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The *Drosophila* embryonic midline is the site of Spitz processing, and induces activation of the EGF receptor in the ventral ectoderm

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SUMMARY

The *Drosophila* EGF receptor (DER) is activated by secreted Spitz to induce different cell fates in the ventral ectoderm. Processing of the precursor transmembrane Spitz to generate the secreted form was shown to be the limiting event, but the cells in which processing takes place and the mechanism that may generate a gradient of secreted Spitz in the ectoderm were not known. The ectodermal defects in *single minded* (*sim*) mutant embryos, in which the midline fails to develop, suggested that the midline cells contribute to patterning of the ventral ectoderm. This work shows that the midline provides the site for Spitz expression and processing. The Rhomboid and Star proteins are also expressed and required in the midline. The ectodermal defects of *spitz*, *rho* or *Star* mutant embryos could be rescued by inducing the expression of the respective normal genes only in the midline cells. Rho and Star thus function non-autonomously, and may be required for the production or processing of the Spitz precursor. Secreted Spitz is the only *sim*-dependent contribution of the midline to patterning the ectoderm, since the ventral defects observed in *sim* mutant embryos can be overcome by expression of secreted Spitz in the ectoderm. While ectopic expression of secreted Spitz in the ectoderm or mesoderm gave rise to ventralization of the embryo, increased expression of secreted Spitz in the midline did not lead to alterations in ectoderm patterning. A mechanism for adjustment to variable levels of secreted Spitz emanating from the midline may be provided by Argos, which forms an inhibitory feedback loop for DER activation. The production of secreted Spitz in the midline, may provide a stable source for graded DER activation in the ventral ectoderm.

Key words: *Drosophila*, EGF receptor, *spitz*, r*homboid*, *Star*, *single minded*

INTRODUCTION

Gradients of diffusible polypeptides play cardinal roles in the determination of a diverse array of cell fates. A variety of mechanisms have been identified for the generation of such gradients. For example, the tissue that produces the diffusible factor may be insensitive to it, while the neighboring tissue is competent to respond to the signals emanating from the producing cells. This mechanism was shown to take place in the case of the *Drosophila* Hedgehog protein which is produced by the *engrailed*-expressing cells, and diffuses to pattern the neighboring cells (Heemskerk and DiNardo, 1994). In the wing imaginal disc, a stripe of *dpp*-expressing cells serves as a source for long-range influence of the secreted protein, which may induce the expression of target genes in a concentration-dependent manner (Nellen et al., 1996; Lecuit et al., 1996). Alternatively, an inactive precursor molecule is produced and its site of activity is defined by the distribution of molecules which take part in processing. For example, the maternal Spätzle precursor is distributed as an inactive protein in the perivitelline fluid surrounding the *Drosophila* embryo. Only upon proteolytic processing that is restricted to the ventral vitelline membrane, will it activate the Toll receptor in the ventral and ventrolateral regions of the embryonic ectoderm (reviewed in Chasan and Anderson, 1993).

In the *Drosophila* ectoderm, the initial determination of fates is induced by the maternal Dorsal pathway (Chasan and Anderson, 1993). Consequently, the embryo is subdivided into distinct domains of gene expression along the dorsoventral axis, including the precursors of the mesoderm, ventral ectoderm, dorsal ectoderm and amnioserosa. Furthermore, within the ventral ectoderm, the nuclear levels of the Dorsal protein may contribute to the induction of different neuroblast cell fates. In the same region, the zygotic *Drosophila* EGF receptor (DER) pathway further specifies and refines ventral cell fates. In *DER/flb* mutant embryos, ventral cell identities are replaced by lateral fates (Raz and Shilo, 1993). Elucidation of the DER signaling pathway has been facilitated by the identification of the *spitz* group genes, which give rise to similar phenotypes and interact genetically with DER (Mayer and Nüsslein-Volhard, 1988; Sturtevant et al., 1993; Raz and Shilo, 1993). The *spitz* gene encodes a TGFα-like molecule which is produced as a transmembrane precursor (Rutledge et al., 1992). Overexpression in the ectoderm of secreted Spitz, but not of the transmembrane precursor, results in ventralization of the embryo. This observation suggests that the levels of secreted Spitz

determine the extent of DER activation in the ectoderm (Schweitzer et al., 1995b).

Different cell fates are monitored in the ventral ectoderm. (1) The ventralmost cells, which experience the highest level of DER activation, express one set of markers, including *orthodenticle* (*otd*). (2) The ventrolateral cells express other markers such as Fasciclin III (Raz and Shilo, 1993). It is thus possible that secreted Spitz is present in a gradient, with the highest concentrations in the ventral midline.

Since the Spitz precursor is ubiquitously expressed (Rutledge et al., 1992), the gradient of DER activation could be regulated by restricted processing of Spitz. Two molecules that may participate in Spitz production or processing were identified as members of the *spitz* group: Rhomboid (Rho) and Star are novel transmembrane proteins containing seven and one transmembrane domains, respectively (Bier et al., 1990; Kolodkin et al., 1994). Genetic epistasis experiments suggested that both proteins function upstream to Spitz, since their mutant phenotypes can be overcome by expression of secreted Spitz (Schweitzer et al., 1995b). However, since high levels of secreted Spitz were used, it was not possible to say whether physiological levels of secreted Spitz would indeed be able to bypass the requirement for Rho and Star. Furthermore, the possible mechanism of action in facilitating Spitz processing and relevant sites of activity of Rho and Star are not known.

It is interesting to note that the *single minded* (*sim*) gene, which is required for the development of the midline cells, has also been assigned to the *spitz* group (Mayer and Nüsslein-Volhard, 1988). Sim is a bHLH-PAS transcription factor that is expressed exclusively in the mesectoderm and midline, and is responsible for the induction of midline cell fates (Nambu et al., 1990, 1991). In *sim* mutant embryos, ventral cell fates are missing, as monitored by cuticle preparations (Mayer and Nüsslein-Volhard, 1988) and lack of expression of ventral markers (Kim and Crews, 1993). An indication for the role of Sim in the context of the *spitz* group is provided by the observation that *spitz*, *rho* and *Star* are expressed in the midline (Rutledge et al., 1992; Bier et al., 1990; Kolodkin et al., 1994; Heberlein et al., 1993). The expression of *rho* was shown to be dependent upon Sim (Nambu et al., 1990). Presumably, expression of *spitz* and *Star* also requires the function of Sim.

In this work, we investigate the sites in which production and processing of Spitz are required in the embryo. The normal function of Spitz appears to be non autonomous: expression of the Spitz precursor only in the midline is sufficient for patterning the ventral ectoderm. Rho and Star also function non autonomously and are required in the midline. Facilitating the expression of *spitz*, *rho* and *Star* is the only *sim*-dependent contribution of the midline to patterning the ventral ectoderm, since the mutant *sim* ectodermal defects can be overcome by expression of secreted Spitz in the ectoderm. These results suggest a mechanism for generating a graded distribution of secreted Spitz, which may subsequently give rise to graded activation of DER in the ectoderm.

MATERIALS AND METHODS

Probes and antibodies

The *otd* probe was kindly provided by R. Finkelstein and the FasIII monoclonal antibodies by T. Volk. Standard procedures were used for RNA in situ hybridization and antibody staining. Enhancer trap lines were stained with X-gal or rabbit anti-β-Gal antibodies (Cappel). Balancer chromosomes carrying *lac*Z-expressing constructs were identified by X-gal staining.

Plasmid constructs

The UAS-*Star* construct was generated by introducing a 3.3 kb *Eco*RI fragment containing the entire coding sequence of the *Star* cDNA into pUAST. The UAS-*rho* construct was generated by introducing the 1.05 kb *Dde*I fragment of the *Rho* cDNA, containing the entire coding region, into the *Eco*RI-*Bgl*II sites of the pUAST vector (Brand and Perrimon, 1993).

Fly strains

Strains carrying the following mutant chromosomes were used: spi^{IIT25}, spi^{OE92}, $rho^{\Delta 38}$, S^{IIN23}, flb^{1F26}, sim^{H9} *and* sna^{II6}. The C50.1S1 enhancer trap line in the *disco* locus, and a *sim* enhancer trap line were used. The following Gal4 inducer lines were used: *twist*-Gal4 (2nd chromosome), *sim*-Gal4 (2nd chromosome), *rho*-Gal4 (2nd chromosome), *Kr*-Gal4/*TM3* (3rd chromosome) and *69B* (3rd chromosome). The following UAS lines were used: UAS-*sspi*4a (2nd chromosome), UAS-*rho*24-2 (2nd chromosome), UAS-*rho*11-1(3rd chromosome), UAS-*mspi*7b (1st chromosome) and UAS-*S*4-3 (3rd chromosome).

To test the effects of induced expression in mutant backgrounds the following lines were constructed: (1) *w; sim*-Gal4, spi^{OE92}/*CyO lacZ* (2) *w*, UAS-*mspi*7b; spi^{OE92}/*CyO lac*Z (3) *w; sim*-Gal4; $rho^{\Delta 38}$/*TM3 lac*Z (4) *w*; UAS-*rho*24-2; $rho^{\Delta 38}$/*TM3 lac*Z (5) *w; sim*-Gal4, S^{IIN23}/*CyO lac*Z (6) *w*; S^{IIN23}/*CyO lacZ;* UAS-*Star*4-3 (7) *w*; *rho*-Gal4; sim^{H9} /*TM3 lac*Z (8) *w*; UAS-*sspi*4a; sim^{H9}/*TM3 lac*Z (9) *w*; S^{IIN23}/*CyO lacZ; 69B*. The flb^{1F26} mutant embryos were grown at 29°C. Embryos were collected at 1 hour intervals at 25°C and aged until stage 11 (6.5 hours after egg lay).

RESULTS

Non-autonomous activity of secreted Spitz and Rhomboid

In order to express secreted Spitz, lines carrying a UAS-*secreted spitz* construct were generated. Upon expression of secreted Spitz in the ectoderm, the tissue in which DER is activated, ventralization of the embryo was observed (Schweitzer et al., 1995b). We next examined whether secreted Spitz expression in neighboring tissues would result in a similar outcome. To do so, secreted Spitz expression was induced by *twist*-Gal4, which is expressed in the mesoderm and midline (Baker and Schubiger, 1996) (see scheme in Fig. 1A). In these embryos, normal invagination of the mesoderm takes place, as monitored by FasIII expression in the visceral mesoderm (not shown). Ventral cell fates in the ectoderm were followed by expression of *otd*, which normally marks the ventralmost cells (Wieschaus et al., 1992; and Fig. 1B). A dramatic ventralization of the embryos is observed; up to 8-10 cell rows express *otd* on each side of the midline (Fig. 1C), comparable to the result obtained following expression of secreted Spitz in the ectoderm. This experiment demonstrates that secreted Spitz can diffuse readily between the mesoderm and ectoderm. Although the mesoderm spreads over the entire ectoderm, expression of *otd* was detected only in the ventral ectoderm. This is likely to result from the fact that by the time secreted Spitz was induced, dorsal cell fates have already been established by the Dpp pathway (see Discussion).

The Spitz precursor is normally expressed in the ectoderm

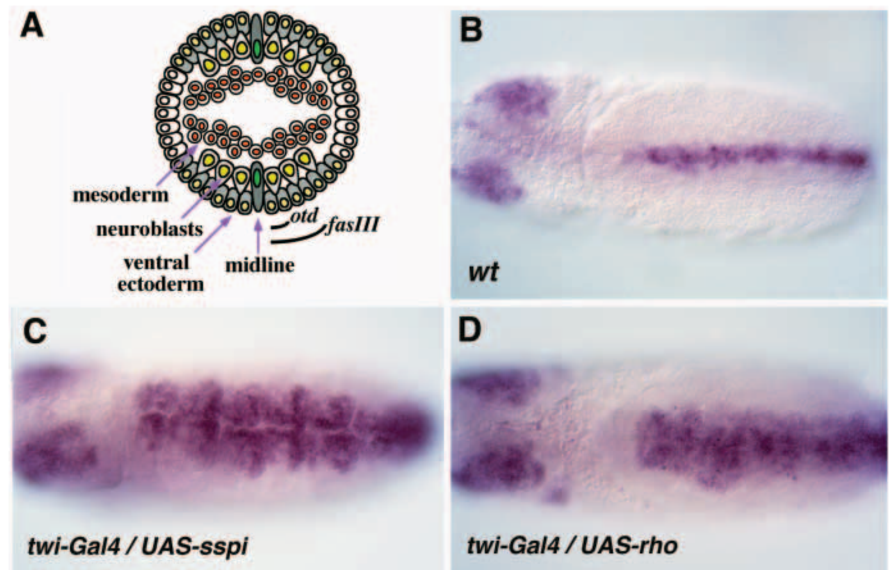

Fig. 1. Secreted Spitz or Rhomboid in the mesoderm induce ventralization of the ectoderm. The non-autonomous effects on ectodermal patterning of secreted Spitz or Rho expressed in the mesoderm were examined. (A) A scheme of a cross section of a germ-band-extended embryo (stage 10/11), showing the different embryonic tissues and the expression domains of *otd* and *fasIII*. (B) *otd* expression in wild-type embryos is restricted to 1-2 cell rows on each side of the midline. (C) In *twist*-Gal4/UAS-*secreted spitz* embryos or (D) *twist*-Gal4/UAS-*rho* embryos, *otd* expression is expanded to 8-10 cell rows on each side of the midline. Anterior is to the left in all figures.

and mesoderm (Rutledge et al., 1992), while Rho is excluded from the mesoderm due to transcriptional repression by Snail (Ip et al., 1992; Nambu et al., 1990). Rho was considered to function as a facilitator of DER signaling and, in view of its multiple transmembrane domains, it was assumed to be required in the same cells as DER. While epistasis studies have suggested that Rho acts upstream to the production of secreted Spitz (Schweitzer et al., 1995b), it was still possible to argue that the high levels of secreted Spitz used may overcome the requirement for facilitation of DER signaling by Rho. Since secreted Spitz expressed in the mesoderm can affect cell fate specification in the ectoderm, it was possible to test whether Rho would also be able to function non-autonomously. Indeed, when Rho was ectopically expressed in the mesoderm and midline, a similar degree of ectodermal ventralization was observed (Fig. 1D). Expression of Star in the mesoderm by *twist*-Gal4 did not give rise to any phenotype (not shown).

Expression of Spitz precursor in the midline patterns the ventral ectoderm

We have shown above that under artificial situations, Spitz and Rho can induce patterning of the ectoderm, in a non-autonomous manner. In view of the ectodermal defects of *sim* mutant embryos and the expression of *spitz*, *rho* and *Star* in the midline, it is plausible that during normal development the midline is the source of secreted Spitz to the ectoderm. The capacity of the Spitz precursor to rescue the *spitz* mutant phenotype in the ectoderm was tested, following induction of UAS-*membrane spitz* exclusively in the midline (by a *sim*-Gal4 construct). FasIII is normally expressed in 4-5 cell rows on each side of the midline in the thoracic segments (Raz and Shilo, 1993; Fig. 2A). In *spitz* mutant embryos, no expression of FasIII is detected in the ventral ectoderm. Upon induction of the normal *spitz* gene in the midline, partial rescue was observed and up to 4 rows of cells express FasIII (Fig. 2C). The incomplete rescue may be accounted for by the fact that

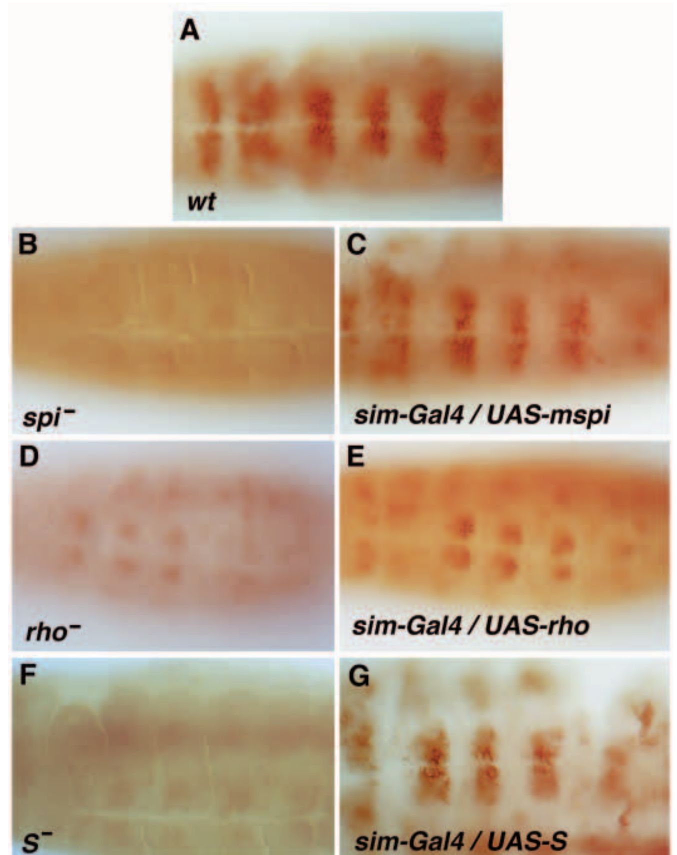

Fig. 2. Spitz, Rhomboid and Star are required in the midline to pattern the ventral ectoderm. (A) In wild-type embryos, expression of FasIII is observed in 4-5 cell rows on each side of the midline of the thoracic parasegments. (B) In spi^{IIT25} mutant embryos, no expression of FasIII in the ectoderm is detected. (C) In spi^{OE92} homozygous embryos in which the expression of UAS-*membrane spitz* was induced by *sim*-Gal4, partial rescue of the phenotype is observed, such that up to 4 cell rows on each side express FasIII. The two *spi* alleles used are null alleles. (D) $rho^{\Delta 38}$ mutant embryo. (E) Induction of UAS-*rho* by *sim*-Gal4 in $rho^{\Delta 38}$ mutant embryos resulted in partial rescue of FasIII expression. (F) S^{IIN23} mutant embryo. (G) Induction of UAS-*Star* by *sim*-Gal4 in S^{IIN23} mutant embryos gave rise to partial rescue.

expression of target genes induced by *sim*-Gal4 (e.g. UAS-*lac*Z) is observed only from stage 9/10 (not shown), which may be later than the time in which *spitz* normally begins to be expressed in the midline. Thus, induction by *sim*-Gal4 may provide expression levels that are lower than the level required for normal activity.

Rhomboid and Star in the midline are sufficient to pattern the ventral ectoderm

In view of the capacity of the Spitz precursor in the midline to pattern the ventral ectoderm shown above, it is possible that additional components of the *spitz* group would also function in the midline. *rho* and *Star* mutant embryos show ectodermal defects similar to *spitz* mutants (Mayer and Nüsslein-Volhard, 1988; and Fig. 2B,D,F). Epistasis experiments have shown that Rho and Star may function before or during Spitz processing (Schweitzer et al., 1995b). To test the possibility that Rho and Star are required in the midline, the rescue of *rho* or *Star* mutant phenotypes was examined following expression of the normal respective gene only in the midline.

While *rho* mutant embryos display no expression of FasIII in the ventral ectoderm, expression of *rho* in the midline was able to partially rescue this defect. In the thoracic segments of the rescued embryos, 1-4 cell rows expressing FasIII can be detected on each side of the midline (Fig. 2D,E). A similar experiment was carried out by expressing *Star* in the midline, in *Star* mutant embryos. Again, 1-3 cell rows expressing FasIII can be monitored on each side of the midline (Fig. 2F,G).

Secreted Spitz is the only *sim*-dependent element contributed by the midline to pattern the ectoderm

We have shown that Spitz is required predominantly or exclusively in the midline. Therefore, *sim* mutants, which are devoid of a functional midline, fail to activate DER and pattern the ectoderm. To test if the early development of the midline itself requires the DER pathway, midline markers were monitored in *DER/flb* mutant embryos. *sim-lacZ* expression shows no aberrations in midline patterning (Fig. 3A). The normal development of the midline is thus independent of the zygotic patterning of the ectoderm through the DER pathway.

It is possible that, in addition to Spitz, the midline may provide other factors that are essential for patterning the ectoderm. This option can be tested experimentally. If the midline is contributing additional *sim*-dependent factors, induction of secreted Spitz in the ectoderm of *sim* mutant embryos should not be able to induce the appearance of ventral cell fate markers. Conversely, if the midline is providing to the ectoderm only a source of secreted Spitz, expression of secreted Spitz in the ectoderm to activate the DER pathway should be epistatic to the *sim* mutant phenotype.

The experiment showed that secreted Spitz in the ectoderm is indeed epistatic to the *sim* mutant phenotype, and the extent of ventralization observed is comparable to the one monitored following a similar expression of secreted Spitz in wild-type embryos (Fig. 3C). This experiment demonstrates that the *sim*-dependent contribution of the midline to the ectoderm is confined to the production and processing of Spitz. In *sim* mutant embryos, Spitz, Rho and Star have been eliminated only in the midline; yet, no expression of the ventralmost markers, such as *otd*, and only rudimentary expression of ventrolateral markers is observed in these embryos (Kim and Crews, 1993; and Fig. 3B). Thus, the severity of the *sim* phenotype implies that the major, if not the exclusive, source of secreted Spitz emanates from the midline.

Rhomboid is the only limiting component of the *spitz* group in the ectoderm

The demonstration that the activity of several *spitz* group genes is normally restricted to the midline was surprising, in view of the fact that the known essential components are also expressed in the ectoderm at the early stages of embryogenesis. *spitz* has maternal transcripts, and the zygotic expression is detected in the ectoderm, mesoderm and midline (Rutledge et al., 1992). *Star* expression was detected in the ectoderm and the midline (Kolodkin et al., 1994; Heberlein et al., 1993). Finally, *rho* is expressed in a highly dynamic pattern. The initial zygotic transcription of *rho* is induced by Dorsal and Twist and repressed by Snail. As a result, *rho* transcripts are detected in the neuroectoderm but are excluded from the mesoderm. The expression of *rho* recedes from the neuroectoderm towards the midline, such that by stage 10 only the midline expression is retained (Bier et al., 1990; Nambu et al., 1990; Ip et al., 1992).

We wanted to test whether *spitz, rho* or *Star* are limiting in the ectoderm. Alternatively, another unknown component required for Spitz processing may be absent from the ectoderm. Each of the three genes was expressed ectopically, in the ectoderm of wild-type embryos. No alterations in ventral cell fates were detected following expression of membrane Spitz (Schweitzer et al., 1995b). In the case of *Star*, ubiquitous ectopic expression in *Star* mutant embryos resulted in an almost complete rescue of the ventral defects (Fig. 4F). No aberrant phenotypes were observed following ectopic expression of *Star* in wild-type embryos (not shown). In contrast, ectopic expression of *rho* by ectodermal inducers such as *rho*-Gal4 or *Kr*-Gal4 resulted in an expansion of ventral cell fates, similar to the one observed following ectopic expression of secreted Spitz (Fig. 4A-D). The effects of Rho expression are dependent upon a functional DER pathway and are not observed in *DER/flb* mutant embryos (Fig. 4E). We can therefore conclude that, at the time in which we induced ectopic expression, Rho is the only limiting component in the ectoderm, with respect to the machinery required to regulate and carry out processing of Spitz.

Patterning of the ventral ectoderm can adjust to variable levels of secreted Spitz from the midline

Having shown that the midline is the major source of Spitz activity, we wanted to test the sensitivity of the system to variable levels of secreted Spitz. One way to increase the amount of secreted Spitz in the midline is to induce the expression of the secreted Spitz construct by *sim*-Gal4 in wild-type embryos. Under these conditions, no expansion of ventral cell fates was detected, as monitored by the expression of *otd* (Fig. 5A). Another procedure to increase the level of secreted Spitz in the midline is to examine *snail* mutant embryos, in which the midline was shown to be dramatically expanded at the expense of the mesoderm (Nambu et al., 1990; Leptin, 1991; Rao et al., 1991). Up to 10 rows of the ventralmost cells express midline markers in the blastoderm stage embryo. Eventually these cells delaminate and the neighboring cells develop as neuroectoderm. Again, under these conditions, normal expression of *otd* was monitored (Fig. 5B).

While ectopic expression of secreted Spitz in the ectoderm or mesoderm gave rise to dramatic ventralization of the ectoderm, an increase in the level of secreted Spitz in the midline had no effect. Judging by the level of induction of UAS-*lac*Z by *sim*-Gal4, the activity of *sim*-Gal4 appears comparable to that of the ectodermal or mesodermal Gal4 lines that were used (not shown). We discuss below how Argos, which provides a negative feedback loop for the DER pathway, may be responsible for adjustments to higher levels of secreted Spitz emanating from the midline.

DISCUSSION

The midline is the source of secreted Spitz

This work has shown that the midline is the source of secreted Spitz for activating DER in the ventral ectoderm. In the absence of functional midline cells (in *sim* mutant embryos), ventral cell fates are not induced. It is formally possible that secreted Spitz triggers DER in the midline and the activation of DER induces the production of unknown secreted proteins that will diffuse, relay the signal and activate a different signaling pathway in the ectoderm. However, we believe this is not the case, since there is evidence that activation of DER normally takes place in the ectoderm: expression of *argos* is induced directly by the DER pathway in embryos and cells. *argos* is expressed, in a DER-dependent manner, in the ventralmost ectodermal cells (Golembo et al., 1996; Gabay et al., 1996). We can therefore suggest that, during normal development, after the midline is formed, it serves as a source for producing secreted Spitz, which will diffuse to the ventral ectoderm. Activation of DER, expressed in all ectodermal cells, will pattern the ventral ectoderm. Production of the midline depends upon cues from the Dorsal protein and the early zygotic genes *twist* and *snail* (Nambu et al., 1990; Leptin, 1991; Rao et al., 1991). It does not depend however, on the DER pathway.

The *sim*-dependent function of the midline in terms of patterning the ventral ectoderm seems to be restricted to Spitz processing, since ectopic expression of secreted Spitz is epistatic to the *sim* mutant phenotype. This work has shown that the Spitz precursor, Rho and Star function non-autonomously in patterning the ectoderm. They are normally expressed in the midline and their expression only in the midline is sufficient to rescue the ectodermal defects of the respective mutant embryos. Rho and Star may be required for the production or processing of the Spitz precursor. The same components are required later in embryogenesis for differentiation of the midline itself (Klämbt et al., 1991).

The production of secreted Spitz in the midline may generate a stable source of gradient for the ectoderm. The actual shape of the secreted Spitz gradient may depend upon several factors, such as the stability of the protein, the diffusion rate, dilution of the protein at a distance from its local source and the extent to which the Spitz protein will be trapped by DER on the ectoderm or by the extracellular matrix. Diffusion of Spitz has also been demonstrated in the eye imaginal disc (Freeman, 1994). While this work has shown that Rho and Star are required in the midline, their mode of action in facilitating Spitz processing remains an open question. They appear to play non-overlapping functions in the process, since both seem to be required in the midline. The ability to obtain ventralization following expression of Rho in the mesoderm suggests that Star or a molecule analogous to Star is normally expressed in the mesoderm.

Why processing of Spitz is restricted to the midline is puzzling, in view of the fact that the known components necessary for Spitz processing are also expressed in the ectoderm, at least at the early stages of embryogenesis. Induction of Rho in the ectoderm gave rise to ectopic ventral fates, suggesting that, at the time of induction or later on, it is the only component limiting or missing for effective Spitz processing. It is possible that at the earlier time in which Rho is normally expressed in the ectoderm, its contribution to Spitz processing is marginal. Alternatively, at this stage, the cells may not be competent to process Spitz, or to respond to secreted Spitz which may be produced in the ectoderm.

The expansion of ventral fates that can be obtained by hyperactivation of the DER pathway (by ectopic expression of secreted Spitz or Rho, or by removal of inhibitory molecules such as Argos), never exceeds 8-10 cell rows on each side of the midline. This result does not necessarily reflect the limits of diffusion of secreted Spitz or the cells in which components of the DER pathway are present, but rather the normal temporal distinction between the establishment of dorsal and ventral fates in the embryo. At 2.5 hours of embryogenesis, the Dpp pathway is triggered in the dorsal ectoderm, and the ventral ectoderm remains naive due to the inhibitory activity of Short gastrulation (Francois et al., 1994). Only later on, presumably after the processing machinery of Spitz begins to function in the midline, does the DER pathway pattern the ventral ectoderm (Raz and Shilo, 1993). Thus, ectopic activation of DER can alter only the fate of cells in the ventral and lateral ectoderm, which have not been previously patterned by Dpp. Under such conditions, the dorsal borders of lateral markers like *disco* retain their normal position (Fig. 4D).

Adjustments to variable levels of secreted Spitz

The ventral ectoderm is exquisitely sensitive to ectopic expression of secreted Spitz when it is provided uniformly, either in the ectoderm or from the mesoderm. Yet, no alterations in patterning were detected when the levels of secreted Spitz were elevated in the midline. We believe that these observations have a profound implication on the way in which graded signaling of the DER pathway is maintained. A central consideration in the regulation of DER activation is not only how the pathway is triggered, but also how signaling is terminated. Graded activation of DER generates a gradient of enzymatic activity of the DER tyrosine kinase and the cytoplasmic kinases triggered by it. If allowed to function over extended time periods, the consequences of DER activation would be similar in all ectodermal cells, regardless of the initial activation levels. Therefore, a special mechanism has evolved in order to define a sharp time window of signaling and to preserve the effects of graded DER activation.

Argos is a secreted polypeptide containing a single EGF repeat (Freeman et al., 1992b), which was shown to inhibit the capacity of Spitz to trigger DER autophosphorylation (Schweitzer et al., 1995a). The mechanism of Argos activity has been highlighted by the observation that transcription of *argos* is induced by the DER pathway (Golembo et al., 1996; Gabay et al., 1996). Thus, activation of DER triggers the

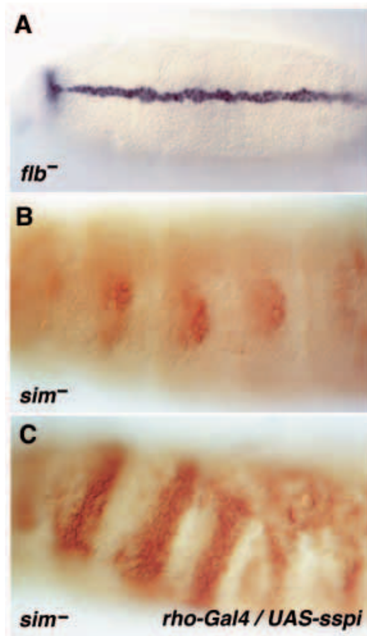

Fig. 3. Secreted Spitz is epistatic to the *sim* mutant phenotype. (A) Midline development does not depend on the DER pathway, as monitored by the expression of *sim-lac*Z in flb^{1F26} mutant embryos. (B) sim^{H9} mutant embryos show significantly diminished expression of FasIII. (C) Induction of UAS-*secreted spitz* by *rho*-Gal4 in the entire neuroectoderm of sim^{H9} mutant embryos is epistatic and results in expanded expression of FasIII. Note: due to the stability of Gal4, the early expression of *rho* in the entire neuroectoderm is retained until stage 12.

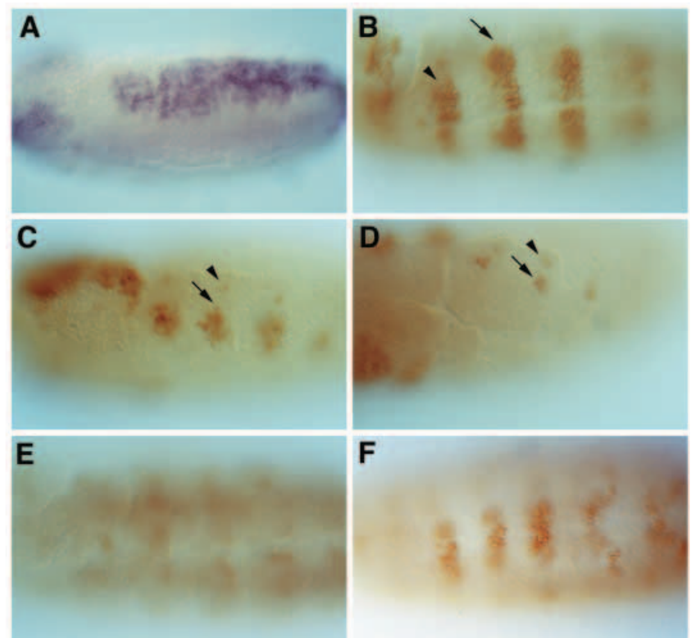

Fig. 4. Ectopic Rho in the ectoderm induces ventralization. (A) Induction of UAS-*rho* expression by *rho*-Gal4 results in expanded expression of *otd*. (B) Induction of UAS-*rho* expression by *Kr*-Gal4 leads to expanded expression of FasIII in parasegments T2 (arrow) and T3, but not in T1 (arrowhead). (C) An enhancer trap in the *disco* locus is expressed in the lateral ectodermal cells. The dorsal border of the *disco* patch is shown by an arrow, and a more dorsal dot expressing *disco* by an arrowhead. (D) Induction of *rho* expression by *rho*-Gal4 results in expansion of the ventral fates at the expense of lateral fates. The dorsal borders of *disco* expression are retained, however (arrow and arrowhead). (E) Induction of ectopic ventral fates by the expression of *rho* in the ectoderm is dependent upon a functional DER pathway. When carried out in flb^{1F26} mutant embryos, no expression of FasIII is detected. (F) The *Star* mutant ectodermal phenotype is rescued by ubiquitous induction of UAS-*Star* expression by the *69B*-Gal4 construct. No ectopic cell fates are induced by ectopic *S* expression, either in wild-type (not shown) or in mutant *S* embryos.

expression of Argos, which is responsible in turn for shutting off the pathway. The effects of Argos are physiologically important not for the cells in which it is produced, but rather in the neighboring cells, which do not express it. Argos thus functions as a signal of lateral inhibition, to restrict the duration or level of DER signaling in the neighboring cells and preserve the graded effects of DER signaling.

We suggest that the inhibitory feedback loop of Argos may account for our observations that, even when higher levels of secreted Spitz are emanating from the midline, normal patterning of the ventral ectoderm is retained. Under these conditions, the ectodermal cells closest to the midline will experience the highest level of DER activation. These cells will subsequently induce Argos, which will shut off signaling in the more lateral cells. As long as the midline is the only source of secreted Spitz, the Argos feedback mechanism is retained even when the absolute levels of secreted Spitz are altered. Thus, the system is extremely flexible in terms of adjusting to variable levels of secreted Spitz from the midline. The situation is completely different if all ectodermal cells are simultaneously encountering secreted Spitz (produced either in the ectoderm or mesoderm). Although Argos will be induced by all ectodermal cells (Golembo et al., 1996), by the time it is made the cells producing it are ventralized and refractive to Argos inhibition. Fig. 6 summarizes the model for Spitz processing in the midline and the role of Argos in retaining the effects of the secreted Spitz gradient.

Similar mechanism of DER activation in other tissues

The DER pathway is extremely pleiotropic, affecting a multitude of crucial developmental decisions. In most of these cases, the same DER signaling 'cassette' is used. It is interesting to examine whether the non-autonomous effects of DER ligands and their processing that were described in this work, also apply to other tissues.

In the ovary, the DER pathway is responsible for patterning both posterior and dorsal follicle cells (González-Reyes et al., 1995; Roth et al., 1995; Price et al., 1989). In this case, DER is triggered by Gurken, another TGFα homologue (Neuman-Silberberg and Schüpbach, 1993). *gurken* is expressed only in

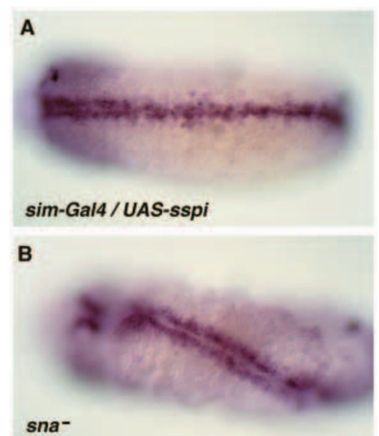

Fig. 5. Patterning of the ventral ectoderm is retained after elevating secreted Spitz levels in the midline. No alterations in the normal pattern of *otd* expression were monitored following an increase in the level of secreted Spitz emanating from the midline. (A) Induction of UAS-*secreted Spitz* by *sim*-Gal4 in wild-type embryos. (B) *snail* mutant embryos in which the midline is expanded at the expense of the mesoderm.

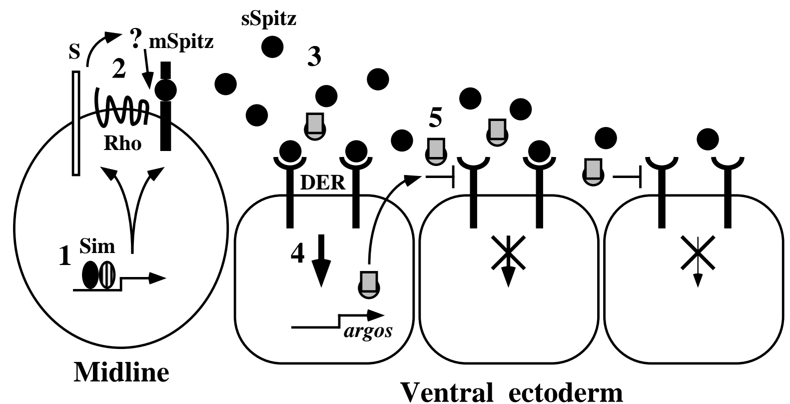

Fig. 6. A model for establishment and maintenance of graded DER activation in the ectoderm. Secreted Spitz is required to activate DER and pattern the ventral ectoderm. The major, if not the exclusive source of secreted Spitz is the midline. In these cells, the expression of *spitz*, *rho* and *Star* depends directly or indirectly on Sim (1), and provides the processing apparatus of the Spitz precursor (2). Secreted Spitz diffuses from the midline, and forms a gradient that is responsible for graded activation of DER in the ectoderm (3). In the cells closest to the midline, the highest level of DER activation will take place. Consequently, expression of *argos* will be induced (4). Diffusion of Argos to the neighboring ectodermal cells will terminate or reduce DER signaling, (5) and thus preserve the graded effects of DER activation. The induction of an inhibitory feedback loop provides a mechanism of adjustment to variable levels of secreted Spitz, as long as they are emanating from the midline.

the germ cells and the transcript is localized to the oocyte nucleus. Gurken triggers DER in the follicle cells, thus acting in a non-autonomous manner. However, it is not known whether Gurken functions as a secreted or a membrane-anchored molecule, and how the expression of *rho* in the dorsal-anterior follicle cells is involved in DER signaling (Ruohola-Baker et al., 1993).

In the eye imaginal disc, Rho and Star are expressed in the same cells, namely R8, R2 and R5 (Freeman et al., 1992a; Kolodkin et al., 1994; Heberlein et al., 1993). These are the same cells in which Spitz is predominantly required (Freeman, 1994; Tio et al., 1994). Furthermore, the capacity of Spitz to diffuse has been demonstrated in this tissue (Freeman, 1994; Tio et al., 1994). Homozygous *Star* patches in the eye contain no R cells (Kolodkin et al., 1994). While loss of *rho* has only a very subtle phenotype in the eye, misexpression of Rho does induce hyperdifferentiation of cells (Freeman et al., 1992a; Freeman, 1994). Finally, Argos is produced by the differentiated cells in the eye disc, and has the capacity to diffuse. Reduction in Argos activity leads to hyperdifferentiation (Freeman et al., 1992b).

In the wing, the DER pathway is responsible for inducing both vein and intervein cell fates (Diaz-Benjumea and Garcia-Bellido, 1990; Diaz-Benjumea and Hafen, 1994). While *DER* is uniformly expressed, the expression of *rho* is restricted to the vein precursors (Sturtevant et al., 1994). Thus, processing of Spitz could be restricted to the veins, where Rho is produced, and provide a source of secreted Spitz gradient to the inter-vein regions. It is interesting to note that *argos* is also expressed in the vein cells (Sawamoto et al., 1994), presumably as a result of induction by high levels of activation of the DER pathway. Reduction in the level of Argos in the wing leads to expansion of the vein region (Sawamoto et al., 1994; Schweitzer et al., 1995a), again demonstrating that Argos is responsible for retaining the graded effects of DER signaling. The regulation of the DER pathway in the wing and ventral ectoderm appear to be highly similar. The major difference is that, in the wing disc, the cells expressing *rho* which are the likely source of the processed ligand, are induced by the DER pathway to become veins. Therefore, they are also the cells in which *argos* expression is observed. In the embryo, the midline which produces the secreted ligand is not affected by the DER pathway at the early stages, and the cells expressing *argos* are therefore the ventralmost ectodermal cells, in which DER activation is maximal.

The analogies between patterning the ventral ectoderm of *Drosophila* by the DER pathway and the induction of vulval cell fates by the EGF receptor in *C. elegans* are striking. This work demonstrated that, in addition to the conserved machinery for activating the receptor and terminating signaling (see Golembo et al., 1996), the mechanisms for generating a graded source of the ligand are also similar. The *C. elegans* Lin-3, a TGF-α homologue, is produced only in the anchor cell (Hill and Sternberg, 1992). Processing of Lin-3 to generate a secreted molecule generates a localized source of ligand, which will induce Let-23, the EGF receptor, in the vulval precursor cells. The distance of a given vulval precursor cell from the anchor cell will dictate the amount of ligand it encounters, and the respective cell fate it will adopt, accordingly (Katz et al., 1995). In the *Drosophila* ventral ectoderm, processing of the ligand, Spitz, is restricted to the midline cells. This creates a stable gradient of secreted Spitz, which will trigger DER in the ectoderm and may induce graded cell fates accordingly.

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REFERENCES

Baker, R. and Schubiger, G. (1996). Autonomous and nonautonomous Notch functions for embryonic muscle and epidermis development in *Drosophila*. *Development* **122**, 617-626.

Bier, E., Jan, L. Y. and Jan, Y. N. (1990). *rhomboid*, a gene required for dorsoventral axis establishment and peripheral nervous system development in *Drosophila melanogaster*. *Genes Dev.* **4**, 190-203.

Brand, A. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Chasan, R. and Anderson, K. V. (1993). Maternal control of dorsal-ventral polarity and pattern in the embryo. In *The Development of Drosophila*

melanogaster (ed. M. Bate and A. Martinez-Arias), pp. 387-424. Cold Spring Harbor: Cold Spring Harbor Press.
Diaz-Benjumea, F. J. and Garcia-Bellido, A. (1990). Behaviour of cells mutant for an EGF receptor homologue of *Drosophila* in genetic mosaics. *Proc. R. Soc. Lond B. Biol. Sci*. **242**, 36-44.
Diaz-Benjumea, F. J. and Hafen, E. (1994). The sevenless signalling cassette mediates *Drosophila* EGF receptor function during epidermal development. *Development* **120**, 569-578.
Francois, V., Solloway, M., O'Neill, J. W., Emery, J. and Bier, E. (1994). Dorsal-ventral patterning of the *Drosophila* embryo depends on a putative negative growth factor encoded by the *short gastrulation* gene. *Genes Dev.* **8**, 2602-2616.
Freeman, M. (1994). The *spitz* gene is required for photoreceptor determination in the *Drosophila* eye where it interacts with the EGF receptor. *Mech. Dev.* **48**, 25-33.
Freeman, M., Kimmel, B. E. and Rubin, G. M. (1992a). Identifying targets of the *rough* homeobox gene of *Drosophila*: evidence that *rhomboid* functions in eye development. *Development* **116**, 335-346.
Freeman, M., Klämbt, C., Goodman, C. S. and Rubin, G. M. (1992b). The *argos* gene encodes a diffusible factor that regulates cell fate decisions in the Drosophila eye. *Cell* **69**, 963-975.
Gabay, L., Scholz, H., Golembo, M., Klaes, A., Shilo, B.-Z. and Klämbt, C. (1996). EGF receptor signaling induces *pointed P1* transcription and inactivates Yan protein in the embryonic ventral ectoderm. *Development* **122**, 3357-3360.
Golembo, M., Schweitzer, R., Freeman, M. and Shilo, B.-Z. (1996). Argos transcription is induced by the *Drosophila* EGF receptor pathway, to form an inhibitory feedback loop. *Development* **122**, 223-230.
González-Reyes, A., Elliot, H. and St. Johnston, D. (1995). Polarization of both major body axes in *Drosophila* by *gurken-torpedo* signalling. *Nature* **375**, 654-658.
Heberlein, U., Hariharan, I. K. and Rubin, G. M. (1993). Star is required for neuronal differentiation in the *Drosophila* retina and displays dosage-sensitive interactions with Ras1. *Dev. Biol*. **160**, 51-63.
Heemskerk, J. and DiNardo, S. (1994). Drosophila hedgehog acts as a morphogen in cellular patterning. *Cell* **76**, 449-460.
Hill, R. J. and Sternberg, P. W. (1992). The gene *lin*-3 encodes an inductive signal for vulval development in *C. elegans*. *Nature* **358**, 470-476.
Ip, Y. T., Park, R. E., Kosman, D., Bier, E. and Levine, M. (1992). The dorsal gradient morphogen regulates stripes of *rhomboid* expression in the presumptive neuroectoderm of the *Drosophila* embryo. *Genes Dev*. **6**, 1728-1739.
Katz, W. S., Hill, R. J., Clandinin, T. R. and Sternberg, P. W. (1995). Different levels of the C. elegans growth factor LIN-3 promote distinct vulval precursor fates. *Cell* **82**, 297-307.
Kim, S. H. and Crews, S. T. (1993). Influence of *Drosophila* ventral epidermal development by the CNS midline cells and *spitz* class genes. *Development* **118**, 893-901.
Klämbt, C., Jacobs, J. R. and Goodman, C. S. (1991). The midline of the Drosophila central nervous system: a model for the genetic analysis of cell fate, cell migration, and growth cone guidance. *Cell* **64**, 801-815.
Kolodkin, A. L., Pickup, A. T., Lin, D. M., Goodman, C. S. and Banerjee, U. (1994). Characterization of *Star* and its interactions with *sevenless* and *EGF receptor* during photoreceptor cell development in *Drosophila*. *Development* **120**, 1731-1745.
Lecuit, T., Brook, W. J., Ng, M., Callajea, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing. *Nature* **381,** 387-393.
Leptin, M. (1991). *twist* and *snail* as positive and negative regulators during *Drosophila* mesoderm development. *Genes Dev.* **5**, 1568-1576.
Mayer, U. and Nüsslein-Volhard, C. (1988). A group of genes required for pattern formation in the ventral ectoderm of the *Drosophila* embryo. *Genes Dev.* **2**, 1496-1511.
Nambu, J. R., Lewis, J. O., Wharton, J. K. A. and Crews, S. T. (1991). The Drosophila *single-minded* gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell* **67**, 1157-1167.
Nambu, J. R., Franks, R. G., Hu, S. and Crews, S. T. (1990). The *single-minded* gene of Drosophila is required for the expression of genes important for the development of CNS midline cells. *Cell* **63**, 63-75.
Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85,** 357-368.
Neuman-Silberberg, F. S. and Schüpbach, T. (1993). The Drosophila dorsoventral patterning gene *gurken* produces a dorsally localized RNA and encodes a TGF alpha-like protein. *Cell* **75**, 165-174.
Price, J. V., Clifford, R. J. and Schüpbach, T. (1989). The maternal ventralizing locus *torpedo* is allelic to *faint little ball*, an embryonic lethal, and encodes the Drosophila EGF receptor homolog. *Cell* **56**, 1085-1092.
Rao, Y., Vaessin, H., Jan, L. Y. and Jan, Y. N. (1991). Neuroectoderm in *Drosophila* embryos is dependent on the mesoderm for positioning but not for formation. *Genes Dev.* **5**, 1577-1588.
Raz, E. and Shilo, B. Z. (1993). Establishment of ventral cell fates in the *Drosophil*a embryonic ectoderm requires DER, the EGF receptor homolog. *Genes Dev.* **7**, 1937-1948.
Roth, S., Neuman-Silberberg, F. S., Barcelo, G. and Schüpbach, T. (1995). *cornichon* and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in Drosophila. *Cell* **81**, 967-978.
Ruohola-Baker, H., Grell, E., Chou, T. B., Baker, D., Jan, L. Y. and Jan, Y. N. (1993). Spatially localized *rhomboid* is required for establishment of the dorsal-ventral axis in Drosophila oogenesis. *Cell* **73**, 953-965.
Rutledge, B. J., Zhang, K., Bier, E., Jan, Y. N. and Perrimon, N. (1992). The *Drosophila spitz* gene encodes a putative EGF-like growth factor involved in dorsal-ventral axis formation and neurogenesis. *Genes Dev.* **6**, 1503-1517.
Sawamoto, K., Okano, H., Kobayakawa, Y., Hayashi, S., Mikoshiba, K. and Tanimura, T. (1994). The function of *argos* in regulating cell fate decisions during *Drosophila* eye and wing vein development. *Dev. Biol*. **164**, 267-276.
Schweitzer, R., Howes, R., Smith, R., Shilo, B. Z. and Freeman, M. (1995a). Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos. *Nature* **376**, 699-702.
Schweitzer, R., Shaharabany, M., Seger, R. and Shilo, B. Z. (1995b). Secreted spitz triggers the DER signaling pathway and is a limiting component in embryonic ventral ectoderm determination. *Genes Dev.* **9**, 1518-1529.
Sturtevant, M. A., O'Neill, J. W. and Bier, E. (1994). Down-regulation of *Drosophila Egf-r* mRNA levels following hyperactivated receptor signaling. *Development* **120**, 2593-600.
Sturtevant, M. A., Roark, M. and Bier, E. (1993). The *Drosophila rhomboid* gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. *Genes Dev.* **7**, 961-973.
Tio, M., Ma, C. and Moses, K. (1994). *spitz*, a *Drosophila* homolog of transforming growth factor-alpha, is required in the founding photoreceptor cells of the compound eye facets. *Mech. Dev.* **48**, 13-23.
Wieschaus, E., Perrimon, N. and Finkelstein, R. (1992). *orthodenticle* activity is required for the development of medial structures in the larval and adult epidermis of *Drosophila*. *Development* **115**, 801-811.

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