cell division (Dong and Jacobs, 1997). In order to determine if the supernumerary glia observed in the HS*rho*1B embryos were derived from mitosis, these embryos were treated with Bromo-deoxyuridine (BrdU) and then stained with an antibody to this synthetic nucleotide. Cells which are in the S phase of the cell cycle incorporate BrdU at much higher levels than do non-dividing cells. Therefore this compound can be used to identify those cells actively preparing to undergo mitosis (see Bodmer *et al.*, 1989). It was determined that the supernumerary MG which arise as the result of ectopic *rho* expression are not created as a result of new cell divisions, since no BrdU labeled cells were detected in the midline of these embryos (Lanoue, 1996).

3.2 Overexpression of *spitz* group genes blocks normal apoptosis of the MG

rhomboid is a member of the *spitz* group, a collection of genes which, when mutated, result in similar phenotypes in many cell and tissue types.

Figure 3. Ectopic expression of Rhomboid results in supernumerary MG.

Embryos containing the AA142 enhancer trap (A and C), which marks the midline glia, or both the HS*rho*1B construct and the AA142 enhancer trap (B,D-F) were labeled with antibodies to β-galactosidase. Embryos A and B are stage 12/0, while D through F represent stage 16-17. All panels in this and successive figures show embryos in saggital view with anterior at left, unless otherwise noted. In wildtype stage 12/0 embryos which have been subjected to 3 heat shocks (A) (Schema A in Figure 2) and stage 16-17 embryos which have been subjected to 6 heat shocks (C) (Schema D in Figure 2) there are approximately 5.3 and 3.7 MG per segment respectively. Stage 12/0 HS*rho*1B embryos which have been heat-shocked 3 times beginning at stage 9 (B) (Schema A, Figure 2) have 7-12 labelled cells per segment, while stage 16-17 embryos which have received 6 heat-shocks from stage 9 (D) (Schema D, Figure 2) have a comparable number of MG. Embryos heat-shocked 3 times, beginning at stage 12 (E) (Schema C, Figure 2) have 4.7 MG per segment by stage 16-17. A similar reduction in MG survival to 4.1 cells per segment, is seen in stage 16-17 embryos which have been heat shocked 3 times, beginning at stage 9 and then allowed to develop to stage 16-17 (F) (Schema B, Figure 2). See Table 1 for a summary of the numerical data.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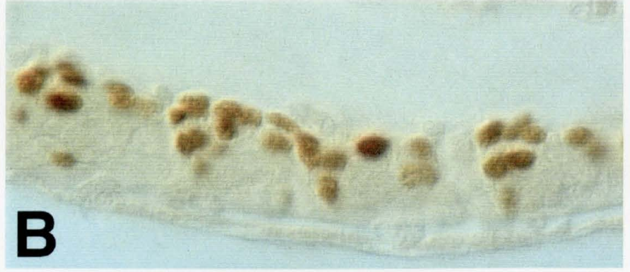
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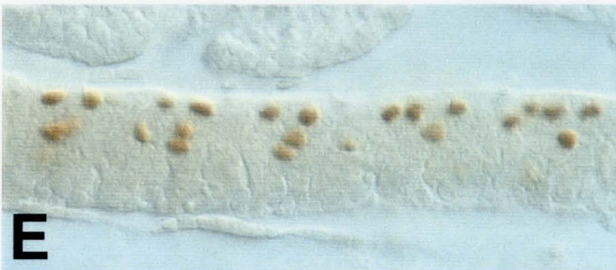
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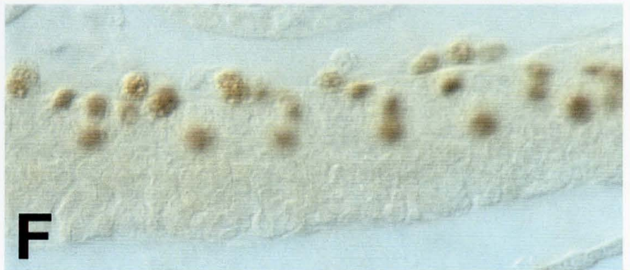
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Table 1: MG number for AA142 or HS*rho*1B; AA142 embryos following heat shock.

Stage and heat shock regime	AA142	HS*rho*1B; AA142
No heat-shock		
Stage 12/0	5.60 ±0.2 (7)**	5.51 ±0.01 (7)
Stage 16-17	3.20 ±0.1 (5)**	3.06 ±0.01 (7)
3 heat-shocks (early)*		
Stage 12/0	5.30 ±0.76 (10)	7.90 ±1.34 (9)
Stage 16-17 (long recovery)*	3.10 ±0.05 (7)	4.11 ±0.63 (13)
3 heat-shocks (late)*		
Stage 16-17		4.73 ±0.36 (10)
6 heat-shocks*		
Stage 16-17	3.68 ±0.39 (12)	6.84 ±0.82 (9)

* : Heat-shock protocols are further describes in methods and materials, see also fig.2
** : Data taken form R. Dong, 1997
The results are represented by mean ± standard deviation (n= number of embryos)

It has been determined that the *spitz* group genes exert their effect by acting through DER and its downstream signal transduction pathways (Schejter and Shilo, 1989; Raz *et al.*, 1990; Raz *et al.*, 1991; Raz and Shilo, 1992; Raz and Shilo, 1993, Sturtevant *et al.*, 1993; Freeman, 1996). Since ectopic expression of *rho* increases the survival of the MG, we investigated the possibility that overexpression of other genes in this pathway might have an identical effect.

Rhomboid is thought to function in *spitz* group signaling by cleaving or facilitating the cleavage of *spitz*, the *Drosophila* TGFα homologue, from its membrane bound form to an activated secreted ligand (Rutledge *et al.*, 1992; Schweitzer *et al.*, 1995; and Freeman, 1994) (see Fig.1) . Thus Rho may act to initiate the signaling cascade (Bier *et al.*, 1990; Schweitzer *et al.*, 1995). sSpi then binds DER and the signal is then transduced through the activation of multiple downstream cytoplasmic signaling pathways (see Fig.1). It is therefore not surprising that overexpression of *sspi* and other genes of the DER/MAPK signaling pathway produce a similar effect to ectopic expression of *rho* using HS*rho*1B. UAS constructs for secreted *spi* (UAS-*sspi*) (Schweitzer *et al.*, 1995), activated *Draf* (UAS-*DRaf*) (Brand and Perrimon, 1991), activated *Ras* (UAS-Ras^{V12}) (Karim and Rubin, 1998), activated *DER* (UAS-DER^{A887T}) (N. Baker, pers. comm.) and *pointedP1* (UAS-*pntP1*) (Scholz *et al.*, 1997) were obtained. Expression of these transgenes was targetted to the developing midline using both *sim*-Gal4 and *sli*1.0-Gal4 as drivers (see materials and methods for a full description of these constructs). Each Gal4 construct drives expression within a subset of cells in the developing midline (Scholz *et al.*, 1997; also see Appendix). Expression of *sim*-Gal4 begins in all mesectodermal cells at stage 9 and gradually becomes restricted to the MG by stage 17 (see Appendix). *sli*1.0

expression begins later, at stage 12, and continues beyond the end of embryogenesis (see Appendix). The expression is slightly more restricted in that only the MG and perhaps the MP1 neurons express this driver (Scholz *et al.* 1997).

Directed misexpression of *sspi*, DER^{A887T} or Ras^{V12} increases the number of AA142 expressing cells in comparison to wildtype embryos which have 3.20 ±0.1 MG per segment at stage 17 (see Table 2). Overexpression of *sspi* using *sli*1.0-Gal4/+; UAS-*sspi*/AA142 (Fig. 4A) results in 7.74 ±0.74 cells per segment (n=11) at stage 17, while use of *sim*-Gal4/+; UAS-*sspi*/AA142 (Fig. 4B) yields 6.5 ± 0.64 MG (n=11). Similar increases in MG number are also observed when an activated form of *DER* is misexpressed using *sli*1.0-Gal4/+; UAS-DER^{A887T}/AA142 (Fig. 4C), 6.62 ±0.74 cells per segment (n=9), and *sim*-Gal4/+; UAS-DER^{A887T}/AA142 (Fig. 4D), 4.87 ±0.8 (n=19). Directed misexpression of an activated form of *Ras* also generates supernumerary MG. *sli*1.0-Gal4/+; UAS-Ras^{V12}/AA142 embryos (Fig. 4E) have 5.92 ±0.64 cells (n=9) and *sim*-Gal4/+; UAS- Ras^{V12}/AA142 embryos(Fig. 4F) have 6.35 ±1.08 (n=10) per segment. Overexpression of UAS-Ras^{wt} produces a negligible effect (data not shown). In all cases the supernumerary MG are regularly distributed throughout the VNC as described for HS*rho*1B; AA142 embryos (Fig. 3). It is possible that *sli*1.0-Gal4, despite its later and more spatially restricted induction of expression, is a stronger driver than *sim*-Gal4. This conclusion is based on the fact that overexpression of UAS-*sspi*, UAS-DER^{A887T}, and UAS-Ras^{V12} using *sli*1.0-Gal4 generates a greater number of supernumerary MG than does overexpression of these same genes using *sim*-Gal4.

Table 2: MG number at stage 16-17 for embryos in which *spitz* group/DER pathway genes have been misexpressed using the Gal4-UAS system.

*Gal4 construct	*sli*1.0-Gal4			*sim*-Gal4		
***UAS construct**	AA142	*aos*^{W11}	*pnt*^{1277}	AA142	*aos*^{W11}	*pnt*^{1277}
sspi	7.74±0.74 (11)	6.49 ±0.47 (9)	7.03 ±0.52 (7)	6.50 ±0.64 (11)	7.32 ±0.54 (8)	6.39 ±0.58 (8)
DER^{A887T}	6.62 ±0.74 (9)			4.87 ±0.80 (19)		
ras^{V12}	5.92 ±0.64 (9)			6.35 ±1.08 (10)		
ras^{V12S35}				4.52 ±0.70 (14)		
ras^{V12C40}				2.60 ±0.30 (14)		
ras^{V12G37}				2.46 ±0.19 (10)		
Draf	4.32 ±0.51 (9)			7.46 ±1.94 (10)	9.35 ±0.99 (8)	5.91 ±0.33 (6)
pntpP1	4.43 ±0.36 (7)			5.58 ±0.30 (10)		

* : All constructs are described in the methods and materials section, also see Appendix 1 for a pictorial description of the *sim*-Gal4 and *sli*1.0-Gal4 expression patterns.
The results are represented by mean ± standard deviation (n= number of embryos).

Since overexpression of the activated ligand, *sspi*, and constitutively activated forms of the receptor DER^{A887T}, and of a cytoplasmic molecule that acts to transduce the signal from the receptor to various downstream signaling pathways, Ras^{V12}, we conducted experiments to determine which of these downstream pathways were being activated by *spitz* group signal transduction.

Misexpression of *Draf* using *sim*-Gal4/+; UAS-*Draf*/AA142 (Fig. 4H) produces a strong effect, yielding 7.46 ±1.94 cells per segment (n=10). However, in most embryos a large fraction of these supernumerary glia appear pushed to the dorsal margin of the nerve cord, as seen in *H99* embryos (see arrow, Fig. 4H). In addition, many late stage embryos contain a high number of apoptotic bodies (see arrow, Fig. 6I). This suggests that the supernumerary glia produced by *Draf* misexpression are not fully differentiated or that they are incompletely protected from PCD. Interestingly, misexpression of *Draf*, in *sli*1.0-Gal4/+; UAS-*Draf*/AA142 embryos (Fig. 4G), is not effective at increasing MG number, as only 4.32 ±0.51 cells per segment (n=9) are observed. Numerous apoptotic profiles are observed in these embryos (see arrow in Fig. 4G). Apparently, late expression of Draf is insufficient to protect most of the MG from undergoing apoptosis.

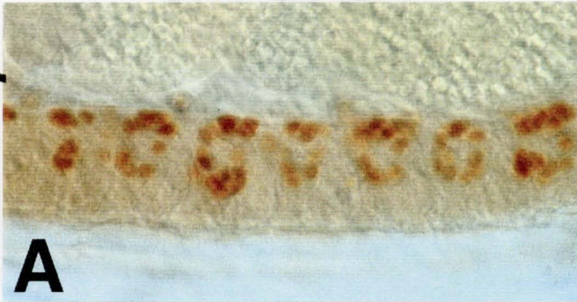
Draf is known to activate MAPK through phosphorylation, (reviewed in Marshall, 1995) and MAPK is known to attenuate Pnt function (O'Neal *et al.*, 1994). Therefore we tested whether overexpression of *pntP1*, a constitutively active form of *pointed* (Klämbt, 1993; O'Neal *et al.*, 1994), was sufficient to increase the survival of the MG.

Figure 4. Overexpression of *spitz* group/DER pathway and MAPK pathway genes increases the number of supernumerary MG.

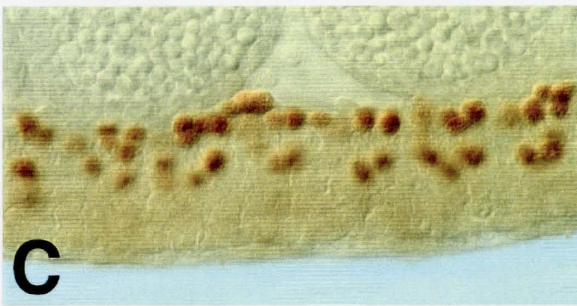
All panels show saggital view of stage 16-17 embryos. Ectopic expression of UAS constructs for *spitz* group/DER pathway and MAPK pathway genes is controlled by *slit*1.0-Gal4 (A,C,E,G,I) or *sim*-Gal4 (B,D,F,H,J) drivers. In UAS-*sspi* embryos (A and B) there is a dramatic increase in the number of MG per segment, regardless of which driver is used. UAS-DER^{A887T} and UAS-Ras^{V12} have a large increase in MG number, however there is a greater increase, in both cases, when *sli*1.0-Gal4 is used (C and E) rather than *sim*-Gal4 (D and F). *sim*-Gal4;UAS-*Draf* (H) shows a similar increase in cell number. Note the dorsal clustering of the surviving MG in these embryos (arrow). *slit*-Gal4;UAS-*Draf* (G) has a considerably lesser effect on supernumerary cell production. Note the high number of apoptotic profiles visible in this late embryo (arrow). UAS-*pnt*P1 embryos show a smaller increase in cell number than does UAS-*Draf*. In comparison, *sim*-Gal4; UAS-*pntP1*/AA142 (J) induces a larger increase than *sli*1.0-Gal4; UAS-*pntP1*/AA142 (I).

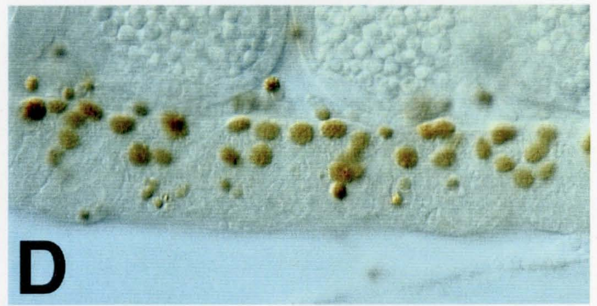
slit-GAL4 *sim-GAL4*

UAS-sSpi



*UAS-DER**





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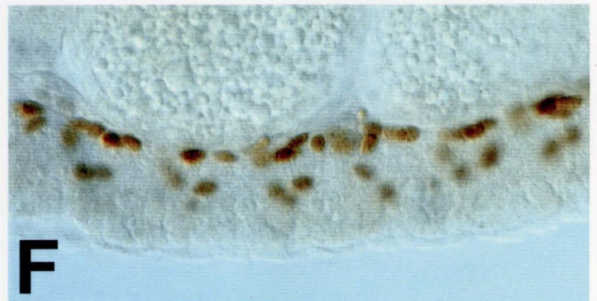

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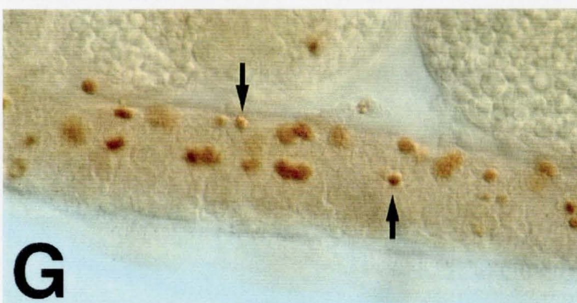
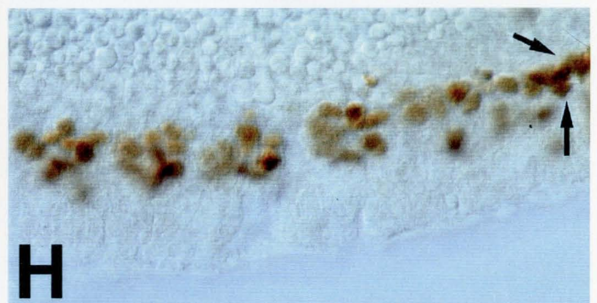
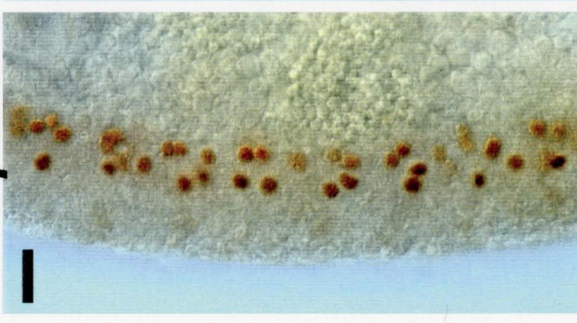




UAS-pntP1



Misexpression of *pntP1* in the mesectoderm using *sim*-Gal4/+; UAS-*pntP1*/AA142 (Fig. 4J) produces 5.58 ±0.3 cells per segment (n=10) while use of *sli*1.0-Gal4/+; UAS-*pntP1*/AA142 (Fig. 4I) gives only 4.43 ±0.36 (n=7). This result is similar to that which was obtained for the misexpression of *Draf*, but is slightly elevated in comparison to wildtype (see Table 1 and Table 2). However, early misexpression of *pnt* using *sim*-Gal4/+; UAS-*pntP1*/AA142 appears less effective at prolonging the life of the MG into the later stages of development than does misexpression of *Draf* under the same conditions. Interestingly, under the control of *sli*1.0-Gal4, both UAS-*pntP1* and UAS-*Draf* appear roughly equivalent in their inability to block cell death, as judged by the number of MG counted in each at stage 16-17. Overexpression of UAS-*pntP2*, which requires phosphorylation to become activated (Brunner *et al.*, 1994), produces no increase in AA142 expressing cells (data not shown).

3.3 MG survival is dependent on coordinated signaling through multiple intracellular signaling pathways

As demonstrated above, overexpression of *sspi*, *DER*^{A887T} and *Ras*^{V12} produce an increase in MG number when directed by either *sli*1.0-Gal4 or *sim*-Gal4 (see Fig. 4). All the MG produced appear normal in their positioning. This data suggests that sSpi, DER^{A887T} and Ras^{V12} are able to induce activation of the full complement of intracellular signaling pathways utilized in normal endogenous *spitz* group signal transduction. Furthermore, it has been established in the literature that mammalian Ras is capable of interacting with multiple downstream signaling pathways including Raf/MAPK and PI3'K/AKT, and perhaps others that are not yet characterized (reviewed in Katz and

McCormick, 1997). Misexpression of *Draf* generates supernumerary MG as well, however this phenotype is dependent upon early expression of the transgene, while overexpression of *sspi*, DER^{A887T}, and Ras^{V12} is not. This suggests that other signaling mechanisms in addition the DRaf/MAPK pathway, are involved in transduction of the survival signal within the MG. Thus we designed experimental protocols to test the relative contribution of different cytoplasmic signaling pathways in this process.

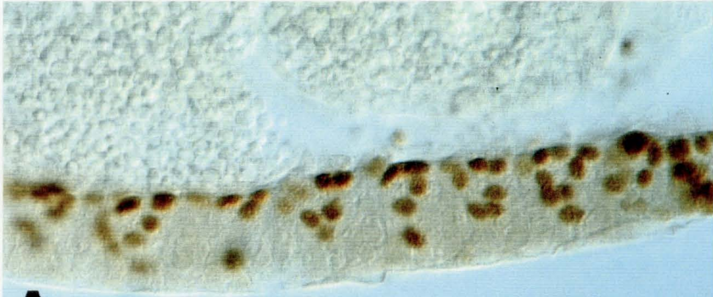
Ras^{V12C40} and Ras^{V12G37} are both activated Ras constructs which have mutations to specific residues in the effector loop region of the protein which, in mammals, suppress the ability of Ras to interact with Raf (reviewed in Katz and McCormick, 1997; Karim and Rubin, 1998). In each case, a dominant negative effect was observed, suggesting that Raf is vital to transduction of the anti-apoptotic signal. Overexpression of these mutant constructs in *sim*-Gal4/+; UAS-Ras^{V12G37}/AA142 (Fig. 5C) and *sim*-Gal4/+; UAS-Ras^{V12C40}/AA142 (Fig. 5D) produce 2.46 ±0.19 (n=10) and 2.60 ±0.30 (n=14) cells per segment respectively. Misexpression of Ras^{V12S35}, a mutant form of Ras which is does not activate the PI3'K/Akt signaling pathway (reviewed in Katz and McCormick, 1997), using *sim*-Gal4/+; UAS-Ras^{V12S35}/AA142 (Fig. 5B), produces 4.52 MG ±0.70 (n=14) MG per segment, a marked decrease from Ras^{V12}, but still an increase over wildtype (Fig. 5A). In addition, numerous apoptotic profiles can be detected (arrow in Fig. 5B), a phenotype frequently observed when only DRaf is overexpressed (see above).

Misexpression of *Dakt/PKB*, a downstream target of PI3'K which acts to prevent cell death by blocking caspase function (Kennedy *et al.*, 1997) in *sim*-Gal4/+; UAS-*Dakt*/+; AA142/+ embryos (Fig. 5E) does not increase the number MG, nor is MG number altered in *sli*1.0-Gal4/+; UAS-*Dakt*/+; AA142/+ (data not shown). Interestingly,

expression of a dominant negative version of Akt (CAAX) does not affect the survival of the MG (data not shown). However, *sim*-Gal4/+; UAS-*akt*/+; UAS-Draf/AA142 embryos show a reduction in the dorsal clustering described for *sim*-Gal4/+; UAS-Draf/AA142 embryos. Misexpression of both genes also appears to decrease the appearance of apoptotic bodies in late stage embryos (data not shown). When *sli*1.0-Gal4 is used to drive expression of both UAS-*Draf* and UAS-*Dakt*, in *sli*1.0-Gal4/+; UAS-*akt*/+; UAS-Draf/AA142 embryos, they appear to act synergistically (Fig. 5F), increasing MG survival over that which is seen in *sli*1.0-Gal4/+; UAS-*Draf*/AA142 or *sli*1.0-Gal4/+; UAS-*Dakt*/+; AA142/+ embryos. This suggests that both *Draf* and *Dakt* act together to mediate survival of the MG.

Figure 5. MG survival is dependent upon coordinated signalling through Draf and Akt.

(A) *sim*-Gal4/+; *Ras*^{V12}/+; AA142/+ embryos have supernumerary glia in comparison to wildtype embryos. (B) *sim*-Gal4/+; UAS-*Ras*^{V12S35}/+;AA142/+, which is postulated to block signalling through PI3'Kinase and AKT, decreases the ability of *Ras*^{V12} to generate supernumerary MG. Note the apoptotic cells in this late embryo (see arrow). (C) *sim*-Gal4/+;*Ras*^{V12G37}/+; AA142/+ or (D) *sim*-Gal4/+; UAS-*Ras*^{V12C40}/+;AA142/+ both of which activate Ras but block signal transduction through Draf, result in a slight decrease in the number of AA142 expressing cells relative to wildtype embryos, a dominant negative effect. (E) Expression of D*akt*, using *sim*-Gal4/UAS-D*akt*; AA142/+ is insufficient to increase the number of MG. (F) However, use of *sli*1.0-Gal4/UAS-D*akt*; AA142/UAS-*Draf* produces more supernumerary MG than the sum of each used independently.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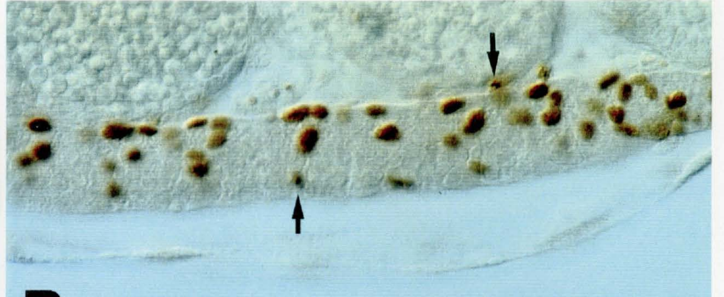
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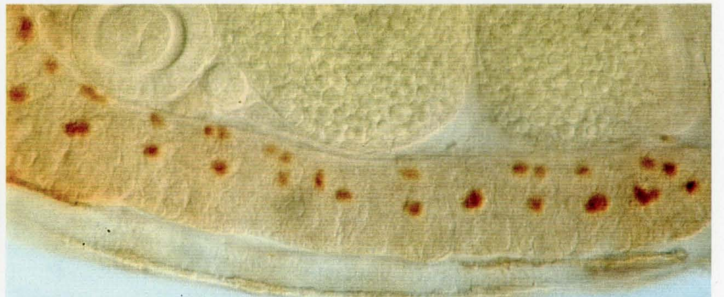
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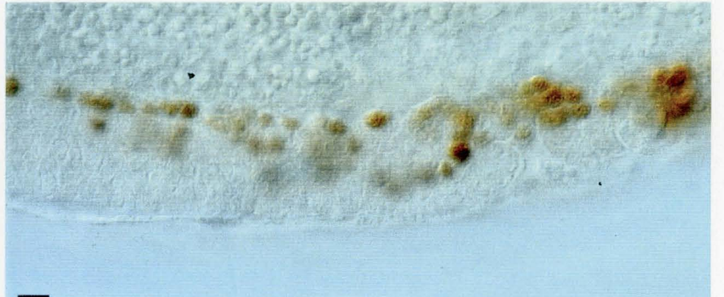

F

3.4 sSpi and Draf upregulate *pnt* and *aos* expression

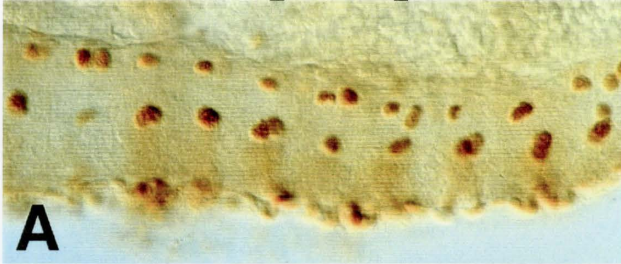
In *H99* embryos glial specific markers such as AA142 and *sli*1.0 are expressed in 11-12 supernumerary MG, while *pnt* and *aos* expression, which are initiated in late stage 12 and only in MG which survive normal PCD, remain limited approximately 3 as in WT (Dong and Jacobs, 1997). *pnt* expression, monitored using the pnt^{1277} enhancer trap, which is a mild hypomorph for this gene, is normally expressed in 2.9 ±0.04 cells per segment at stage 16-17 (Dong, 1997) (Fig. 6B). Homozygotes have a slightly reduced cell number (Perz, 1994). 7.03 (n=7) cells per segment express *pnt* in *sli*1.0-Gal4/+; UAS-*sspi*/pnt^{1277} embryos (Fig. 6D, see Table 2), 6.39±0.58 (n=8)cells per segment in *sim*-Gal4/+; UAS-*sspi*/pnt^{1277} embryos (Fig. 6F, see Table 2) and 5.91 ±0.33 (n=6) cells per segment in *sim*-Gal4/+; UAS-*Draf*/pnt^{1277} embryos (Fig. 6H, see Table2). In WT embryos aos^{w11} is expressed in a similar number of midline cells, 3.2 ±0.1 per segment (Stemerdink and Jacobs, 1997 and Fig. 6A). Like pnt^{1277}, aos^{w11} is a hypomorphic allele. Homozygotes have 5.1 ±0.2 *aos* expressing cells per segment (Stemerdink and Jacobs, 1997). In *sli*1.0-Gal4/+; UAS-*sspi*/aos^{w11}embryos, 6.48 ±0.47 (n=9) cells per segment express *aos* (Fig. 6C), while 7.32 ±0.54 (n=8)cells per segment express this negative regulator of DER signaling (see introduction) in *sim*-Gal4/+; UAS-*sspi*/aos^{w11} embryos (Fig. 6C) and 9.35 ±0.99 (n=8) cells per segment express *aos* in *sim*-Gal4/+; UAS-*Draf*/aos^{w11} embryos (Fig. 6G and I). It is useful to note that some of the *aos* expressing cells in *Draf* embryos have accumulated in the dorsal part of the nerve cord (Fig. 6G) and that some embryos have a high number of apoptotic profiles (Fig. 6I) as seen for AA142 (Fig. 4).

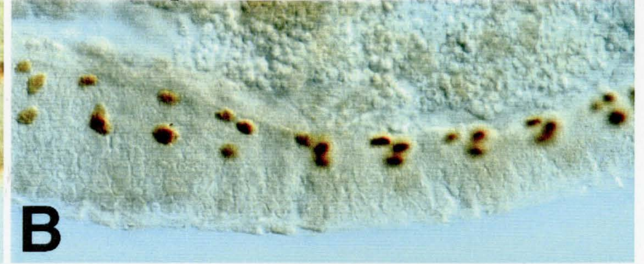
Figure 6. Directed expression of sSpitz or Draf induces expression of *argos* and *pointed* in all surviving MG.

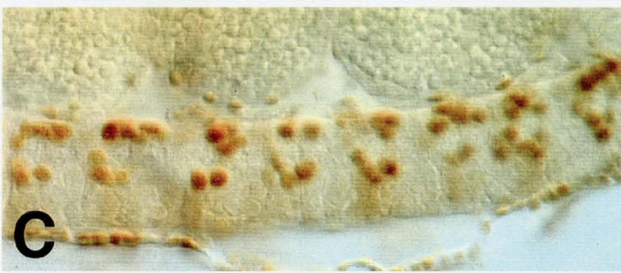
Embryos expressing the *pnt*^{1277} (B,D,F,H) or *aos*^{w11} (A,C,E,G,I) enhancer traps were stained with antibodies to β-galactosidase. All panels are of stage 16-17 embryos. In wildtype embryos, approximately 3 cells per segment express *argos* (A) or *pointed* (B). Directed misexpression of *sspi* dramatically increases this number. (C) *sli*1.0-Gal4/+; UAS-*sspi*/*aos*^{w11}, (D) *sli*1.0-Gal4/+; UAS-*sspi*/*pnt*^{1277}, (E) *sim*-Gal4/+; UAS-*sspi*/*aos*^{w11}, (F) *sim*-Gal4; UAS-*sspi*/*pnt*^{1277} all contain an increased number of cells. UAS- *raf* also leads to an increase in the number of MG expressing these markers, however these cells either sit dorsally in the VNC or undergo apoptosis late in embryogenesis. (G and I) *sim*-Gal4/+; UAS-*Draf*/*aos*^{w11} and (H) *sim*-Gal4/+; UAS-*Draf*/*pnt*^{1277} (H) shows an increase in cell number. Note the high number of apoptotic bodies (arrow in I).

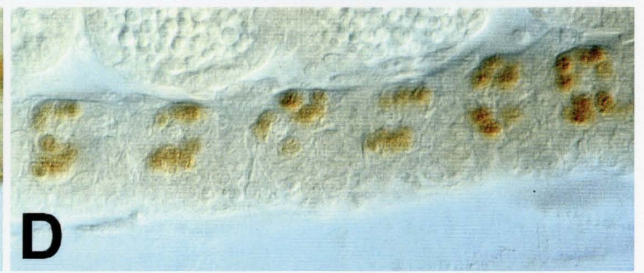
aos[w11]/+ | *pnt[1277]/+*

UAS-sSpi
Slit-GAL4

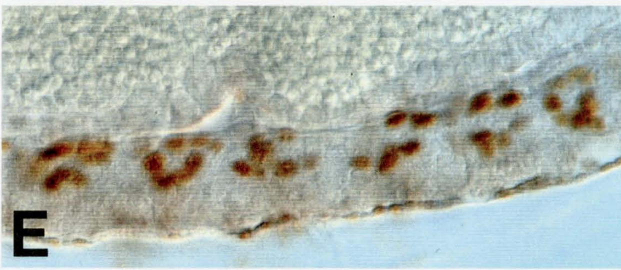




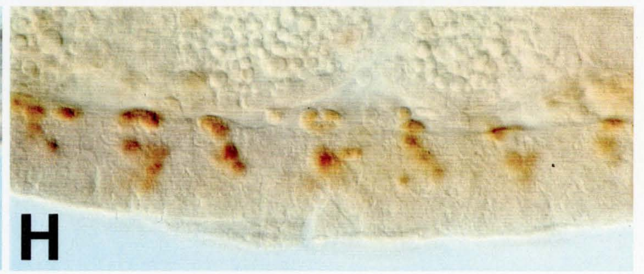


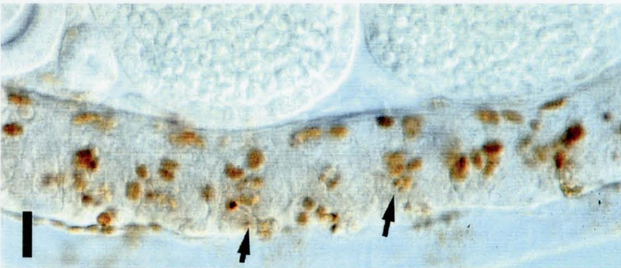


UAS-sSpi
sim-GAL4



*UAS-raf**
sim-GAL4





This demonstrates that *pnt* and *aos* expression can be upregulated by the *spitz* group/DER pathway, and that Draf is sufficient for this signaling, even though it may not be sufficient for complete differentiation or survival of the MG (see above).

3.5 *rhomboid* expression can be induced by the *spitz* group signaling and by DRaf

During the course of normal development, *rho* expression is detected in the entire neurectoderm of blastoderm embryos. Mesectodermal expression of *rho* begins around stage 8, as determined by mRNA *in situs* or the enhancer trap X81 (Bier *et al.*, 1990, Lanoue, Gordon and Jacobs, unpublished results). The midline expression at this stage is very similar to that observed for *single-minded* (Bier *et al.*, 1990; Xiao *et al.*, 1996). It should be noted that detection of *rho* expression, using anti-β-galactosidase staining in X81 as an indicator, is temporally less sensitive than detection using antisense mRNA probes for *rho* (Lanoue, Gordon and Jacobs, unpublished results), due in part to the perdurance of β-galactosidase, and to the lag between mRNA transcription and translation of the protein. Stage 13-14 X81 embryos show that *rho* is expressed in a subset of the mesectodermal cells at this stage (Fig. 7A).

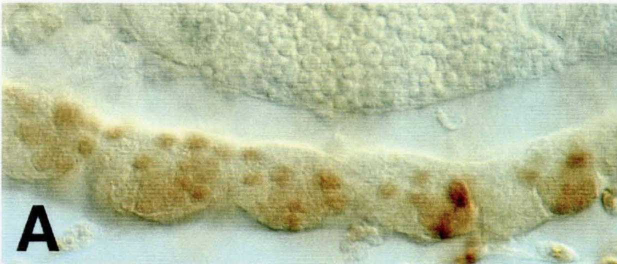
Expression of *rho*, because of its temporally and spatially restricted pattern, is postulated to act to define the region in which *spitz* group signaling will be activated (Bier *et al.*, 1990; Schweitzer *et al.*, 1995). This is thought to occur through the facilitation of Spitz cleavage (Schweitzer *et al.*, 1995). It is uncertain what signals exist to induce and regulate expression of *rho*. It is known from studies on oogenesis that induction of *rho* expression is dependent upon signaling through DER (Sapir *et al.*, 1998). Furthermore, it has been demonstrated that subsequent activation of the *spitz* group/DER signaling

pathway acts to upregulate *rho* expression, thus creating an amplification loop (Sapir *et al.*, 1998). The question of initiation of signaling is not addressed here, however experiments were conducted to determine if the *spitz* pathway is capable of increasing the number of midline cells which express *rho* during midline development. This could help to determine if the *spitz* group/DER pathway could influence *rho* expression, as seen during oogenesis (Sapir *et al.*, 1998).

Ectopic expression of Rho in HS*rho*1B; X81 embryos, after 3 early heat-shocks beginning at stage 9 (see materials and methods) (Fig. 7B), or directed misexpression of sSpi using *sim*-Gal4/+; UAS-*sspi*/X81 (Fig. 7C) results in an increase in the number cells expressing *rho* at late stage 13, as detected by X81. This result was confirmed for *sim*-Gal4/+; UAS-*sspi*/+ embryos using anti-sense *rho* probes (data not shown). Upregulation of *rho* in these embryos is not restricted to the MG, as are increases in *aos* or *pnt* expression (Fig. 6). *sim*-Gal4/+; UAS-*Draf*/X81 embryos show a more subtle increase in the X81 expression pattern (Fig. 7D). *Draf*, a cell autonomous factor, is restricted to cells expressing *sim* and thus can only exert a direct effect within this subset of cells. Therefore only cells which express the transgene can have increased expression of *rho*. As noted, a subtle increase in the *rho* expression pattern is observed in these embryos. It is possible that increased levels of *rho* expression in the cells receiving the autonomous signal via the UAS-*Draf* transgene is able to create a small non-autonomous effect which could raise *rho* expression levels in neighbouring cells very subtly. This is an indirect effect which results from upregulation of Rho function and not from misexpression of *Draf* itself.

Figure 7. *rhomboid* expression in the embryonic midline is upregulated by overexpression of *spitz* group genes or Draf.

Stage 13-14 embryos carrying the enhancer trap X81 which marks expression of *rhomboid* were stained with antibodies to β-galactosidase. (A) In wildtype embryos, the number of cells expressing the X81 enhancer trap begins to decrease by early stage 13 and is restricted to a subset of mesectodermal cells by stage 13-14. (B) *sim*-Gal4/+;UAS-*sspi*/X81 and (C) HS*rho*1B;X81/+ embryos which have received 3 heat shocks, beginning at stage 9, show a dramatic increase in the number of X81 expressing cells compared to wildtype. Note that elevated *rhomboid* expression is not restricted to the MG. (D) *sim*-Gal4/+;UAS-*Draf*/X81 embryos also show an increase, however the number of extra cells expressing the enhancer trap appears reduced when compared to (B) and (C).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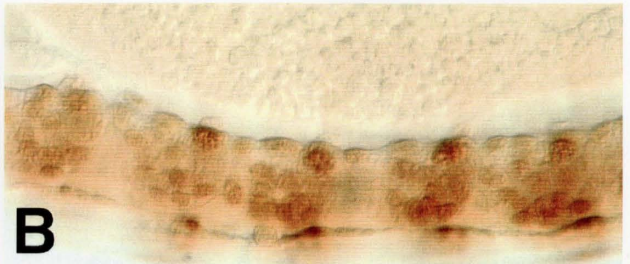
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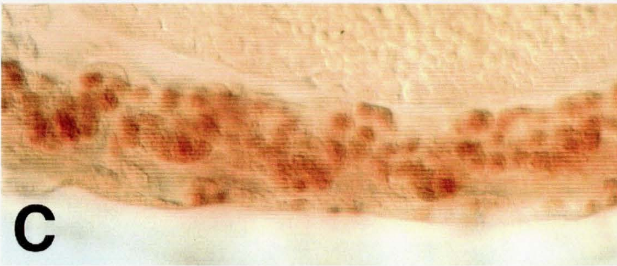
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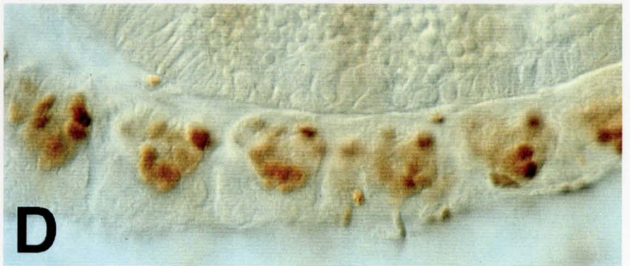
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3.6 *spitz* group mutations suppress the effects of ectopic Rho expression

In WT embryos, which have been subjected to heat-shock, approximately 5.30 ±0.76 MG are detected at stage 12/0, based on AA142 expression (Fig. 3A, Table 1). Loss of function of any of the *spitz* group genes results in a dramatic reduction in surviving MG (Klämbt and Jacobs, 1991; Sonnenfeld and Jacobs, 1994). At stage 12/0, *spi*, *rho*, *S* and *flb* mutant embryos which have been subjected to 3 heat-shocks, beginning at stage 9, have significantly reduced numbers of MG. rho^{38},AA142 embryos contain 2.38 ±0.18 MG per segment (n=7) (Fig. 8A, see Table 3), spi^{1}; AA142 embryos have 2.38 ±0.45 (n=10) (Fig. 8C, Table 3), S^{11N23}; AA142 embryos contain 2.47 ±0.50 AA142 expressing cells (n=7) (Fig. 8E, Table 3), and flb^{2E03}; AA142 embryos have 2.22 ±0.47 (n=11) (Fig. 8G, Table 3).

The effect of ectopic *rhomboid* expression was assessed in these mutants, in order to determine the epistatic relationship between *rho* and the other members of the *spitz* group. Ectopic expression of *rho* in HS*rho*1B; AA142 embryos results in a dramatic increase in MG number (Fig. 3B, Table 1). In general, this phenotype was suppressed in *spitz* group/DER pathway mutants, suggesting that these genes are epistatic to *rho*.

HS*rho*1B; rho^{38},AA142 embryos (Fig. 8B, Table3) show some suppression of the *spitz* group phenotype, generating 4.73 ±0.53 MG per segment (n=11). Although the number of MG is far lower than that which is normally seen in HS*rho*1B embryos, this represents a partial rescue of the rho^{38} phenotype. In HS*rho*1B; spi^{1}; AA142 embryos (Fig. 8F, Table 3) some degree of rescue was also observed in terms of MG number. These embryos contain 4.44 ±0.66 cells per segment which express AA142 (n=9).

Table 3: MG numbers at stage 13 for *spitz* group mutants and attempted rescues using HS*rho*1B.

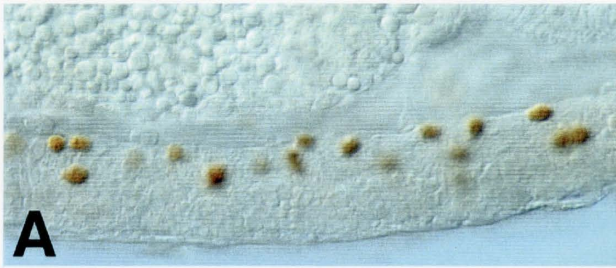
spitz* group mutant	*mutant*; AA142*	HS*rho*1B; *mutant*; AA142*
spitz^{1}	2.38 ±0.45 (10)	4.44 ±0.66 (9)
Star^{IIN23}	2.47 ±0.50 (7)	3.34 ±0.21 (8)
faint little ball^{2E03}	2.22 ±0.47 (11)	2.15 ±0.61 (14)
rhomboid^{38}	2.38 ±0.18 (11)	4.73 ±0.53 (11)

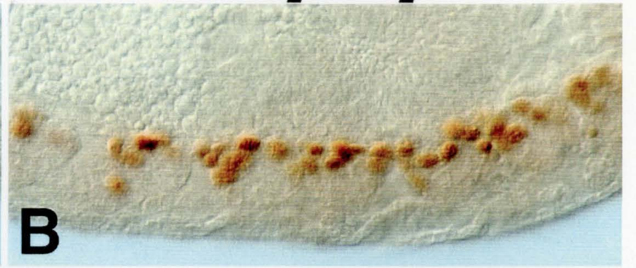
*See materials and methods and results for complete description.
The results are represented by mean ± standard deviation (n= number of embryos).

Figure 8. Rhomboid is epistatic to other members of the *spitz* group/DER pathway. AA142 expression is examined in embryos carrying the HS*rho*1B construct and also homozygous for a null allele of a gene form the *spitz* group pathway. All embryos homozygous for *spitz* group mutations show a decrease in surviving MG at stage 12/3. (A) *rho*^{38}, AA142, (C) *spi*^{1}; AA142, (E) *S*^{11N23}; AA142, and (G) *flb*^{2E03}; AA142 all have phenotypes characteristic of *spitz* group mutants. (B) HS*rho*1B; *rho*^{38},AA142 shows some suppression of the *spitz* group phenotype, although the number of MG is far lower than that which is normally seen in HS*rho*1B embryos. (D) HS*rho*1B; *spi*^{1}; AA142 and (F) HS*rho*1B; *S*^{11N23}; AA142, show a less noticeable suppression of the mutant phenotype, while (H) HS*rho*1B; *flb*^{2E03}; AA142 shows almost no ability to rescue the phenotype. Note than most of the glia rescued by HS*rho*1B appear clustered in the dorsal region of the VNC.

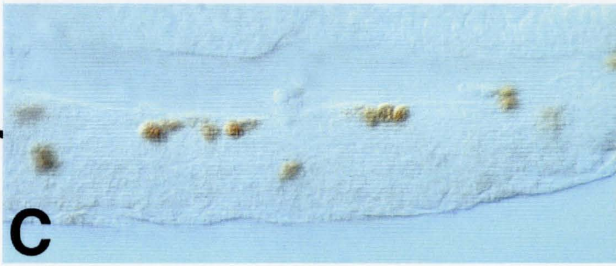
HS[rho]

rho

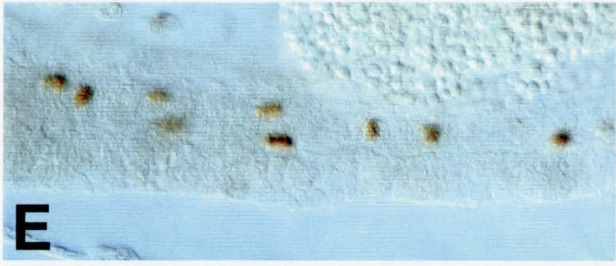


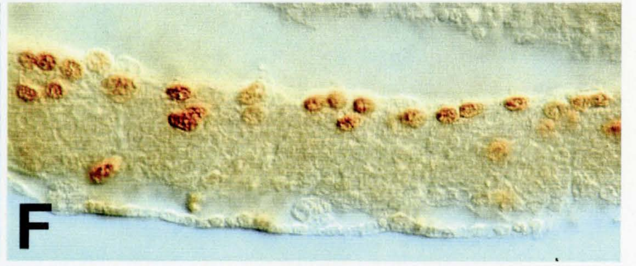


spi

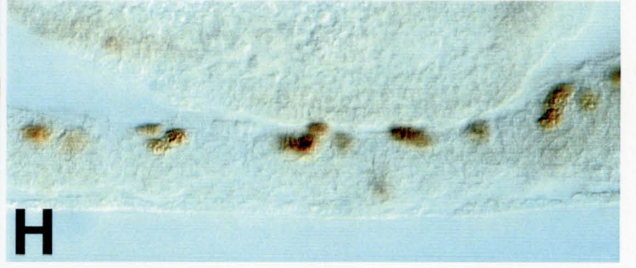


S





flb



It should be noted that this is far below the normal number seen in HS*rho*1B embryos and that the cells appear clustered in the dorsal region of the VNC. Interestingly, *sim*-Gal4/UAS-*sspi*; *rho*^{38}-AA142 embryos(data not shown) have a similar number of MG per segment in comparison with *sim*-Gal4; UAS-*sspi*/AA142 embryos (see Fig. 4B). Combined, these data support the model that Rho acts in the signaling cell, perhaps in the cleavage Spitz, and not in the receiving cell (Schweitzer *et al.*, 1995; Golembo *et al.*, 1996).

HS*rho*1B; S^{l1N23}; AA142 embryos (Fig. 8F) have a mild increase in MG number relative to S^{l1N23} mutant embryos (Fig. 8D)(see Table 3). 3.34 ±0.21 cells per segment (n=8) were counted in these embryos, still well below the ectopic Rho phenotype. HS*rho*1B; *flb*^{2E03}; AA142 embryos (Fig. 8H, Table 3) have no increase in MG number relative to *flb*^{2E03} mutant embryos, at 2.15 ±0.61 cells per segment (n=14). HS*rho*1B; S^{l1N23}; AA142 embryos have a small increase in MG number, relative to S^{l1N23}; AA142 embryos, however this number is still well below that observed in HS*rho*1B; AA142. Similarly, HS*rho*1B; *flb*^{2E03}; AA142 embryos show do not show a any increase in MG number over *flb*^{2E03}, AA142 mutant embryos. These data suggest that both DER and S are required to mediate signaling initiated by Rhomboid.

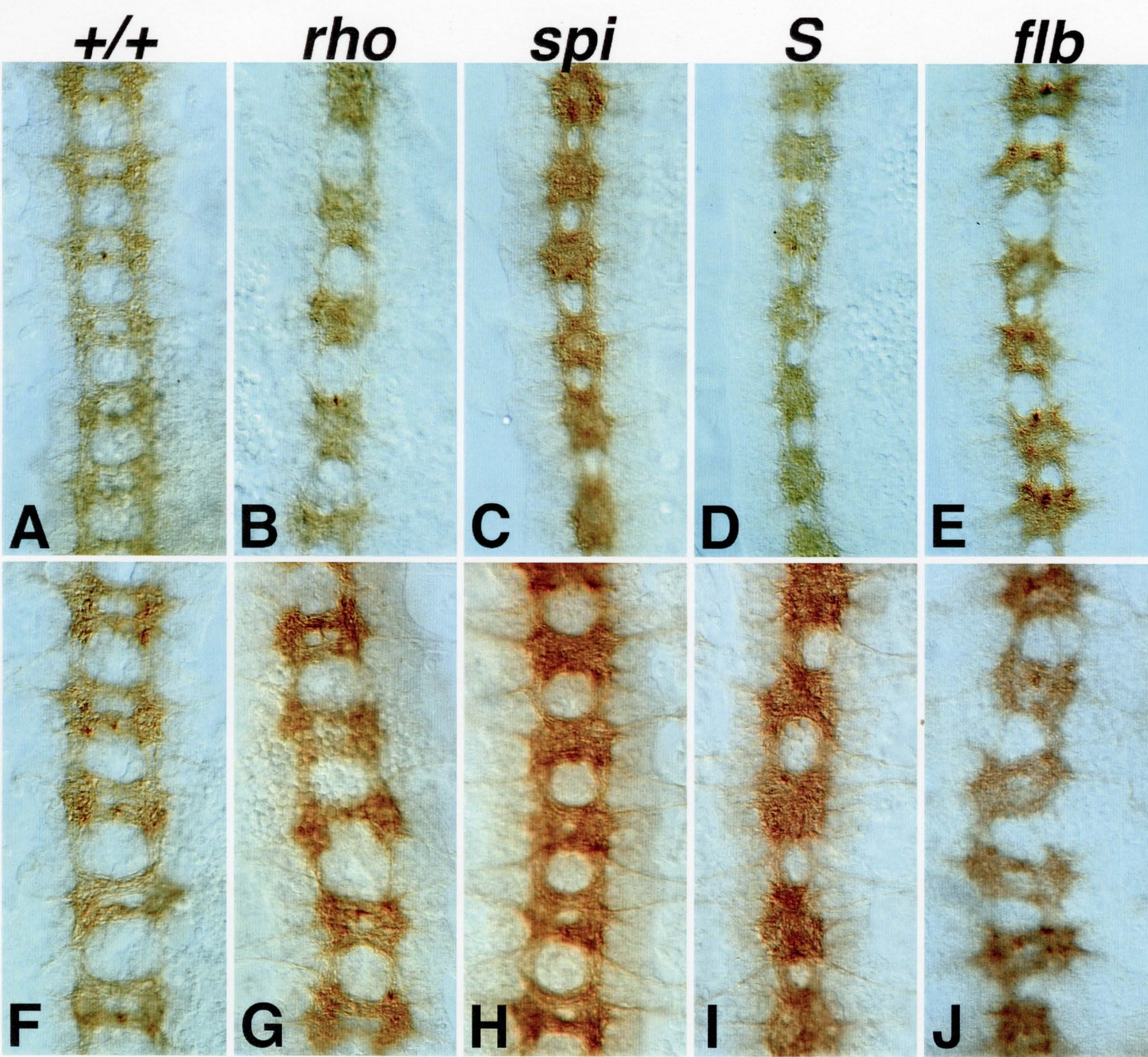
In addition to a reduction in MG number, embryos homozygous for spitz group/DER pathway mutations have defects in their axon tracts which can be assessed with the use of α-102, a monoclonal antibody whose antigen is found on both the longitudinal and commissural axons (Klämbt and Jacobs, 1991; Sonnenfeld and Jacobs, 1994). HRP cytochemistry of α-102 stained, WT embryos have well separated commissures and regularly spaced longitudinal tracts (Fig. 9A). Overexpression of rho

using HS*rho*1B (Fig. 9B) or sSpi through *sim*-Gal4/+; UAS-*sspi*/+ (data not shown) has no apparent effect on axon tract morphology, indicating that the presence of supernumerary glia does not affect axon tract development. *spitz* group mutant nerve cords display a variety of defects including fused commissures, and both collapse and thinning of the longitudinal tracts (Fig. 9C, 9E, 9G, 9I and Klämbt and Jacobs, 1991). It is believed that these defects occur as the result of the loss of MG function.

Ectopic expression of *rho* in a *rho* hypomorph in HS*rho*1B; rho^{38}, AA142 embryos partially rescues the axon tract phenotype (Fig. 9D), however there is some variation among segments. In HS*rho*1B; spi^{1}; AA142 embryos the collapsed longitudinals tracts (see Fig. 9E) appear significantly restored, however the commissures are still fused (Fig. 9F). This suggests that ectopic *rho* expression is able to partially rescue the mutant phenotype. This correlates well with the MG phenotype which also is partially rescued in these embryos (Fig. 8D). Overexpression of *rho* is embryos mutant for *S* or *flb* has no appreciable effect on the morphology of the VNC (Fig. 9H and J). The axons tracts of HS*rho*1B; S^{IIN23}; AA142 and HS*rho*1B; flb^{2E03}; AA142 embryos appear no different from those of S^{IIN23} or flb^{2E03} embryos (Fig. 9G and I). This suggests that both Star and DER function are required to mediate signaling initiated by Rhomboid.

Figure 9. Rhomboid overexpression does not correct Commissure malformation in embryos mutant for *spitz* group genes.

Stage 13 embryos were stained with the monoclonal antibody 102 which labels commissural and longitudinal axons. (A) Shows a frontal view of a wildtype embryo with normal, well separated commissures and regular spacing between the longitudinal tracts. (B) Induction of ectopic *rhomboid* expression using HSrho1B does not appear to affect the development of the nerve cord. (C) rho^{38}, (E) spi^{1}, (G) S^{l1N23}, and (I) flb^{2E03} show varying degrees of commissure fusion and collapse of the ventral nerve cord, typical of *spitz* group embryos. S^{l1N23} appears the most severe of all. (D) HS*rho*1B;rho^{38} shows a significant rescue of the mutant phenotype. (F) HS*rho*1B;spi^{1} shows rescue of the collapsed nerve cord, but not of the fused commissures. (H) HS*rho*1B; S^{l1N23}, and (J) HS*rho*1B;flb^{2E03} do not appear to compensate for any aspect the mutant phenotype.


+/+
rho
spi
S
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A
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D
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HS[rho]
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DISCUSSION

4.1 *rhomboid* acts through the *spitz*/DER signaling pathway to increase MG number

Experiments reported here clearly demonstrate that ectopic *rhomboid* expression is sufficient to cause a significant increase in MG number. Furthermore, the data illustrates that *rho* expression must be maintained until the end of embryogenesis in order to prevent these cells from undergoing apoptosis. It is of considerable interest to determine the mechanism by which ectopic Rhomboid acts to block cell death in the MG lineage.

Recent investigations into *spitz* group function suggest two possible modes of action for Rhomboid. In the first model, Rho is proposed to act in a cell autonomous manner to amplify or mediate signaling between its ligand Spitz and the *Drosophila* homologue of the EGF receptor family, DER (Bier *et al.*, 1990; Sturtevant *et al.*, 1993). The second, and perhaps more widely accepted model suggests that Rho facilitates or carries out cleavage of Spi from its membrane bound form, which is thought to be non-functional, to an activated secreted form which acts non-autonomously (Schweitzer *et al.*, 1995). Mammalian TGF-α and Gurken, another ligand of DER, are believed to function in an analogous manner (Massagué, 1990; Sapir *et al.*, 1998). It is still not clear in the model proposed by Schweitzer *et al.* whether Rho acts autonomously or non-autonomously to generate the secreted signaling molecule. As mentioned, ectopic Rho generated with the HS*rho*1B construct has a unambiguous effect on MG survival. Conversely, autonomous expression of *rho* in the MG generated in *sli*1.0-Gal4/+; UAS-*rho*/AA142 embryos gives only a very subtle increase in cell number. These data suggest that Rho does not activate signaling in an autonomous manner. Two issues weaken this

conclusion. Firstly, *sli*1.0 expression is not initiated until stage 11, at the earliest (Scholz *et al.*, 1997), whereas HS*rho*1B expression is induced at stage 9. Furthermore, when HS*rho*1B expression is induced by 3 heat-shocks beginning at stage 12, rather than stage 9, the effects on MG survival are dramatically reduced. Therefore it can not be ruled out that initiation of *rho* expression earlier than stage 11 is required for MG survival. However, misexpression of *rho* from stage 9 using *sim*-Gal4 to drive UAS-*rho* did not result in any significant increase in MG number. Secondly, we have not determined the relative levels of expression derived from each construct. It is quite possible that high levels of Rho are required to generate the effect observed when using HS*rho*1B (see methods), and insufficient levels are generated using UAS-*rho* as a source. It is known that under wildtype conditions, Rhomboid is targeted to plaques at the membrane by a unknown mechanism (Sturtevant *et al.*, 1996). These plaques of Rho are associated in some cell types with proteins, such as Armadillo, which are commonly localized to cell junctions (Sturtevant *et al.*, 1996). HS*rho* expression generates more Rhomboid protein than can be properly localized. This results in more uniform distribution of Rho in the plasma membrane (Sturtevant *et al.*, 1996). The heat shock protocol adopted here has been shown to increase levels of Rho throughout the plasma membrane, not just within plaques. It is possible that *rho* mediated *spitz* group signaling is only capable of generating enough signal to create supernumerary glia under this condition. *sim*-Gal4 induces expression, in a greater number of midline cells, although at a apparently lower level than *sli*1.0 (see results and below) in a pattern similar to that observed for normal *rho* expression (Bier *et al.*, 1990; Xiao *et al.*, 1996). However, no significant increase in MG number is detected. Further experiments which drive high levels of UAS-*rho*

expression in a more widespread pattern, such as the entire neurectoderm, will be required to resolve this issue.

The data may be insufficient to conclude whether Rho functions autonomously or non-autonomously but it is sufficient to indicate that the capacity of Rho to function depends, at least partially, on the other *spitz* group genes. Epistatic analysis demonstrates that the increased survival of the MG induced by ectopic *rho* expression requires sufficient levels of Spi, S and DER. In embryos mutant for either *spi*, *S*, or *DER*, HS*rho*1B was unable to rescue the wildtype MG number, although some increase was observed over the mutant phenotype. Furthermore, ectopic *rho* expression is unable to fully rescue the axon pattern defects characteristic of these mutants (see Klämbt *et al.*, 1991).

Overexpression of *rho* fails to produce a HS*rho*1B phenotype in rho^{38} mutant embryos, however the number of MG in these embryos is relatively close to wildtype cell number in contrast to the other mutations assessed. In addition, the axon tracts appear almost normal in some segments, although variations are observed between neuromeres. It is somewhat unexpected that a full rescue of the HS*rho*1B phenotype is not observed in these embryos. One possible explanation for this result is that low levels of Rho are required at some earlier stage to assure specification of a full complement of MG progenitors. *rho* expression data supports this conclusion. In wildtype embryos, *rho* is first expressed at the cellular blastoderm stage, while ectopic *rho* expression was not introduced in these embryos until later at stage 9 (see Bier *et al.*, 1990, Lanoue, Gordon and Jacobs, unpublished results).

In embryos mutant for *spi* there is a marked decrease in the number of MG present, even at stage 12/0. Noticeable defects in the development of the axon tracts also exist and include a thinning of the nerve cord caused by a medial collapse of the longitudinal axon tracts and a thickening, and incomplete separation of the commissures. These defects are relatively minor in comparison to phenotypes observed for *flb* and *S* (see fig. 6). Epistatic analysis confirms that Spi is required for Rho function. The *spi* mutant phenotype of HS*rho*1B; *spi*; AA142 embryos demonstrate that loss of Spi function almost completely blocks the ability of ectopic Rho expression to rescue the loss of MG phenotype in these embryos. This suggests that Rho functions upstream or in tandem with Spi. In addition, overexpression of *sspi* in a *rho* mutant background results in an increase in MG number, and appears to rescue the mutant axon tract phenotype. This further strengthens the hypothesis that Rho acts upstream of Spi. Thus is possible that Spi acts in the cleavage of membrane bound Spi as proposed in Schweitzer *et al.* 1995. Interestingly, in observation of the axon tracts of HS*rho*1B; *spi*; AA142 embryos it was concluded that the longitudinal tracts appear to be correctly positioned, not collapsed medially as seen in embryos mutant for *spi*. The commissures remain fused, or at least significantly thickened, in almost all segments examined. Therefore, it is possible that Rho could have some function independent of Spi. However, it is more likely that *spi* has a maternal affect (its' embryonic expression is ubiquitous) and that this accounts for these observations (Rutledge *et al.*, 1992). In this case, low levels of maternal Spi could be activated by *rhomboid* expression, rescuing some aspects of the phenotype. It is interesting that the medial collapse of the longitudinal tracts was rescued, but not the fusion of the commissures. I hypothesize that longitudinal tract separation is an earlier function of the

MG than is commissure separation. In *spi* mutant embryos that have been partially rescued by ectopic expression of *rho* using HS*rho*1B, the maternally contributed Spi is very limited, and only sufficient to perpetuate MG survival via DER activation for a short period. Consistent with this model, I suggest that late stage HS*rho*1B; *spi*; AA142 embryos would contain almost no glia, since they would have undergone apoptosis due to a deficit of *spitz* group signaling. This, in turn, accounts for the observed CNS phenotype. Further experiments will be required to provide evidence for this theory.

In the case of *spi* and *flb* mutant embryos, ectopic expression of *rho* was able to partially rescue the axon tract phenotypes (see results), however no change was observed in *S* mutant embryos. *Star* embryos appear to have a more severe phenotype than either *rho* or *spi* embryos (see Fig.7). Based on the spatially restricted expression pattern of this gene (see Kolodkin *et al.*, 1994), it is possible *S* could have less mitigation by a maternal contribution than either *rho* or *spi*. Alternatively, *S* could have another function outside of its proposed role in *spitz* group signaling (see Schweitzer *et al.*, 1995). A recent study uncovered mutations of *S* as a enhancers of *sin(a)*, a repressor of *tramtrack* (*ttk*), in the development of the ommatidial subunit in the eye (Neufeld *et al.*, 1998). *tramtrack* encodes a transcription factor which is a negative regulator of neuronal development in the eye and in the CNS, where it is expressed by the MG (Xiong and Montell, 1993; Giesen *et al.*, 1997). It appears that both *sin(a)* and *phylopod* negatively regulate Tramtrack function by targeting this protein for degradation, thus removing a negative regulator of neuron specific target genes (Li *et al.*, 1997). No other member of the *spitz* group was detected in this study, and it was determined that mutations of *ras*, *sos* and *GAP*, which lie

downstream of *DER* in this pathway and which probably mediate the cell survival signal through DRaf and Akt (see below), do not affect this function of *sin(a)*. Therefore it is possible that Star functions in blocking repression of Ttk during development of the eye and perhaps elsewhere in development. Furthermore, it is interesting that the mutant *Star* axon phenotype, as detected by staining with α-102, is more severe in terms of the degree of commissure fusion and longitudinal axon collapse, than the other *spitz* group mutants. It appears that this phenotype is closer to that described for *ttk* than for *spi*, *rho* or *flb* (see Giesen *et al.*, 1997, Fig. 6G). In addition, *S* mutant embryos that have had PCD blocked by the *H99* deficiency, have an unusual phenotype not present in other *spitz* group mutants that have been denied apoptosis. In these embryos, the MG migrate to either the dorsal or ventral margin of the nerve cord, whereas mutant glia in other *spitz* group mutants tend to migrate exclusively to the dorsal region (Dong and Jacobs, 1997, Fig. 8). This is of interest since it appears that MG in *ttk* embryos survive until late embryogenesis, but have defects in their ability to migrate properly, and tend to "wander" in the nerve cord. I propose that Star functions autonomously in the cells receiving the *spi* signal at the receptor, working to integrate the survival signal, and transducing an anti-repression signal to the Ttk proteolysis machinery. Additional experiments, involving cell autonomous misexpression of *S*, will be required to confirm this hypothesis.

The axon tract phenotype of *flb* mutant embryos is also very severe, however the collapse of the longitudinal tracts is less pronounced than in *S*. *flb* mutant nerve cords display breaks in the longitudinal axon tracts and commissures that are not formed perpendicularly to the longitudinal axons, defects not routinely seen in mutants for other *spitz* group genes. This disorganized and highly erratic axon phenotype may be the result

of the loss of EGF receptor function elsewhere in the ventral nerve cord (VNC) or in the patterning of the neighbouring ventral ectoderm (Raz and Shilo, 1993; Golembo *et* al., 1996; Skeath, in press). Ectopic expression of *rho* using HS*rho*1B in this background does not significantly improve these defects. It seems likely that Rho function is dependent on DER activity, as proposed in the literature (Sturtevant *et al.*, 1993; Schweitzer *et al.*, 1995).

4.2 *Spitz* group genes and the DER signaling pathway function to block apoptosis of the Midline Glia

Analysis of embryos homozygous for the *H99* deficiency, which uncovers *rpr*, *hid*, and *grim* has demonstrated that in the absence of normal cell death, supernumerary Midline Glia (MG) are created which express glial specific markers such as AA142 and *sli*1.0 (Dong and Jacobs, 1997; Zhou *et al.* 1997). Ectopic expression of *rho* employing HS*rho*1B has been determined to have the same effect on otherwise WT embryos. In embryos homozygous for the *H99* deficiency, 11.2 cells survive until stage 17 and express MG specific genes (Dong and Jacobs, 1997). In HS*rho*1B embryos that have been heat-shocked 6 times, an average of only 6.84 MG survive at stage 17, although many segments contain up to 12 AA142 expressing cells (data not shown). Therefore ectopic expression of rho using the HS*rho*1B construct is less efficient at rescuing the (MG) than impairing the machinery of apoptosis.

Experiments were conducted to determine if the anti-apoptotic effect observed for HS*rho*1B was an isolated phenomenon or one that could be attributed to *spitz* group signaling in general. Epistatic analysis confirms the hypothesis that Rho acts upstream of Spi, perhaps in activation of the ligand (see above). Misexpression of UAS-

sspi using either *sli*1.0-Gal4 or *sim*-Gal4, which generates a ubiquitous signal, results in a similar increase in MG number to that which is observed in HS*rho*1B embryos. This confirms previously published results of Scholz *et al.*, 1997. In addition, misexpression of DER^{A887T} and Ras^{V12}, activated variants of both DER and Ras, gave similar results. Thus it seems most likely that Rho and Spi act together to transmit a survival signal to the MG acting through the EGF receptor. This signal is subsequently passed on to the small monomeric G-protein Ras, which then can activate other cytoplasmic signaling molecules.

As demonstrated in the results, misexpression of *spitz* group/DER pathway genes with *sli*1.0-Gal4 appears to have a more pronounced effect than does *sim*-Gal4. This is interesting since the latter construct induces expression of any UAS construct at an earlier stage throughout the mesectoderm (Wharton and Crews, 1993??). The most likely explanation is than *sli*1.0-Gal4 is able to induce a higher expression level than is *sim*-Gal4. In addition, this data clearly demonstrates that most, if not all, of the supernumerary glia (see Dong and Jacobs, 1997) remain rescuable until at least late stage 11 or early stage 12 when *sli*1.0-Gal4 expression is initiated.

Interestingly, ectopic *rho* expression beginning later than stage 9 results in a less than optimal MG survival rate. If expression is generated late, beginning at stage 12, then only the 6 original MG present at this stage in WT appear to survive. It is therefore possible that *rho* expression is required earlier than *spi* expression in order to generate the same effect. This temporal lag could allow for Rho translocation to the membrane and the subsequent cleavage of Spi within the same cell or in neighbouring cells.

Our experiments have demonstrated that sustained *rho* amplification or DER pathway signaling is required for the MG to survive to the end of embryogenesis. If

HS*rho*1B expression is induced and subsequently stopped for a prolonged period of time, any extra glia generated will undergo apoptosis. In WT embryos, it is thought that up-regulation of expression of the inhibitor *argos* (*aos*) in a subset of the developing MG leads to a down-regulation in DER mediated signaling in the non-surviving MG (Stemerdink and Jacobs, 1997). Therefore, it is logical to assume that once the ectopic source of *spitz* group signaling is removed, Aos terminates DER mediated signaling in these supernumerary glia and they subsequently undergo apoptosis. Since we know that overexpression of *spitz* group/DER pathway genes causes up-regulation of both *pnt* and *aos* (see below), this data suggests that these supernumerary glia, despite being fully, or almost completely differentiated as MG, are not insulated from apoptosis since removal of the ectopic signal leads to cell death. Therefore there must be something special about the subset glia which survive that allows them to become refractory to the effects of Aos.

4.3 DER acts through multiple cytoplasmic pathways to induce survival and differentiation of the MG

Release of Spitz blocks apoptosis in the MG through activation of DER which transduces a survival signal through cytoplasmic signal transduction pathways. Overexpression of *sspi*, in addition to blocking PCD in these cells, up-regulates expression of *pnt* and *aos* thereby inducing differentiation (Klämbt, 1993; Klaes *et al.*, 1994). This is in contrast to the *H99* deficiency, which prevents cell death more effectively, but does not increase the number of *pnt* or *aos* expressing cells. Therefore it appears that *spitz* group signaling has dual functions in MG development, preventing premature cell death and assuring proper differentiation.

As demonstrated, misexpression of an activated form of Ras, a cytoplasmic G-protein which interacts with the activated EGF receptor (reviewed in Katz and McCormick, 1996; and Marshall, 1995), is also capable of blocking cell death in the MG lineage. In mammalian systems, Ras can activate a number of downstream effectors including PI3'K and Draf (reviewed in Katz and McCormick, 1997). Mutations to activated Ras which remove its ability to interact with DRaf completely block the ability of Ras to mediate survival of extra MG. This suggests that DRaf function is essential to transduction of the *spi*/DER survival signal. Overexpression of *DRaf*^{act} from stage 9, using *sim*-Gal4, causes a marked increase in MG number in most embryos that is comparable to the effect generated by *sspi* overexpression. It is also sufficient to induce at least part of the differentiation program as evidenced by the increases in the number of *pnt* and *aos* expressing cells in these embryos. Misexpression of *pntP1*, a constituatively activated form of Pointed, also rescues MG from cell death, but not as effectively as DRaf^{act} misexpression. It is possible that failure to down-regulate Yan, which acts antagonistically to Pnt, accounts for this difference. Despite the fact that DRaf is effective at up-regulating the expression of *pnt* and *aos*, it does not completely mimic the effects of sSpi overexpression. DRaf^{act} misexpression may result in an increase in cell number, however many of these supernumerary glia are "stacked" along the dorsal margin of the VNC. This phenotype is quite similar to that which is observed in embryos which are homozygous for the *H99* deficiency (Dong and Jacobs, 1997; Zhou *et al.*, 1997). It is observed that in a fraction of these *sim*-Gal4; UAS-*DRaf*^{act}/AA142 embryos the amount of stacking is reduced, and that there are less MG. Instead, a portion of the supernumerary glia appear to be in the process of undergoing programmed cell death, as

indicated by the high number of apoptotic profiles in the nerve cords of these embryos. I postulate that DRaf expression is sufficient to transduce at least part of the *spi* signal, but may be insufficient to perform all the functions required for the survival and proper differentiation of the MG. In *sim*-Gal4; UAS-*DRaf*^{act} embryos, a fraction of supernumerary MG still receive endogenous *spitz* signal. However, some of the supernumerary glia do not receive this endogenous signal, and these are the cells which normally become stacked. This explanation is also applicable to embryos homozygous for the *H99* deficiency. It is possible that this behaviour is associated with improper migration or differentiation, or incomplete protection from PCD. In a fraction of the *sim*-Gal4; UAS-*DRaf*^{act} embryos, these supernumerary MG do not accumulate dorsally but undergo apoptosis instead, which accounts for the drop in cell number or the appearance of apoptotic profiles in these embryos (see results). If misexpression is introduced at a later stage, using *sli*1.0-Gal4 as a driver, DRaf^{act} seems to be incapable of blocking cell death, even though Spi, DER^{A887T} and Ras appear slightly more effective when using this driver. This indicates that Spi must be acting through other cytoplasmic signaling pathways in addition to DRaf to account for its ability to rescue embryos at later stages.

A mutation which affects the interaction of Ras with PI3'K decreases the survival effect generated by Ras misexpression but does not completely repress the function of activated Ras. In mammals, PI3'K is capable of up-regulating PKC function, which in turn can cause phosphorylation of MAPK, a major target of DRaf signaling (reviewed in Carpenter and Cantley, 1996). So it is possible that PI3'K could work by indirectly stimulating the Raf/MAPK pathway. Alternatively, PI3'K may act through up-regulation

of DAkt, a *Drosophila* homologue of Akt/PKB, which is a potent inhibitor of apoptosis that acts by blocking caspase function (Kennedy *et al.*, 1997).

Overexpression of Akt has no effect on MG number, demonstrating an inability to independently block cell death in this tissue. However, co-expression of both *Dakt* and *DRaf^{act}* using *sli*-Gal4 results in a synergistic effect on MG number, suggesting that both the DRaf/MAPK and PI3'K/AKT pathways function together to assure MG survival. Additionally, co-expression of both kinases using *sim*-Gal4 appears to decrease the stacking phenotype described earlier. Therefore it seems likely that this phenotype is associated with incomplete protection from some of the effects of PCD. I propose that overexpression of Akt in embryos homozygous for the *H99* deficiency could correct the stacking phenotype observed in these embryos as well.

In *Drosophila*, Akt appears to act downstream or independently of *rpr*, *grim* or *hid* function (Staveley *et al.*, 1998). Loss of function at the *akt* locus is compensated for by overexpression of p35, a viral inhibitor of caspase function, therefore it seems likely that Akt may function similarly in mammals and *Drosophila*. Interestingly, *p35* expression does not appear to influence MG number (J.R. Jacobs, personal communication).

I propose that the genes within the *H99* deficiency act to up-regulate the transcription or function of the caspases. Expression of DRaf^{act} sufficiently early in development blocks this function by preventing the initiation of caspase activity. If expression of UAS-*Draf^{act}* is induced later, it is incapable of preventing the cells from undergoing apoptosis since the caspase cascade has already begun. Why Akt expression doesn't block cell death at this point is unknown. It is possible that the level of UAS-*Dakt* expression is simply not high enough to counteract the effect of increasing caspase

concentration in the absence of DRaf activation, which would decrease the amount of caspases being activated. It is also possible that Akt can only inhibit a subset of the caspases, or that additional factors exist to inhibit DAkt function. In this case, expression of DRaf^{act} serves to remove this repression. Misexpression of both *DRaf^{act}* and *Dakt* rescues many MG from apoptosis, but is not as effective as *sspi* misexpression in this capacity. It is possible that other factors, such as DJNK (see Scholz *et al.*, 1997) or other targets of PI3'K, like PKC, must be activated to fully respond to the *spi* signal.

It has been reported that overexpression of *ras* down-regulates expression of *hid* and can abrogate cell death mediated by overexpression of this gene (Kurada, Bae and White, personal communication). Since overexpression of *sspi* up-regulates Ras function, it is likely that *spitz* signaling acts to block *hid* mediated cell death by acting at the level of transcription. The same situation does not exist where *rpr* is concerned. Our experiments demonstrate that *rpr* mRNA levels are not affected by *sspi* overexpression. Since *rpr* probably functions cooperatively with *hid* and *grim* to mediate MG apoptosis in normal embryos (Zhou *et al.*, 1997) and is effective at inducing apoptosis of the MG independently when overexpressed (J.R. Jacobs, pers. comm.), it seems likely that *spi* must block Rpr function through a non-transcriptional mechanism in order to mediate cell survival. Recent evidence demonstrates the Ced-9 or Bcl-2 can block Rpr induced apoptosis *in vitro* (Hisahara *et al.*, 1998). In mammals, Raf1 phosphorylation results in activation of Bcl-2 survival function (reviewed in Reed, 1997a and 1997b). Therefore it is possible that such a mechanism exists in flies and functions in MG development.

4.4 *spitz* group genes and DRaf up-regulate expression of *rhomboid*

It is clear from the results that ectopic expression of Rho and misexpression of sSpi or the serine/threonine kinase DRaf can lead to an up-regulation in *rhomboid* expression, as assayed using the X81 enhancer trap or anti-sense RNA *in situs*. In oocyte development, it has been demonstrated that signaling through DER initiates *rho* expression which then can act on Spi, upregulating this signaling pathway. Subsequent Spi release then up-regulates DER signaling in responsive surrounding cells which then acts to increase *rho* levels. This creates an amplification loop (reviewed in Wasserman and Freeman, 1997). In this pathway, Gurken, another *Drosophila* EGF ligand, acts to initiate this signaling cascade (Sapir *et al.*, 1998).

I propose that a similar loop exists in the developing mesectoderm. An initiating signal may exist which causes up-regulation of *rho* expression. Preliminary data suggests that *Vein*, a *Drosophila* neuregulin homologue, may be capable of performing this function in the developing midline (Lanoue, Gordon and Jacobs, unpublished results).
This leads to an amplification loop similar to that which is seen in oocyte development. Overexpression of *rho* or *sspi*, which can act non-autonomously, increase the size and spatial limits of this initiating signal and thus increases the number of cells involved in the loop. In addition, sustained ectopic expression of these genes results in a greater number of cells expressing *rho* at later stages. In these embryos, the effect of *aos*, which probably acts to down-regulate the signal in the peripheral cells (Golembo *et* al., 1996; Freeman, 1997), is nullified by the sustained overexpression of *rho* or *sspi*.

Misexpression of UAS-$DRaf^{act}$, which acts cell autonomously, using *sim*-Gal4 as a driver, does not significantly increase the spatial pattern of *rho* expression. However, it

appears that this signal can sustain *rho* expression at higher levels, in a subset of the mesectodermal cells, later into development. The very fact that the spatial expression pattern is not increased in these embryos, suggests that these extra cells which can be induced to express *rho* by overexpression of Rho or sSpi, require threshold levels of signaling in order to be responsive. The signal induced within the $DRaf^{act}$ expressing cells is apparently not strong enough to generate levels of *rho* expression that are sufficient to create a ubiquitous signal through Spi release. Therefore neighbouring cells are not induced to upregulate *rho* expression and thus expand the number of cells responding to the amplification loop. In wildtype embryos this requirement for strong signal may function to restrict the spatial pattern of *rho* expression.

Cells positioned at the center of this spatial pattern presumably receive higher levels of *spi* signaling than do cells located on the periphery. In a similar model that which was proposed in Stemerdink and Jacobs, 1997, these prospective MG could be the first to achieve the threshold level of signaling required to initiate expression of *aos*. These cells could then secrete this negative regulator of DER signaling and effectively down-regulate *spitz* group signaling in these cells (Schweitzer *et al.*, 1995; reviewed in Freeman, 1997). Since an absence of signal results in induction of the apoptotic program, the centrally located cells would, in effect, kill the peripheral cells. Presumably, signaling levels within this central cluster of cells would be high enough to counteract the inhibitory effect of Aos on DER mediated signaling. Alternatively, induction of *pointed* or of other target genes by higher levels of *spitz* group signaling could be sufficient to insulate these cells from apoptosis brought on by a drop in the level of signal. The latter proposal is more attractive based on the fact that Aos causes a decrease in *DER* mRNA levels (Stemerdink

and Jacobs, 1997), which would eventually make all cells subjected to this stimulus (or lack thereof) unable to respond to *spi* group signaling. Misexpression of *spitz* group/DER pathway genes results in an increase in *pnt* and *aos* expression in all surviving MG. Since it has been demonstrated that high levels of Aos can kill all MG in the VNC at a given stage (Stemerdink and Jacobs, 1997), it seems likely that higher levels of *spitz* group/DER signal can induce resistance to PCD upon these cells.

4.5 Summary and Future Work

I have demonstrated that ectopic expression of *rho* is sufficient to generate supernumerary MG by blocking PCD in this lineage. In addition, I have also demonstrated that Rho must function with or through the other members of the *spitz* group/DER signaling pathway in order to create this effect. Epistatic analysis has confirmed that Rho acts before Spi in this signaling pathway. Therefore it is possible that the hypothesis that Rho acts to facilitate cleavage of Spi, proposed by Schweitzer *et al.*, 1995, is valid. Epistatic analysis also demonstrates that Star function is required in order for ectopic *rho* expression to generate supernumerary MG. It has been proposed that S functions with Rho to facilitate cleavage of Spi. In the future, it would be interesting to determine whether ectopic expression of *Star* results in a midline phenotype comparable to the one described for ectopic *rho* expression.

I have presented data which suggests, although not conclusively, the Rho acts in a non-autonomous manner to generate a signal. Using a similar experimental design, it would be possible to determine the difference between ectopic expression of *S* using a

Figure 10. Summary of signal transduction pathways associated with MG survival and differentiation

Model for *spitz* group/DER pathway function in the survival and differentiation of the MG. It is proposed here that Rho functions in the release of Spi from the membrane. This is in agreement with previously published reports (Schwietzer *et al.*, 1995). This secreted form of Spi is then able to activate DER mediated signaling in neighbouring cells. Activation of the receptor may depend on the function of Star. Subsequently, downstream cytoplasmic signal cascades are activated which affect gene expression, and perhaps also modify the activity of other proteins (both outcomes are shown in black). Activation of these pathways has the net affect of promoting cell survival and activating MG specific gene expression, which results in differentiation of the cells. At the same time, neuronal differentiation is repressed by blocking degradation of Tramtrack (shown in blue). See text for further details.

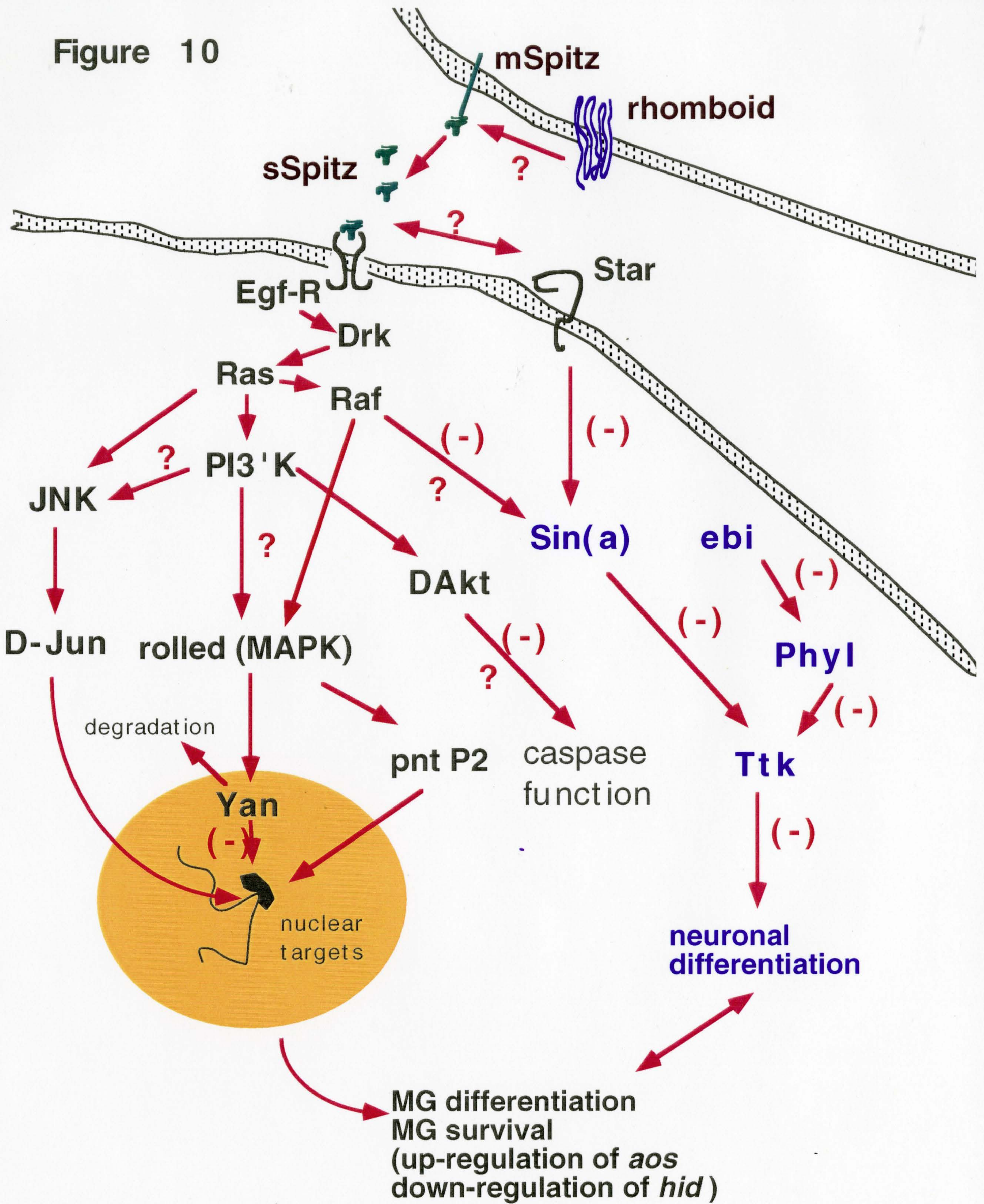
Figure 10
mSpitz
rhomboid
sSpitz
?
?
Star
Egf-R
Drk
Ras
Raf
(-)
(-)
?
PI3'K
JNK
?
Sin(a)
ebi
?
(-)
DAkt
(-)
D-Jun
rolled (MAPK)
(-)
Phyl
?
(-)
degradation
pnt P2
caspase
function
Ttk
Yan
(-)
(-)
nuclear
targets
neuronal
differentiation
MG differentiation
MG survival
(up-regulation of aos
down-regulation of hid)

heat-shock construct and directed misexpression using a UAS-*Star* construct coupled with an appropriate Gal4 driver. This could help to determine if S acts autonomously, within the cells receiving the signal, or in a non-autonomous manner to generate the signal.

I have suggested an alternate role for Star in mediating repression of the proteolytic machinery involved in the degradation of Tramtrack, a transcription factor instrumental in repression on the neuronal differentiation program. Future work must be done to test this hypothesis.

Misexpression of other genes of the *spitz* group/DER signaling pathway is also sufficient to generate supernumerary MG. These extra glia originate from MG precursors which are prevented from undergoing PCD. This process requires the function of the Draf/MAPK pathway, but may also involve the action of other cytoplasmic signaling networks, although to a lesser degree. It has been suggested that the *spitz* group/DER pathway acts by down-regulating mRNA levels of *hid*, which is positive effector of cell death (Kurada, Bae and White; pers. comm.). This result must be confirmed. Yan is proposed to up-regulate *hid* expression (Kurada, Bae and White; pers. comm.). It should be determined if Pnt has the opposite affect. In addition, our results demonstrate that transcription of *rpr*, a key cell death gene, is not affected by directed misexpression of *sspi*. The determination of the mechanism by which *spitz* group/DER pathway signaling impinges upon Rpr function is a possible topic for future research. The effect of *spitz* group/DER pathway signaling on the expression of *grim*, another cell death gene, proposed to be involved in MG apoptosis, must also be investigated.

Finally, it has been observed that overexpression of *spitz* group/DER pathway signaling genes results in increases in the number of cells expressing target genes

associated with MG differentiation, such as *pointed* and *argos*. *rhomboid* expression is also up-regulated by increased *spitz* group signaling, suggesting a mechanism whereby initially low levels of DER signaling can be amplified to generate a localized concentration of signal, in the form of sSpi (reviewed in Wasserman and Freeman, 1997). It is of considerable interest to determine the mechanism by which this amplification loop is initiated in the developing midline.

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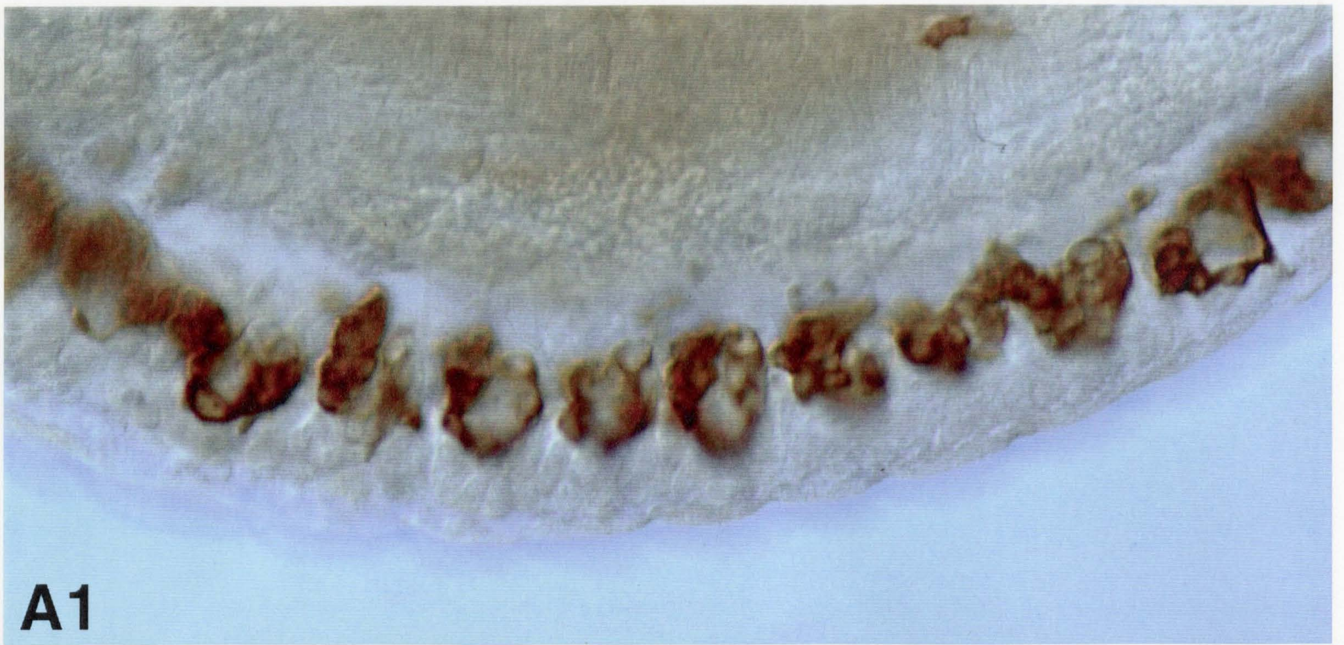
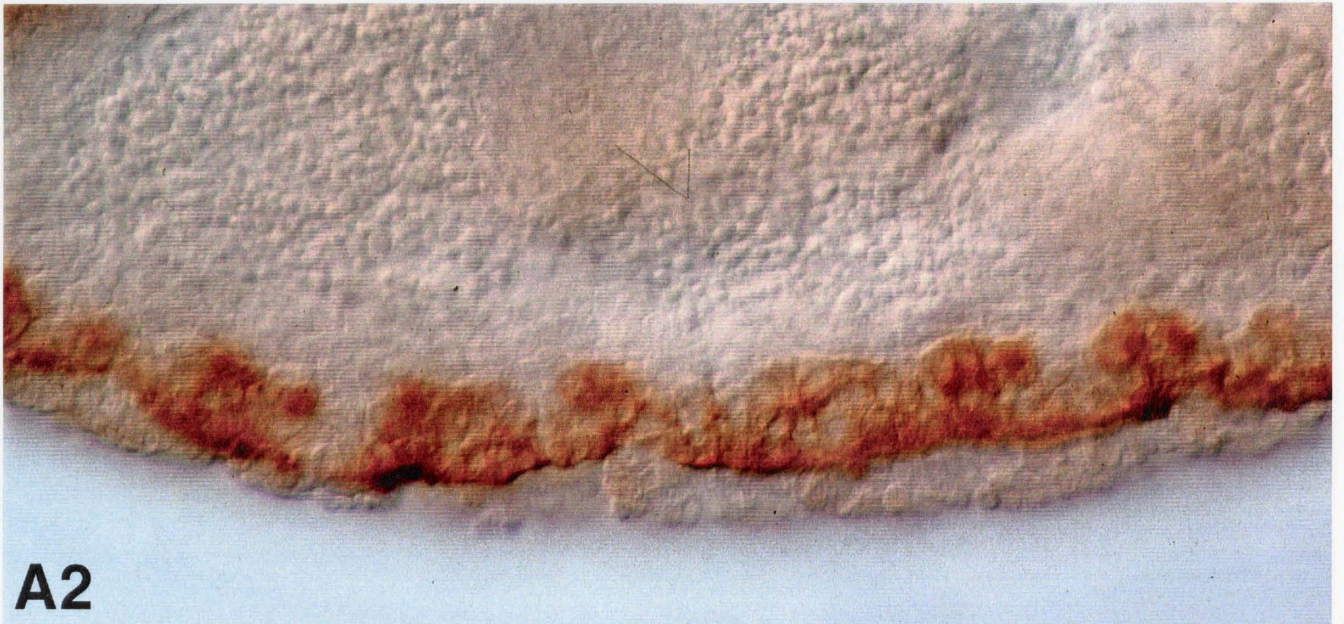
APPENDIX

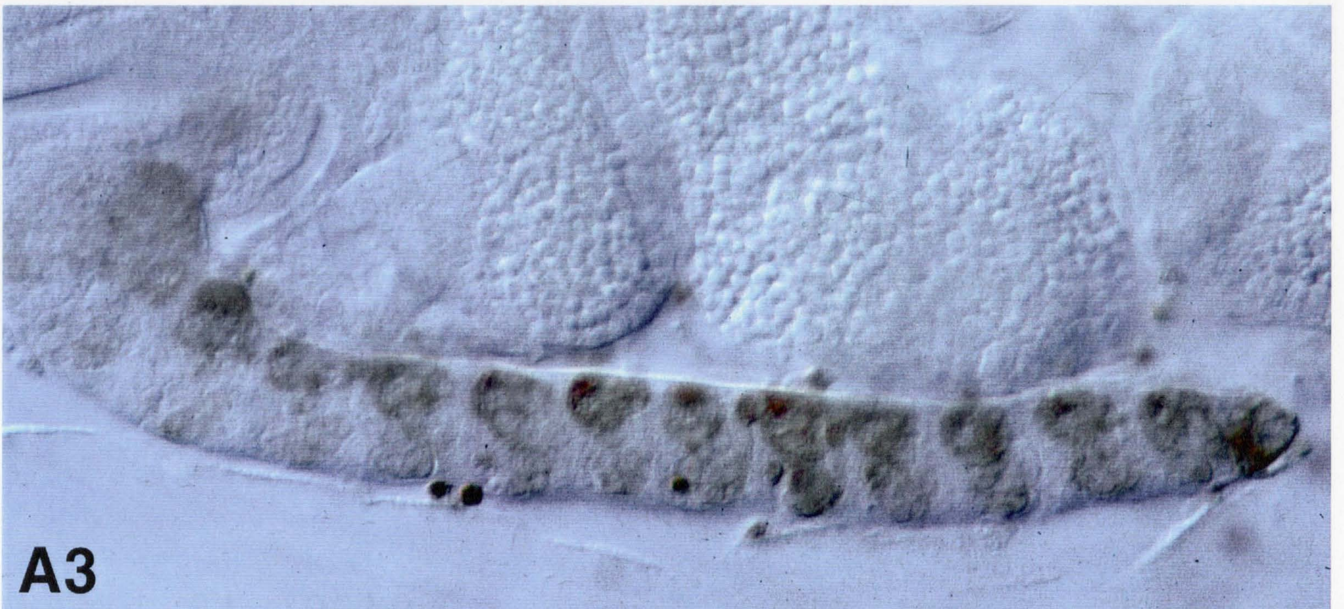
APPENDIX 1

Expression patterns of *sli*1.0-Gal4 and *sim*-Gal4

The expression patterns of *sli*1.0-Gal4 and *sim*-Gal4 are represented. Both Gal4 driver lines have been crosses to UAS-tau-LacZ and then stained immunocytologically using anti-ß-galactosidase (see materials and methods for a full description). (A) Saggital view of a stage 13 *sli*1.0-Gal4 embryo. Expression is detected in the presumptive MG as well as the MP1 (Scholz *et al.*, 1997). This expression begins at stage 11 and continues through the end of embryogenesis. *sim*-Gal4, stage 10 (B) and stage 16 (C). Expression of this transgene is similar to the wildtype expression pattern observed for *single-minded* (Wharton and Crews, 1993). Anti-ß-galactosidase labelling is first detected at about stage 9 in all of the cells of the mesectoderm. Later, expression becomes restricted to the MG lineage.


A1


A2


A3