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Argos and Spitz group genes function to regulate midline glial cell number in *Drosophila* embryos

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SUMMARY

The midline glia of the *Drosophila* embryonic nerve cord undergo a reduction in cell number after facilitating commissural tract morphogenesis. The numbers of midline glia entering apoptosis at this stage can be increased by a loss or reduction of function in genes of the spitz group or *Drosophila* EGF receptor (*DER*) pathway. Argos, a secreted molecule with an atypical EGF motif, is postulated to function as a DER antagonist. In this work, we assess the role of *argos* in the determination of midline glia cell number. Although all midline glia express *DER*, *argos* expression is restricted to the midline glia which do not enter apoptosis. Fewer midline glia enter apoptosis in embryos lacking *argos* function. Ectopic expression of *argos* is sufficient to remove all *DER*-expressing midline glia from the nerve cord, even those that already express *argos*. *DER* expression is not terminated in the midline glia after spitz group signaling triggers changes in gene expression. It is therefore likely that an attenuation of DER signaling by Argos is integrated with the augmentation of DER signaling by Spitz throughout the period of reduction of midline glia number. We suggest that signaling by Spitz but not Argos is restricted to adhesive junctions. In this manner, midline glia not forming signaling junctions remain sensitive to juxtacrine Argos signaling, while an autocrine Argos signal is excluded by the adhesive junction.

Key words: *Drosophila*, glia, EGF Receptor, *rhomboid*, *spitz*, *argos*, apoptosis

INTRODUCTION

Generation of the intricate cytoarchitecture of a mature nervous system depends in part upon the initial establishment of a simple pattern or scaffold, along which the first axon tracts are established. Subsequently, these pathways are followed by axons from neurons that arise later (Singer et al., 1979; Silver et al., 1982; Jacobs and Goodman, 1989a). Once the mature axon tract architecture is established, the scaffold is no longer required. Cells that contributed to the scaffold are either removed or differentiate into glial support cells. For example, neurons of the subplate of the neocortex establish pathways used by neurons of the lateral geniculate body to innervate the visual cortex. Subplate neurons undergo apoptosis after establishment of the mature pathway (Allendoerfer and Shatz, 1994). Similarly, the 'glial sling' across the septum provides the substratum upon which callosal axons cross the midline. Subsequent to establishment of the callosum, cells of the glial sling die (Hankin et al., 1988). It remains unclear what factors regulate the survival of these transient cells.

The midline glia (MG) of the *Drosophila* ventral nerve cord are transient cells, which function in mid embryogenesis to establish the position and morphology of the commissural axon tracts of the nervous system. These cells also produce D-netrin and Commissureless proteins, both implicated in growth cone guidance (Mitchell et al., 1996; Tear et al., 1996). During commissural tract morphogenesis, a subset of the MG play a role in the separation of the anterior and posterior commissures by migrating and interposing themselves between axon fascicles traversing the midline (Klämbt et al., 1991). Subsequent to establishment of commissure morphology, a subset of the MG undergo apoptosis, leaving 3.2 MG per segment at hatching (Sonnenfeld and Jacobs, 1995). These numbers do not change until the middle of the third instar, when new MG arise to provide about 24 MG per segment. These MG participate in the remodeling events of metamorphosis. During pupation all the MG enter apoptosis (Perz, 1994).

The reduction of MG numbers from 6 to 3.2 between stages 13 and 16 of embryogenesis occurs stochastically – in different segments, 2, 3 or 4 MG survive. MG in different positions relative to the commissures survive embryogenesis, provided one of the surviving MG resides between the anterior and posterior commissures (Sonnenfeld and Jacobs, 1995). This represents the earliest stochastic cell loss event documented in *Drosophila* embryogenesis.

The genetic basis of determination and early differentiation of the MG is not clear. The expression of genes of the MG lineage is regulated by genes required by all mesectodermal cell (MEC) lineages, such as *single-minded* and segment polarity genes (Nambu et al., 1990; Jacobs, unpublished observations). In early stage 12, up to 12 MECs express genes encoding structural proteins characteristic of differentiated MG (Dong and Jacobs, 1997). However, only 6 of these MECs participate in commissure establishment, which occurs during

stages 12 and 13 (Sonnenfeld and Jacobs, 1995). More is known about the genetic basis of MG survival. Genes of the spitz group and the *Drosophila* EGF receptor (*DER*) are not required for early MG functions, such as the generation of commissural axon fascicles. When function of these genes is lacking, however, the MG fail to migrate, separation of the anterior and posterior commissural tracts fails to occur and all the MG subsequently enter apoptosis (Klämbt et al., 1991; Sonnenfeld and Jacobs, 1994).

In the developing eye, spitz group function appears to promote cell survival through DER activation (Freeman, 1994b, 1996; Schweitzer et al., 1995a). It is not evident whether MG survival may be stochastically regulated by different levels of Spitz signaling. Recent characterisation of the secreted protein Argos demonstrates that DER signaling is attenuated by this EGF analog (Freeman et al., 1992, 1994a; Sawamoto et al., 1994; Schweitzer et al., 1995b; Okabe et al., 1996), suggesting a possible mechanism for the developmental reduction of MG numbers.

In this report, we characterise the role of *argos* in regulation of MG survival during embryogenesis. The time course and pattern of expression of *argos* suggest that expression of *argos* in surviving MG acts to promote apoptosis in neighbouring MECs. Misexpression of *argos* can also promote apoptosis in *argos*-expressing MG. This suggests that DER may integrate agonist Spitz and antagonist Argos binding during the entire period of the determination of MG number.

MATERIALS AND METHODS

Fly strains and heat-shock conditions

The *argos*$^{\Delta 7}$ amorph and *argos*^{w11} enhancer trap, kindly provided by C. Klämbt, were described in Freeman et al. (1992). Two *argos* misexpression constructs, each containing 4 copies of a *hsp70-argos* transposon were the gift of M. Freeman (Freeman, 1994a) and H. Okano (Sawamoto et al., 1994). All experiments with mutants and *hs-argos* constructs were performed with stable balanced lines. Balancer chromosomes were detected embryonically with a *P[actin-lacZ]* reporter transposon provided by C. Goodman. *hs-argos* dosage was monitored in stable double mutant lines by examining eyes from heat-shocked pre-pupae. *Elp*^{B1}, a *DER* hypermorph, was provided by N. Baker (Baker and Rubin, 1989). A. Brand provided UAS*D-raf* (Brand and Perrimon, 1994) and G. Boulianne generated the C38 GAL4 enhancer trap (Yeh et al., 1995). The *slit 1.0 lacZ* reporter (Wharton and Crews, 1993) and AA142 enhancer trap (Klämbt et al., 1991) have been previously characterised. Remaining mutant strains, including the wild-type Canton-S were obtained from the Bloomington Stock Centre.

Heat shocks were performed upon 0-4 hour egg collections on apple juice agar that were aged to stage 12 to 15, when collection plates were floated in a 37°C water bath. Unless otherwise described in the results, each heat shock was 45 minutes followed by 45 minutes of recovery.

Histology

Embryo fixation and immunocytochemistry was performed as described (Patel, 1994) employing anti-β-galactosidase polyclonal antibody (Cappel), and monoclonal 102 and 1D4 (anti Fasciclin II, provided by C. Goodman), imaged with HRP-conjugated secondary antibodies (Jackson Immunoresearch). HRP immunochemistry was completed before in situ hybridisation to RNA probes, which was performed according to Tautz and Pfeifle (1989) with modifications by D. Mellerick (Mellerick and Nirenberg, 1995). The vector for generation of *DER* probe was generously provided by E. Bier (Sturtevant et al., 1994). Each cell count was averaged for abdominal segments 1 through 7 for each embryo, then averaged for '*n*' embryos. Averages are presented with standard error.

Embryos were dissected in Schneider's media (Gibco) before fixation for electron microscopy on Thermanox plastic and tissue was processed according to published protocols (Klämbt et al., 1991; Sonnenfeld and Jacobs, 1995). Embryonic staging follows criteria of Klämbt et al. (1991).

RESULTS

Argos is a negative regulator of MG survival

Numerous genes whose expression is restricted to the 3 MG per segment in mid and late embryogenesis (after stage 15) begin expression in 8 to 12 MECs per segment between stages 10 and 12 (Sonnenfeld and Jacobs, 1995). Among the genes sharing this time course of expression are genes encoding structural proteins associated with MG function (*slit*, *D-netrin*, *commissureless*) as well as some of the genes in the spitz signaling pathway, such as *Star* and *rhomboid* (Bier et al., 1990; Kolodkin et al., 1994; Rothberg et al., 1990; Harris et al., 1996; Tear et al., 1996; Sonnenfeld and Jacobs, 1994). Of the early population of 8-12 MECs per segment, only 6 MECs participate in commissure separation and ensheathment. The further decrease in MG number during and after commissure separation can be monitored with the *AA142* enhancer trap, which begins *lacZ* expression at stage 12/5 in 5-6 cells per segment, declining to 3.2 MG per segment at stage 15 (Fig. 1). This decline in the number of MG cells is due to apoptosis, which begins at stage 13, after commissure separation (Sonnenfeld and Jacobs, 1995).

In contrast to this pattern of MG gene expression is the

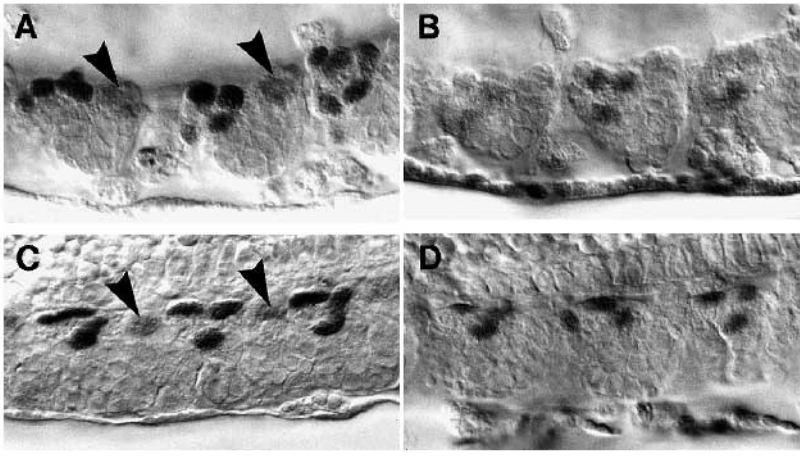

Fig. 1. Onset of *argos* expression in the MG lineage. Expression of the MG lineage-specific *AA142* enhancer trap (A,C) is compared to expression of the *argos*^{w11}/+ enhancer trap (B,D) during commissure separation, at stage 13 (A,B) and when developmental reduction of MG numbers is nearly complete at stage 15 (C,D). Arrowheads in A and C indicate the MGP cells that subsequently undergo apoptosis. The more heavily labeled cells in A and C correspond to MGM and MGA, which also express *argos*^{w11}/+. The MGP do not express *argos*^{w11}/+. In this and all subsequent figures, sagittal views with anterior at left and dorsal at top are presented.

expression of *argos*, monitored with the *argos*^{w11} enhancer trap. *argos* expression in the MG begins later than any other gene restricted to the MG lineage. Unlike other MG genes, the number of *argos*-expressing cells increases during embryogenesis. Expression is first detected at stage 12/0 in 2.6±0.1 (*n*=10) cells per segment, and rising to 3.2±0.1 MG per segment by stage 17 (*n*=10 embryos, Figs 1, 2A). This unique pattern of expression suggests that *argos* function reflects or is involved in the process that restricts MG numbers developmentally.

argos^{w11} is a previously characterised P element insertion, which generates a hypomorphic recessive phenotype, evidenced by a minor yet consistent increase in photoreceptor and cone cell number in the adult eye (Freeman et al., 1992). We wished to determine whether changes in *argos* function might also act to regulate MG number. A reduction in *argos* function increases MG numbers. *argos*^{w11} enhancer expression, detected in a wild-type number of MG when heterozygous (3.2±0.1, *n*=10), is detected in 5.1±0.2 cells per segment (*n*=10) in *argos*^{w11} homozygotes (Fig. 2A, B). Segments where 6 MG survive are often encountered. An amorphic allele *argos*$^{\Delta 7}$ was generated by imprecise excision of the P element from *argos*^{w11} (Freeman et al., 1992). Embryos homozygous for *argos*$^{\Delta 7}$ also have 5.3±0.2 (*n*=10) *slit*-expressing MG per segment at stage 16 Fig. 2C). The X55 enhancer trap, normally expressed in the transient MGP cells, labels no MG in wild type or in *argos*$^{\Delta 7}$ homozygotes at stage 16. Therefore we believe that these MG arise from the MGA and MGM lineage (data not shown).

In the converse situation, ectopic Argos decreases MG number. Two different constructs, in which *argos* expression is driven by the hsp70 promoter (Freeman et al., 1992; Sawamoto et al., 1994), reduce the number of MG that survive embryogenesis (Fig. 2D). Between 30 and 120 minutes after heat shock, increased numbers of apoptotic profiles were detected bearing perdurant *lacZ* immunoreactivity from MG enhancer trap expression. The most sensitive period for alteration of MG number by ectopic expression of *argos* was during the natural time of reduction of MG numbers, between stages 12 and 16. A single 30 minute heat shock between stages 12/0 and stage 14, with 2 copies of the *hs-argos* construct was sufficient to reduce MG numbers observed at stage 16 (using the P*[HSargos]* transformants generated by Sawamoto et al., 1994). After two 45 minute heat shocks separated by 45 minutes of recovery, 0.36±.04 MG survive. Three heat shocks were sufficient to direct all MG to apoptosis. 45 minute heat shocks before stage 12, and heat shocks stage 16 or later had little or no effect on MG number. Nevertheless, these heat-shock treatments caused embryonic lethality, suggesting other vital functions were affected by ectopic *argos* expression. Our heat-shock treatments did not affect MG number or embryo viability in wild-type embryos. Ectopic expression of *argos* in larvae had no effect on MG number or viability. These data suggest that the survival of all MG is negatively regulated by Argos production, during the natural period of apoptosis in the MG lineage.

MG loss compromises axon ensheathment

Developmental loss of the MG evoked by ectopic *argos* is reminiscent of the phenotypes of null mutations in the spitz pathway, such as *Star* and *rhomboid*. In these mutations, the MG fail to migrate to separate the axon bundles of the anterior and posterior commissures. These cells subsequently undergo apoptosis (Klämbt et al., 1991; Sonnenfeld and Jacobs, 1994). This produces a 'fused commissure' phenotype, in which the posterior boundary of the anterior commissure and anterior boundary of the posterior commissure are not delineated. If ectopic *argos* expression reproduces the MG death phenotype, does it similarly generate a phenotype with other aspects of the spitz group phenotype?

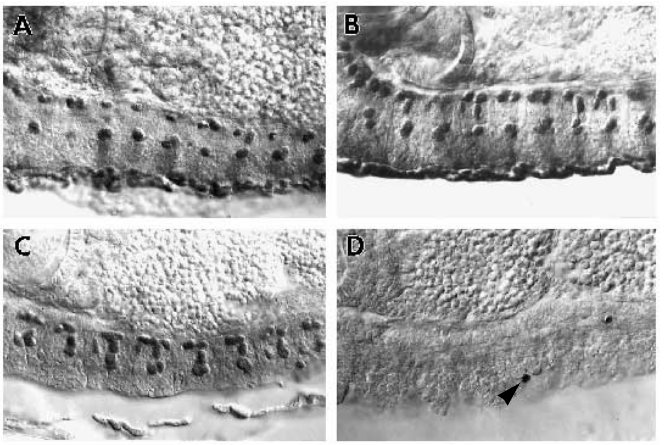

Fig. 2. Argos function modifies MG number. (A) During stage 17, wild-type numbers of MG express the *argos*^{w11} enhancer trap in heterozygotes; (B) however, the number of expressing cells are higher in *argos*^{w11} homozygotes. (C) MG cell numbers expressing the *P[slit1.0lacZ]* MG reporter in embryos homozygous for an amorphic allele of *argos* (*argos*$^{\Delta 7}$) are also higher than wild type. (D) Misexpression of *argos* directed by four copies of *P[hs-argos]* (see text) dramatically reduces the number of *P[slit 1.0 lacZ]* cells. Apoptotic labeled cells may be seen (arrowhead).

The morphology of the axon tracts at stage 16 was examined in *hs-argos* embryos subjected to three 45 minute 37°C heat shocks, separated by 45 minutes, beginning at stage 12/0. This represents the earliest stage when *argos* enhancer trap expression in the midline can be detected. This treatment was sufficient to direct all MG towards apoptosis. Similar to spitz group mutants, some narrowing of the nerve cord was seen. The commissural tracts were less uniform, and slightly wider than wild type (Fig. 3). Commissure fusion typical of spitz group mutations was very rarely seen. Thickening of the commissures after mis-expression of *argos* resembles a phenotype of *roundabout*, a mutant that shows perturbations in axon guidance at the midline (Seeger et al., 1993). The *roundabout* phenotype is effectively assessed by the pattern of Fasciclin II distribution. Fasciclin II antibody normally labels the axon surface in a subset of the longitudinal tracts of the CNS. Fasciclin II expression normally does not traverse the midline. However, in *roundabout* mutants, Fasciclin II immunolabeling is found on some commissural axons (Seeger et al., 1993). Subsequent to ectopic *argos* expression, Fasciclin II expression was found on some axons crossing the commissures, suggestive of a weak *roundabout* phenotype (Fig. 3).

Ultrastructural examination of MG-depleted embryos revealed that, in contrast to wild type, axons of the commissural tracts were not ensheathed, with some indication of membrane damage or loss on the exposed dorsal surface of the commissure (Fig. 4). Other glia did not migrate to the commissures to compensate for the loss of the MG. However, the

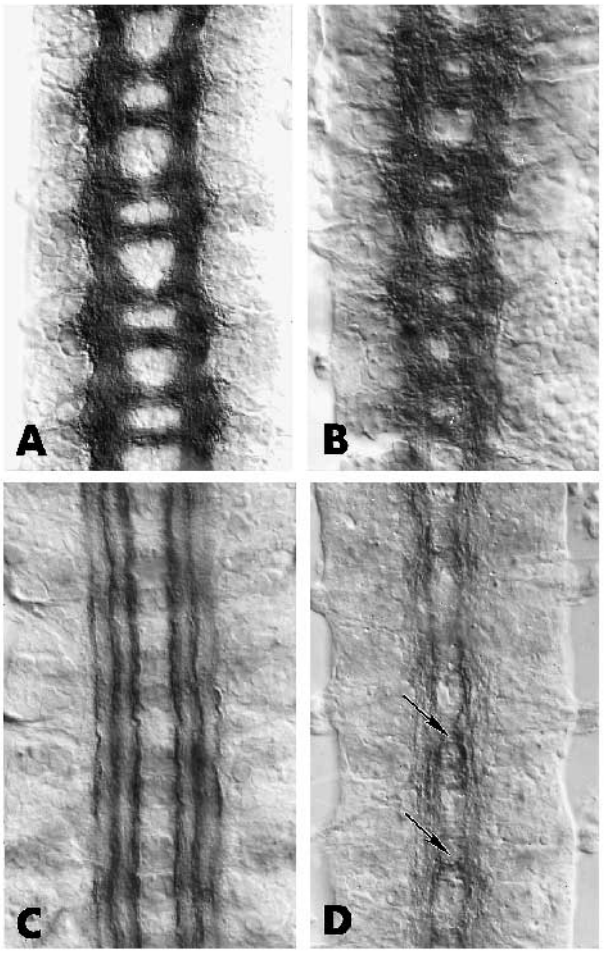

Fig. 3. Argos mis-expression alters commissure regularity. CNS axon tract morphology shown with (A,B) HRP histochemistry of 102 and (C,D) Fasciclin II immunolabeling. (A,C) Wild-type and (B,D) *hs-argos* stage 12/3 embryos were subjected to three 45 minute heat shocks and aged to stage 16 before fixation and immunolabeling. (D) Arrows indicate Fasciclin II mis-expression on commissural axons.

commissures remained well separated, due to the interposition of dorsal neurons between the anterior and posterior commissures. Ultrastructural analysis and examination of *reaper* mRNA expression after heat-shock treatment revealed no changes in the lateral halves of the CNS of *HS-argos* embryos relative to wild type, although other effects of ectopic *argos* on neurons remain possible (data not shown).

The MG express both *DER* and *argos* before they migrate to separate the commissures (Figs 1, 7). It is possible that the MG may still initiate commissure separation before entering apoptosis, so that subsequent medial migration of cells such as the RP neurons is sufficient to maintain morphology. This is in contrast to embryos mutant for spitz group genes, where initial migration of the MG does not occur and commissure fusion results (Klämbt et al., 1991).

Spitz pathway function regulates MG survival

The spitz pathway has been shown to function by activating the *Drosophila* EGF-receptor (Golembo et al., 1996b; Freeman, 1997). DER activation is sufficient to initiate expression of *argos* in Schneider S2 cells stably transformed with *DER* (Golembo et al., 1996a). Here, we addressed the question of whether spitz pathway function, DER signaling and *argos* expression interact to regulate MG number.

To that end, we have examined MG differentiation and survival in embryos mutant for each of the spitz group genes, *DER*, and some members of the *Ras* pathway that mediate DER signaling (Sonnenfeld and Jacobs, 1994; Lanoue and Jacobs, unpublished results). Fig. 5 summarises these effects for one gene from each group, compared to wild type (Fig. 5A). In embryos homozygous for a null mutation in *rhomboid*, a spitz group gene, very few MG survive to stage 16 (0.9±0.1 per segment, n=8, Fig. 5B). Conversely, ectopic expression of *rhomboid* increases MG number to above normal levels (7MG per segment, Fig. 5C). Embryos homozygous for a DER hypomorph, flb^{IIE07} lack all MG (Fig. 5D). Conversely, embryos homozygous for a gain-of-function allele of *DER* (Elp^{B1}) have slightly more MG than normal until at least stage 15 (4.0±0.1 per segment, n=12; Fig. 5E). MG number is normal in Elp^{B1} first instar larva (3.1 per segment, data not shown) suggesting that reduction in MG numbers is delayed in this mutant. Misexpression of a constitutively active form of Raf (*UAS D-raf1*; Brand and Perrimon, 1994) in a subset of MECs, using the C38 GAL4 enhancer trap (Yeh et al., 1995) also results in more MECs expressing a MG-specific enhancer trap (Fig. 5F).

Spitz pathway function is required for argos expression

The data described above suggest that the spitz-DER signaling pathway acts to increase the numbers of MG that survive to late embryogenesis, while *argos* appears to act as a negative regulator of survival. Embryos doubly mutant for mutations that reduce or abolish spitz pathway function and mutant for $argos^{w11}$, manifest a spitz group phenotype of commissural tract fusion (data not shown) and in MG position and survival (Fig. 6). The $argos^{w11}$ enhancer trap is activated weakly or not at all in spitz pathway mutants. This demonstrates that the spitz pathway functions upstream of *argos* and that *argos* expression is dependent upon spitz pathway function. This analysis is consistent with similar assays in the ventral ectoderm, the eye imaginal disc, chordotonal organs and other tissues (Freeman, 1994a; Golembo et al., 1996a; Okabe et al., 1996).

DER mRNA levels decline after *argos* signaling

In vitro assays suggest that *argos* expression is activated by DER signaling and that Argos binding to DER reduces the level of DER signaling (Golembo et al., 1996a). How might this function to regulate MG numbers during development? Mutant analysis has demonstrated that DER signaling is required for MG survival. Perhaps when a sufficient number of DER molecules present on the surface of a presumptive MG bind Argos, the level of DER signaling drops sufficiently to trigger apoptosis.

We have examined *DER* mRNA expression in MG exposed to increased levels of Argos. Wild-type and *hs-argos* embryos were doubly labeled for *slit* 1.0 *lacZ* reporter expression and the presence of *DER* transcript 45 minutes after being subjected to a single 60 minute heat shock. At stages 12/0 and 15, MG normally express detectable levels of *DER* transcript (Fig. 7A,C). If subjected to ectopic Argos (at stage 12/3 and 14), the MG subsequently shift from their wild-type

position, and all *DER* transcript labeling disappears. Loss of *DER* expression occurs before the nuclear condensation typical of apoptosis is apparent. *DER* mRNA levels were lower in MG lacking cell contact with other MG (data not shown). The results of similar experiments on stage 16 embryos were less dramatic, as levels of detectable *DER* transcript decline gradually during development, however, MG express *DER* mRNA at least until hatching. Shutdown of transcription and degradation of RNA are early indicators of apoptosis (Kerkhoff and Ziff, 1995). Expression of *reaper* (*rpr*) is also a useful early indicator of apoptosis (Dong and Jacobs, 1997). However, in situ hybridisation with a *rpr* RNA probe did not clearly indicate an increase of expression in the midline (data not shown).

Transient glia do not express *argos*

Increased levels of Argos secretion were sufficient to direct all MG to apoptosis. Is MG apoptosis normally triggered by *argos* expression? We noted that *argos* expression within the MG lineage differed from other MG lineage-specific gene expression because the numbers of *argos*-expressing cells did not decline during embryogenesis. This suggests that transient MG that enter apoptosis are those that did not express *argos*. This interpretation was supported by comparison of the time course of $argos^{w11}$ enhancer trap expression with expression of other genes in the MG. Expression of mRNA of genes specific to the MG lineage, such as *slit* and *DER*, began at the start of stage 12. In late stage 12 $argos^{w11}$/+ embryos, enhancer trap expression was first detected in one or two of the 8-12 *slit* and *DER*-expressing MECs (Fig. 8A,B). This expression begins before the migration of the MG that separate the commissures. Three MG-expressing the *argos* enhancer trap were seen per segment at stage 14, however 4-5 MG were identified by *slit* or *DER* transcript detection. MG not expressing $argos^{w11}$ were displaced away from the commissural tracts, suggesting that they may have been in early stages of removal from the nerve cord (Fig. 8C,D). During stage 16, all *slit* and *DER*-expressing glia also expressed the *argos* enhancer trap (Fig. 8E,F). *argos* expression was never detected in apoptotic or displaced MG, suggesting that *argos* expression identifies MG that persist to larval stages.

DISCUSSION

Functions of embryonic MG

A role for the MG in separation and ensheathment of the commissures has been indicated in earlier studies (Jacobs and Goodman, 1989a). The migration of MG between early commissural axons in wild-type embryos indicates a morphogenetic function of the MG (Klambt et al., 1991). Fusion of the anterior and posterior commissures, characteristic of spitz group mutations, is correlated with the early apoptosis of all

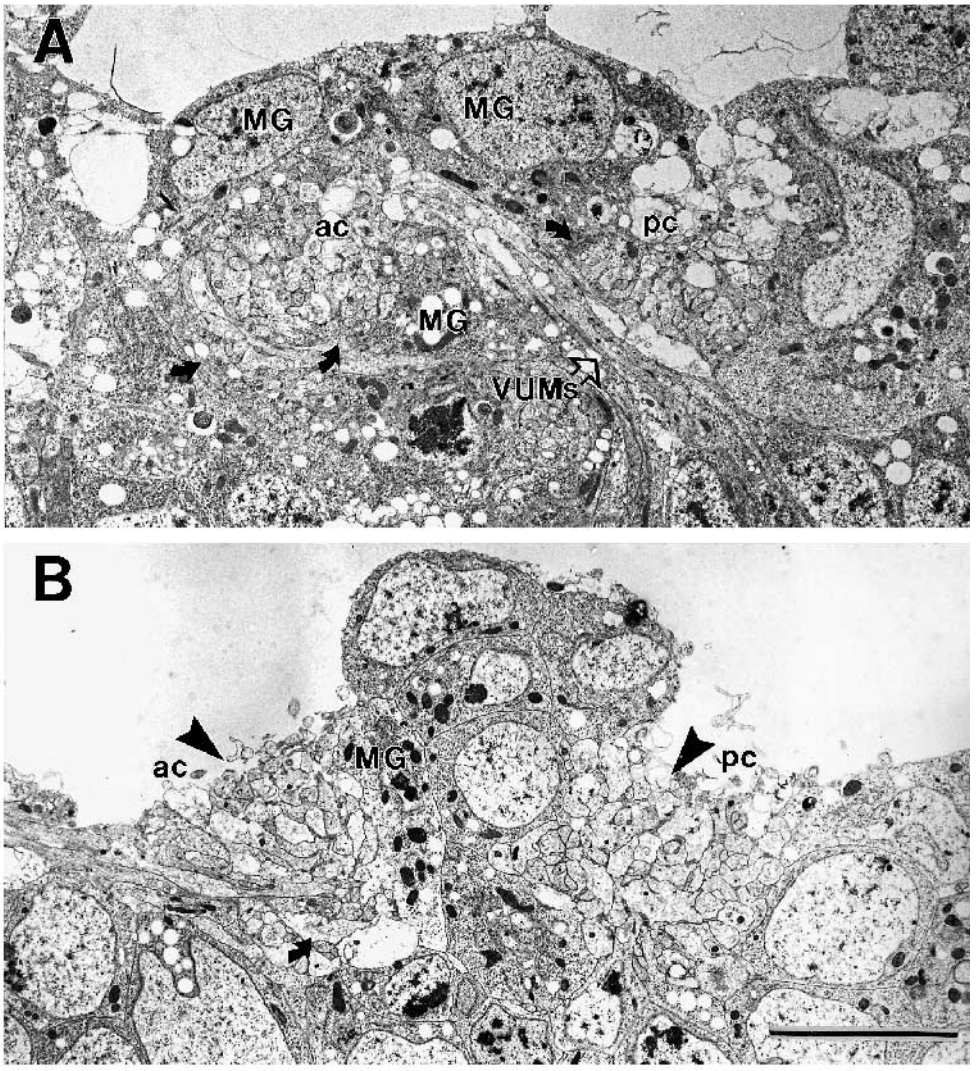

Fig. 4. Argos misexpression results in desheathment of commissural axon tracts. (A) Wild-type embryos subjected to heat shock have normal numbers of MG ensheathing the axons of the commissural tracts. (B) After heat-shock activation in *hs-argos* embryos, MG loss and desheathment of commissural axons is evident, (see arrowheads). A single MG persists in this segment. MG are identified by nuclear size, position and presence of lamellipodia ensheathing commissural axons (curved arrows). The anterior commissure (ac) posterior commissure (pc) and midline axons of the VUM neurons (VUMs) are identified. Anterior is at left. Calibration: 4 μm.

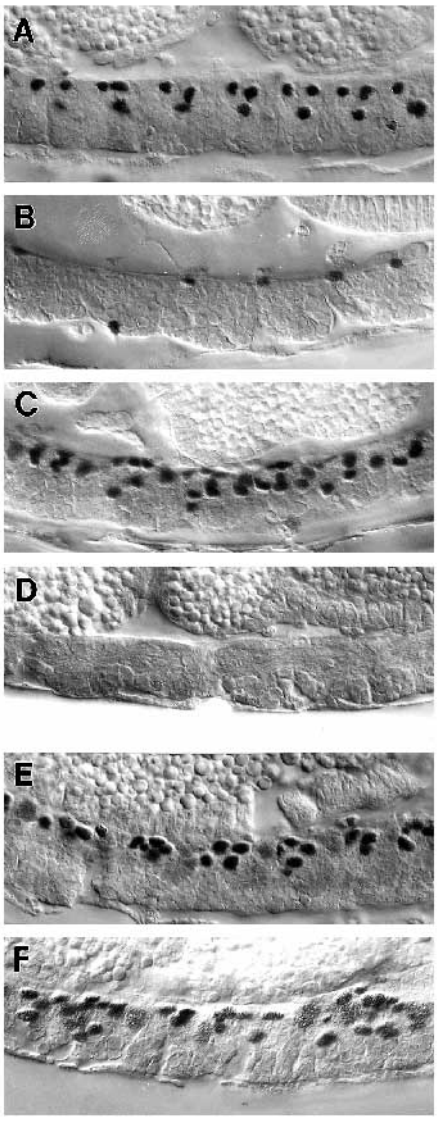

Fig. 5. Spitz pathway function regulates MG number. MG numbers indicated by *AA142* enhancer trap expression are contrasted in different genetic backgrounds that alter the level of spitz pathway signaling. (A) A wild-type embryo at stage 16, averaging 3.2 per segment. (B) Embryos homozygous for an amorphic allele of *rhomboid (rho^{38})* at the same stage average 0.9 MG per segment. (C) After misexpression of *rhomboid* in *hs-rho* embryos, 7-8 AA142-expressing cells per segment are observed. (D) Embryos homozygous for a DER hypomorph, *flb^{IIE07}* lack all MG, while (E) embryos homozygous for the DER hypermorph, *Elp^{B1}*, a slight increase in the number of AA142-expressing cells to 4.0 MG per segment is seen. (F) If an activated form of *D-raf* (*P[UAS D-raf1]*) is expressed in a subset of the mesectoderm by the C38 *P[GAL4]* enhancer trap, the number of AA142-expressing cells is also increased relative to wild type.

MG, which further supports this view (Sonnenfeld and Jacobs, 1994). The MG also express genes encoding extracellular matrix proteins, such as Slit and putative axon guidance cues such as D-netrin and Commissureless (Rothberg et al., 1988; Mitchell et al., 1996; Harris et al., 1996; Tear et al., 1996). Mutations in these genes severely perturb the guidance of axons that normally cross the midline (Seeger et al., 1993).

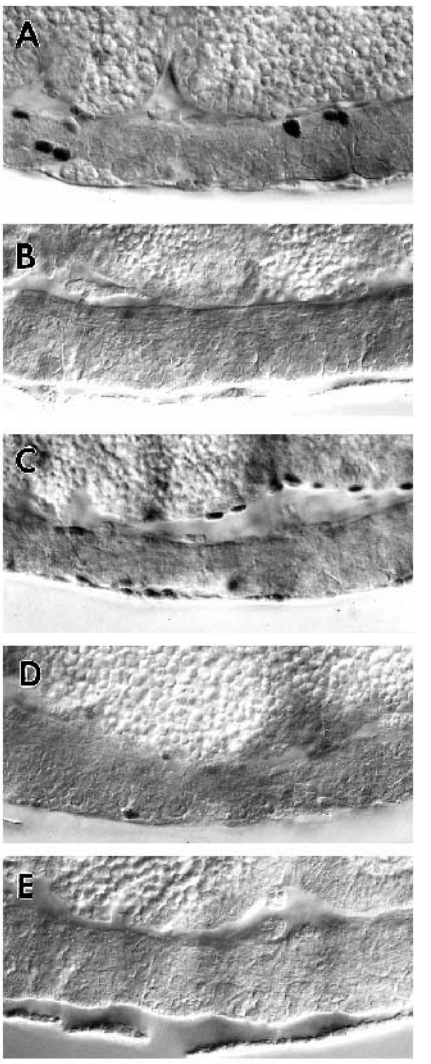

Fig. 6. Hypomorphic or null mutations in the Spitz pathway mutations are epistatic to *argos^{w11}*. AA142 enhancer trap expression are examined in embryos doubly homozygous mutant for *argos^{w11}*, which would increase in MG number, and alleles of the spitz group which would decrease in MG number. Double mutants *rhomboid*, (*rho^{38}*, *argos^{w11}* in A), *Star* (*S^{IIN23}*; *argos^{w11}* in B), *spitz* (*spi^{E55}*; *argos^{w11}* in C), *DER* (*flb^{2EO7}*; *argos^{w11}* in D) and *pointed* (*pnt^{9J}*, *argos^{w11}* in E) show the MG number phenotype typical of the spitz group mutation.

Misexpression of *argos* later than stage 12 has revealed more subtle MG functions, because early roles in commissure establishment progress undisturbed, while the MG do not survive to perform later functions. Late removal of the MG results in desheathment of the commissural tracts and disruption of the glial component of the blood-brain barrier. Although the commissures remain intact and well separated, superficial axons are exposed to the hemolymph and the wall of the gut, where movements of larva would likely result in axon damage. MG removal during mid-embryogenesis also produces a mild phenotype typical of the *roundabout* mutant, which is the misplacement of Fasciclin II upon axon segments traversing the midline (compare with Seeger et al., 1993). This reflects either pathfinding errors or a change in the distribution of Fasciclin

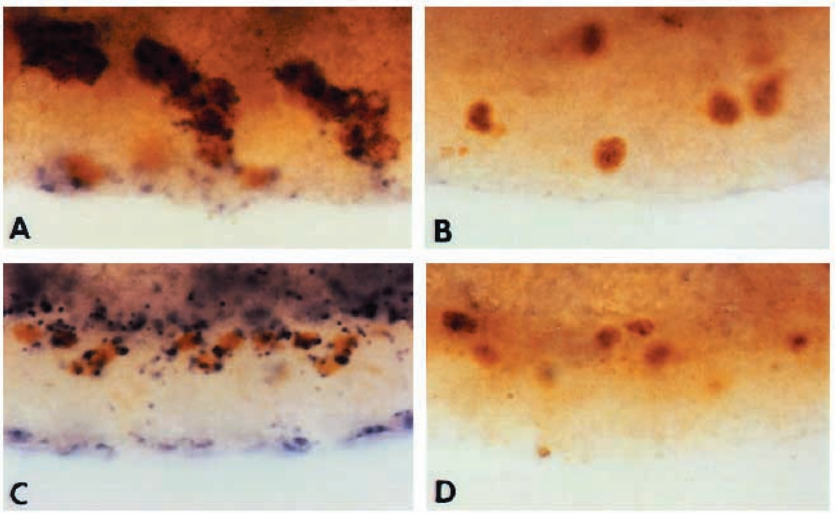

Fig. 7. Misexpression of *argos* reduces DER mRNA levels. (A,C) Wild-type and (B,D) *hs-argos* embryos subjected to one heat shock beginning at stage 12 (A,B) and stage 14 (C,D) are doubly labeled for *slit1.0lacZ* expression (brown) and *DER* mRNA (blue). All *slit 1.0*-expressing cells express DER in wild-type embryos (A,C), however, DER transcript levels are dramatically reduced after heat shock in *hs-argos* embryos (B,D).

II on normally projecting axons. Although this phenotype might result from the effects of ectopic *argos* on neuronal differentiation, targeted MG ablation employing the GAL4 system results in similar changes in Fasciclin II distribution (John and Brand, personal communication). This suggests that the MG have a continuing role in attraction and repulsion of axons from the midline, even after initial axon tracts have been successfully established.

argos functions to regulate MG number

Previous studies have identified the embryonic MG as a transient cell type. There may be 12 MEC precursors competent to differentiate into MG, which will express MG lineage markers if DER signaling is hyperactivated or if cell death is blocked (Sonnenfeld and Jacobs, 1995; Dong and Jacobs, 1997). However, in wild-type embryos, establishment and morphogenesis of the commissures begins at late stage 12 with 6 MG per segment. Once form has been established, by stage 16, 3 MG per segment remain for ensheathment of the commissures. Consequently, approximately half of the MG undergo apoptosis between stage 13 and 16 of embryogenesis. A remarkable feature of the reduction in MG number is a stochastic pattern of cell removal, suggestive of a response to a non-autonomous factor (Sonnenfeld and Jacobs, 1995).

The period of normal MG apoptosis and MG sensitivity to misexpression of *argos* is coincident with the first hours of *argos* expression in the MG. Higher levels of *argos* expression direct more MG to apoptosis. The earliest detectable effect of increased Argos signaling in the MG is the removal of *DER* mRNA. Rapid removal of mRNAs is an event associated with apoptosis in other cell types (Kerkhoff and Ziff, 1995). *argos* expression in the MG is dependent upon DER signaling and opposes spitz group function. It is therefore likely that *argos* expression participates in the developmental pruning of MG numbers, by repressing the spitz pathway that functions to promote MG survival. A loss of *argos* function does not eliminate embryonic MG apoptosis, suggesting that other factors, perhaps including the level of Spitz signaling, also influence MG survival.

Role of Argos in cell signaling

Genetic and molecular studies demonstrate that *argos* activity both opposes and depends upon DER signaling, suggestive of a role in negative feedback or lateral inhibition of DER signaling between cells (Schweitzer et al., 1995b; Golembo et al., 1996a,b). *argos* functions to restrict cone cell and photoreceptor number during eye disc development, restrict wing vein domains during wing disc development, define position in

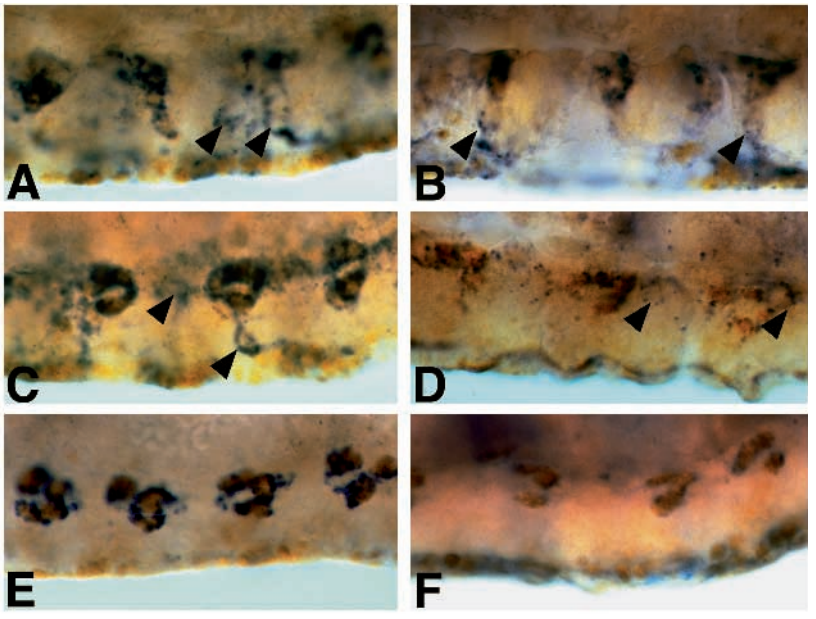

Fig. 8. Argos in expressed only in MG which survive embryogenesis. Expression of the $argos^{w11}$/+ enhancer trap (brown) is contrasted with expression of *slit* mRNA (blue, in A,C,E) and *DER* mRNA (blue, in B,D,F) at (A,B) stage 12, (C,D) stage 14 and (E,F) stage 16. Note the presence of MG-expressing *slit* or *DER* at stages 12 and 14 not labeled for *argos* expression (arrowheads).

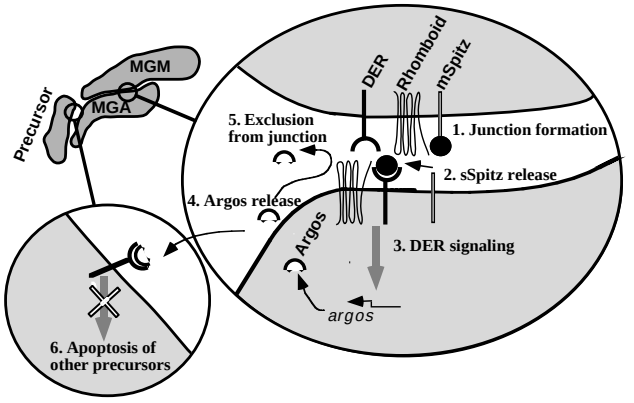

Fig. 9. A model for suppression of autocrine Argos signaling during MG signaling. Rhomboid, DER and components of the membrane skeleton associate in juxtacrine signaling junctions between migrating Midline Glia (1). Within this junction, Rhomboid facilitates transmembrane Spitz signaling, or the release of secreted Spitz (sSpitz) from membrane Spitz (mSpitz), which then activates DER, also localised within the junction. Sufficient DER signaling triggers Argos production (3), which is secreted (4). Released Argos has a minimal effect upon signaling within junctions because of restricted inward diffusion (5), yet Argos effectively reduces DER signaling outside of junctions, such as distant MG with reduced junction formation.

the ventral ectoderm, restrict chordotonal organ number and restrict MG number during embryogenesis (Sawamoto et al., 1994; Freeman, 1994a; Golembo et al., 1996a,b; Okabe et al., 1996; Scholz et al., 1997; this study).

It has been suggested in ommaditial development that photoreceptors, cone cells and pigment cells produce Argos in succession after each responds to Spitz released by neighbouring, previously determined cells (Freeman, 1996, 1997). Spitz has been postulated to induce differentiation in short-range signaling in the developing ommatidium, while subsequent Argos release then acts as a long-range inhibitor of differentiation (Freeman, 1997).

It is likely that during the specification of MG number in the embryonic nerve cord, a subset of the equivalent cells receive sufficient DER activation by Spitz to begin *argos* expression. The source of Spitz is likely to be within the group of equivalent cells. The subsequent release of Argos by activated MG may work as a long-range inhibitor of DER activation, to restrict the number of equivalent cells acquiring MG fate (Sawamoto et al., 1994; Schweitzer et al., 1995b; Scholz et al., 1977; this study).

Autocrine and lateral signaling through DER?

Embryonic DER can be activated by EGF-like proteins Spitz and Vein (Schweitzer et al., 1995a; Schepp et al., 1996). Spitz is broadly distributed in embryonic tissue in a transmembrane form; its activity depends upon spatially restricted Star and Rhomboid, which appear to localise Spitz release between adhering cells that express spitz group proteins (Rutledge et al., 1992; Ruohola-Baker et al., 1993; Sturtevant et al., 1996). It has been proposed that Rhomboid functions to release an active Spitz fragment from the transmembrane form, which then acts locally (Schweitzer et al., 1995a). Spitz may then bind to DER on the same cell, or diffuse to activate adjacent cells (Golembo et al., 1996b). DER agonist Vein, and the DER antagonist Argos, are expressed in a spatially restricted pattern and are released in a diffusable form (Freeman, 1994a; Schepp et al., 1996). Vein contains an Ig-like domain suggestive of interaction with other extracellular proteins, which may limit the extracellular domain of signaling (Schepp et al., 1996). DER signaling within any cell is therefore dictated by relative exposure to different locally restricted or diffusable ligands.

Activation of *argos* in premigratory MG suggests that DER signaling begins in some MG by stage 12/3. *argos* expression can induce apoptosis in the MG from then until at least late stage 15, representing nearly 5 hours of development. During this period, all MG express *DER* mRNA and remain responsive to ectopic Argos. Elevated levels of *argos* production will direct even *argos*-expressing MG to apoptosis, indicating that the DER signaling pathway remains functional in MG-expressing argos. Therefore, although *argos*-expressing MG do not ordinarily enter apoptosis, it appears that all MG continue to integrate relative levels of DER activation and inhibition until at least stage 15. This period of integration of DER signaling in the MG is relatively longer than in the eye or the ventral ectoderm (Freeman, 1996; Golembo et al., 1996b). This would suggest that *argos*-expressing MG depend upon continued Spitz activation of the DER at levels higher than adjacent non-*argos*-expressing MG to overcome possible autocrine inhibition by released Argos.

It is likely that the short-range signaling of Spitz and the long-range inhibition by Argos demonstrated in the eye also characterises function among MG precursors. Spatial restriction of signaling by released Spitz may depend upon its restricted diffusion, perhaps by a rapid rate of sequestration by receptors. Alternatively, signaling may be restricted by close apposition sometimes encountered between communicating cell surfaces (Fagotto and Gumbiner, 1996; Lambie, 1996). In *C. elegans*, activation of the EGF-like receptor LET-23 is enhanced by proteins that appear to associate with adherens junctions (Hoskins et al., 1996; Simske et al., 1996). This may also be the case with the vertebrate EGF receptor, which interacts with β-catenin in cadherin-based adherens junctions (Hoschuetzky et al., 1994). Direct evidence of such an interaction is lacking in *Drosophila*. However, the phenotype of a hypermorphic allele of *DER* is suppressed by mutations in *coracle*, which encodes a membrane skeleton protein associated with septate junctions in *Drosophila* (Fehon et al., 1994).

We suggest that Rhomboid functions to sustain Spitz signaling within adhesive domains of the cell membrane where DER is concentrated. Rhomboid localises to Armadillo-containing junctions on or near the apical surface of epithelial cells, where it encounters the apical surface of other *rhomboid*-expressing cells (Sturtevant et al., 1996). Armadillo, a *Drosophila* β-catenin homologue, and *D*E-cadherin, expressed by the MG, are associated with adherens junctions (Cox et al., 1996; Oda et al., 1994). Loss of *rho* function results in a loss of cell-to-cell contiguity in some mesectodermal lineages, implicating Rhomboid in an adhesion function (Sonnenfeld and Jacobs, 1994). If Rhomboid facilitates signaling by the transmembrane form of Spitz, or by stimulating Spitz release from the membrane (Schweitzer et al., 1995a; Golembo et al., 1996b), then apposition of junctions between cells may not only localise the Spitz signal but then also restrict the diffusion

of Argos into this zone of signaling (Fig. 9). This would make cells less sensitive to autocrine inhibition.

In this model, juxtacrine Spitz signaling, spatially limited to junctions between adhering MG cells, determines which MG express *argos* first. Released Argos subsequently attenuates DER signaling of other cells with fewer or smaller junctions. In the absence of *argos* function, all MG escape this inhibition, but not all MG survive, perhaps because the level of Spitz signaling remains insufficient. Support for this model requires proof that Rhomboid and DER co-localise to junctions between MG, and that released Argos does not interfere with signaling between adhering MG.

It is evident that tissue differences in the spatial and temporal pattern of Argos and Spitz release and *DER* gene function defines the role of *argos* signaling. Further clarification of the functional role of Star and Rhomboid in DER signaling should provide a better understanding of how Spitz, Vein and Argos signals are integrated by the receptor.

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