

The CNS midline cells control the *spitz* class and *Egfr* signaling genes to establish the proper cell fate of the *Drosophila* ventral neuroectoderm

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ABSTRACT The *spitz* class genes, *pointed (pnt)*, *rhuboid (rho)*, *single-minded (sim)*, *spitz (spi)* and *Star (S)*, as well as the *Drosophila* epidermal growth factor receptor (*Egfr*) signaling genes, *argos (aos)*, *Egfr*, *orthodenticle (otd)* and *vein (vn)*, are required for the proper establishment of ventral neuroectodermal cell fate. The roles of the CNS midline cells, *spitz* class and *Egfr* signaling genes in cell fate determination of the ventral neuroectoderm were determined by analyzing the spatial and temporal expression patterns of each individual gene in *spitz* class and *Egfr* signaling mutants. This analysis showed that the expression of all the *spitz* class and *Egfr* signaling genes is affected by the *sim* gene, which indicates that *sim* acts upstream of all the *spitz* class and *Egfr* signaling genes. It was shown that overexpression of *sim* in midline cells fails to induce the ectodermal fate in the *spi* and *Egfr* mutants. On the other hand, overexpression of *spi* and *Draf* causes ectopic expression of the neuroectodermal markers in the *sim* mutant. Ectopic expression of *sim* in the *en*-positive cells induces the expression of downstream genes such as *otd*, *pnt*, *rho*, and *vn*, which clearly demonstrates that the *sim* gene activates the EGFR signaling pathway and that CNS midline cells, specified by *sim*, provide sufficient positional information for the establishment of ventral neuroectodermal fate. These results reveal that the CNS midline cells are one of the key regulators for the proper patterning of the ventral neuroectoderm by controlling EGFR activity through the regulation of the expression of *spitz* class genes and *Egfr* signaling genes.

KEY WORDS: *Drosophila* ventral neuroectoderm, CNS midline cells, *spitz* class genes, *Egfr* signaling genes, cell fate determination

Introduction

How an egg is diversified into a multicellular organism is one of the fundamental questions in developmental biology. Since *Drosophila* is complex enough to serve as a model for understanding the molecular mechanisms of vertebrate development and is amenable to sophisticated genetic analysis, this study aims to understand molecular and cellular basis of the patterning of the *Drosophila* ventral neuroectoderm along the dorsoventral axis.

Dorsoventral patterning of the *Drosophila* embryo is initiated by the maternal regulatory transcription factor, *dorsal (dl)* (Roth *et al.*, 1989). Gradient activity of *dl* from the ventral to the dorsal region, established by the *spätzle/Toll* signaling pathway, subdivides the ventral region into three distinct subdivisions: mesoderm, ventral neuroectoderm and dorsal epidermis (Fig. 1; Rusch and Levine, 1996). After gastrulation, the establishment of neural diversity begins with the formation of potential neuronal clusters from the ventral neuroectoderm by the positional cues from several seg-

ment polarity and dorsal-ventral genes (Goodman and Doe, 1993; Baht, 1998; Cornell and Von Ohlen, 2000). A single cell, selected from the neuroectodermal cluster, delaminates inward to be a neuroblast, and the cells at the external surface develop into the epidermis. This selection procedure is performed by the proneural and neurogenic genes (Campos-Ortega, 1993). The proneural genes are expressed as a cluster of six to eight neuroectodermal cells, and then their expression remains at the singled-out cell to form the enlarged neuroblast (Campuzano and Modolell, 1992). The neuroblast, formed by the proneural genes, prevents the other cells within a cluster from becoming neuroblasts by lateral inhibition mediated by the neurogenic genes (Lehmann *et al.*, 1983). During germ band extension, about 30 neuroblasts in each hemisegment generate 350 embryonic neurons and 30 glial cells (Schmidt *et al.*, 1997; Schmid *et al.*, 1999). These neurons develop

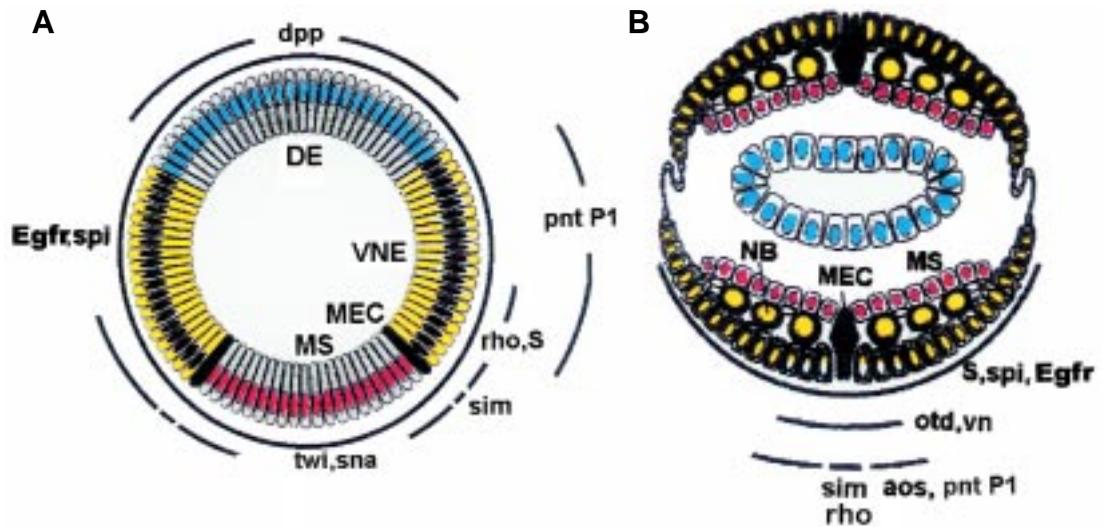
Abbreviations used in this paper: CNS, central nervous system; EGFR, Epidermal Growth Factor Receptor; MAPK, Mitogen Activated Protein Kinase.

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Fig. 1. A schematic diagram of the development of the ventral neuroectoderm, and the expression of the *spitz* class and *Egfr* signaling genes in the ventral neuroectoderm during two successive developmental stages.

(A) During the cellular blastoderm stage, the highest *dpp* gradient activity defines the dorsal epidermis (DE). The mesectodermal cells (MEC), shown in black, separate the presumptive mesoderm (MS), specified by *sna* and *twi*, from the ventral neuroectoderm (VNE) that is defined by *rho*, *pnt P1*, *S* and *vn*. *sim* is expressed in the mesectoderm

(MEC), and *spi* and *Egfr* are expressed in the entire ectoderm. **(B)** During germband elongation, the MEC becomes juxtaposed at the ventral midline and delaminates into the neuroblast layer. *rho* expression becomes restricted to the midline. *aos* and *pnt P1* are expressed in the ventral-most ectoderm, and *otd* and *vn* are expressed in both the midline and ventral-most ectoderm. Dorsal is up and sectional views are shown.



into the ventral nerve cord and subesophageal ganglion (Goodman and Doe, 1993; Campos-Ortega and Hartenstein, 1997).

Although cell fate determination of the ventral neuroectoderm along the anteroposterior axis is relatively well known, cellular and molecular basis of the cell fate determination along the dorsoventral axis has not yet been clearly revealed. A group of the genes, involved in the cell fate determination along the dorsoventral axis, are the *spitz* class genes. They are expressed in the appropriate regions of the ventral neuroectoderm before and during neurogenesis, as shown in Fig. 1 and known to be required for the proper development of the ventral neuroectoderm (Rusch and Levine, 1996). The *spitz* class genes were found by the phenotypic similarity of mutants: loss of the denticle belts in the ventral-most region and the defects in the ventral part of the CNS (Mayer and Nüsslein-Volhard, 1988). The *spitz* class genes include *pnt*, *rho*, *sim*, *S*, *spi* and *sichel* (*sic*). *Egfr* signaling genes, *aos*, *Egfr*, *otd*, and *vn*, together with the *spitz* class genes, are also required for the fate determination of the ventral neuroectodermal cells through the differential activation of the EGFR signaling pathway (Freeman, 1997; Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997; Skeath, 1998; Udolph et al., 1998; Yagi et al., 1998). It is known that *Egfr* is required for the establishment of the ventral neuroectodermal cell fate via the graded activation, which is regulated by the processing of the ligand Spi by transmembrane proteins, Rho and S, and the CNS midline cells serve as a signaling center for generating Spi ligand (Golembo et al., 1996). *aos* and *vn* encode additional inhibitory and weakly activating ligands for *Egfr*, respectively. Based on the study which shows that activation of the *Egfr* is triggered by Spi in the ventral-most ectoderm, and that this activation can be inhibited by Aos in the lateral ectoderm (Schweitzer et al., 1995b; Gabay et al., 1996), it was proposed that Aos negatively regulates EGFR signaling to generate the EGFR activity gradient in the ventral neuroectoderm. It was also shown that *vn* is induced by the Spi/EGFR signaling pathway to provide a weakly activating ligand for the cell fate determination of the lateral neuroectoderm (Golembo et al., 1999; Wessells et al., 1999).

It was demonstrated that *sim* is required for differentiation of the midline cells and regulates many genes which are necessary for the development of the CNS midline cells (reviewed by Crews, 1998). The *sim* mutant shows not only the loss of all neural and glial cells in the midline, but also the lack of expression of the ventral epidermal, NB, and lateral neuronal markers, though the *sim* gene is expressed only in the midline and mesodermal cells (Kim and Crews, 1993; Raz and Shilo, 1993; Kim, 1995; Gabay et al., 1996; Golembo et al., 1996; Lürer et al., 1997; Menne et al., 1997; Zhou et al., 1997; Lee et al., 1999; Yu et al., 1999; Chang et al., 2000). Extensive studies on the role of *sim* in the fate determination of the ventral neuroectoderm proposed that *sim* promotes the generation of the EGFR activity gradient in the ventral neuroectoderm by negative and positive feedback cycles that are driven by a negative ligand, Aos, and positive ligands, Spi and Vn. Despite comprehensive studies on the roles of the *sim*, *spitz* class, and *Egfr* signaling genes in the cell fate determination of the ventral neuroectoderm, it is not clearly established how the CNS midline cells, specified by the *sim* gene, influence the expression of each individual *spitz* class and *Egfr* signaling genes to determine the proper cell fate of the ventral neuroectoderm. In addition, the hierarchical relationship among the *sim*, *spitz* class and *Egfr* signaling genes in the proper cell fate determination of the ventral neuroectoderm has not been clearly elucidated.

In this study, the roles of the CNS midline cells in the fate determination of the ventral neuroectoderm were investigated by analyzing the ventral neuroectodermal markers in the *sim* mutant. This analysis showed that the *sim* gene acts upstream of all the *spitz* class and the *Egfr* signaling genes. It was shown that *sim* induces the ventral neuroectodermal fate by activating the EGFR signaling pathway which involves the *spitz* class and *Egfr* signaling genes. Ectopic expression of *sim* in the *en*-positive cells induces the downstream genes such as *otd*, *pnt*, *rho* and *vn*. These results demonstrate that the CNS midline cells are one of the key regulators for the proper patterning of the ventral neuroectoderm by regulating the *Egfr* activity through the *spitz* class and *Egfr* signaling genes.

Results

sim acts upstream of all the *spitz* class and *Egfr* signaling genes in the fate determination of the ventral neuroectoderm

sim is known to be a master regulator in the proper patterning of the ventral neuroectoderm and CNS midline cells (reviewed by Crews, 1998). Considering the observation that the CNS midline cells, specified by *sim*, contact with the ventral neuroectoderm, mesoderm and later with the ventral neuroblasts and epidermis, ventral neuroectodermal and mesodermal cells near the midline cells may interact with the midline progenitors through cell-cell interaction. These findings suggest that *sim* acts early to regulate many genes which are required for the proper patterning of the ventral neuroectoderm.

To elucidate the hierarchical relationship of the *sim* gene in relation to the other *spitz* class and *Egfr* signaling genes in the proper patterning of the ventral neuroectoderm, the expression of *spitz* class genes was analyzed in the *hs-sim* and *sim* mutants by *in situ* RNA hybridization. Since *rho* is known to be a target gene of *sim* (Nambu *et al.*, 1990), the expression of *rho* in the midline (Fig. 2J) is abolished in the *sim* mutant at stage 11 (Fig. 2K). Conversely, it is greatly expanded to 10-12 cells in the ventral region of the *hs-sim* mutant (Fig. 2L). Like the *rho* gene, the ectodermal expression of *otd*, *pnt*, *vn*, and *aos* genes (Fig. 2 D,G,M,P) is drastically reduced in the *sim* mutant (Fig. 2 E,H,N,Q), and their expression is expanded by overexpression of the *sim* gene (Fig. 2 F,I,O,R). In addition, the expression level of *spi* in the neuroblast layer and ectoderm of each segment is much reduced in the *sim* mutant embryos at stages 9 (Fig. 3C,D) and 11 (Fig. 3 G,H), compared to that in the intersegmental ectoderm of the wild-type embryos (Fig. 3 A,B,E,F). However, *en* expression level of wild-type and *sim* mutant embryos remains almost the same. On the other hand, the expression of the *sim* gene is not affected in the other *spitz* class mutants such as the *spi* and *otd* mutants (Fig. 2 A-C). These data indicate that *sim* directly or indirectly regulates the following *spitz* class and *Egfr* signaling genes: *aos*, *otd*, *pnt*, *rho*, *spi*, and *vn*.

S and *spi* regulate the proper expression of *rho* gene in the ventral neuroectoderm

rho expression appears in two longitudinal ventral domains of the neuroectoderm at cellular blastoderm stage (Bier *et al.*,

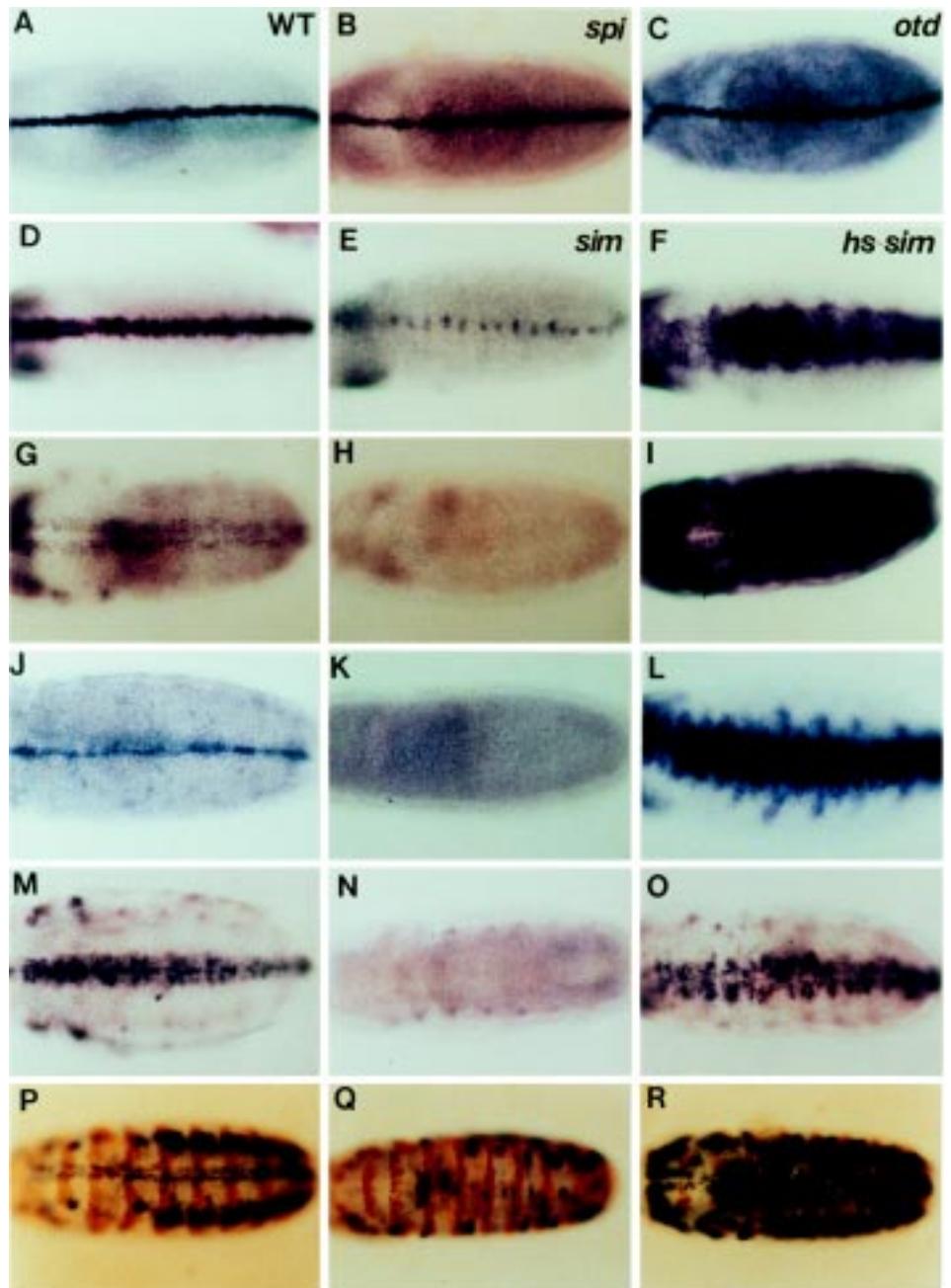


Fig. 2. The *sim* positively regulates the expression of the *spitz* class and *Egfr* signaling genes.

The expression of *sim* in wild-type embryos (A) is not altered in *spi* (B) and *otd* (C) mutant embryos. The expression of *otd* (D-F), *pnt* (G-I), *rho* (J-L), *vn* (M-O), and *aos* (P-R) is abolished in the *sim* mutant (E,H,K,N,Q) and expanded in the *hs-sim* mutant (F,I,L,O,R) at stage 11. This result indicates that *sim* acts upstream of these genes. In all panels, anterior is to the left, and the ventral view is shown.

1990). Slightly later, *rho* expression becomes narrower and delimited by a sharp ventral boundary with a periodically repeated pattern along the anteroposterior axis (Fig. 4 A,D). During gastrulation, *rho* expression becomes restricted in a single stripe in either side of the midline, and two stripes are juxtaposed at the midline as the ventral furrows close (Fig. 4 G,J). To determine the relationship of *rho* to the other *spitz* class genes, *rho* expression was analyzed in the *spitz* class

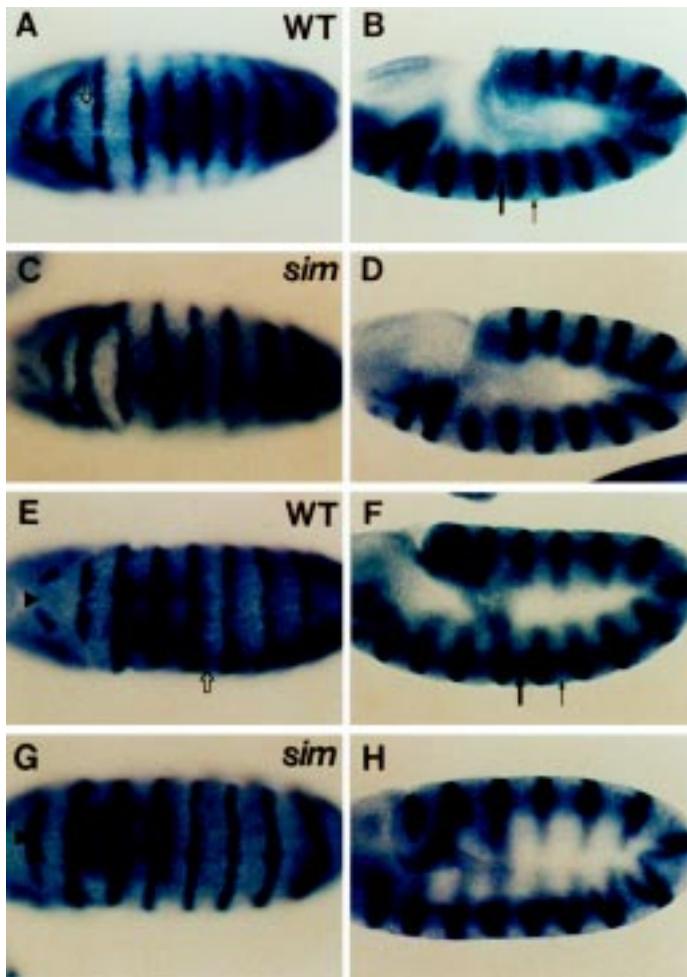


Fig. 3. *spi* expression in the ventral neuroectoderm is regulated by *sim*. (A–D) *spi* expression at stage 9. (E–H) *spi* expression at stage 11. *spi* expression in the ventral ectoderm (empty arrows) of wild-type embryo at stage 9 (A) and stage 11 (E) is almost abolished in the *sim* embryo (C, G). *spi* expression in the sagittal NB layer (thick arrows) and ectoderm (thin arrows) of wild-type embryos at stage 9 (B) and stage 11 (F) is much reduced in *sim* embryos (D, H). (A, C, E, G) shows the ventral view with the ventral midline marked with an open arrowhead. (B, D, F, H) shows the sagittal view of embryos with anterior to the left.

mutants. In *S* and *spi* mutants at stage 5, *rho* expression becomes much reduced, fails to show sharp ventral boundary (Fig. 4 B,C) and remains discontinuous till stage 11 (Fig. 4 H,I), whereas it is not affected in the *pnt* as well as in *aos* and *otd* mutants (data not shown). In contrast, *rho* expression in the ventral neuroectoderm expands along the anteroposterior axis in the gain-of-function strains, *sca-Gal4/UAS-S* and *sca-Gal4/UAS-spi* (Fig. 4 E,F) during the blastoderm stage. At stage 11, *rho* expression in the midline cells of wild-type embryos (Fig. 4 G,J) increases after *S* and *spi* were overexpressed in the entire neuroectoderm of *sca-Gal4/UAS-S* and *sca-Gal4/UAS-spi* embryos (Fig. 4 K,L). These results indicate that the *S* and *spi* play important roles in the proper expression of the *rho* gene in the ventral neuroectoderm and mesectoderm before and after gastrulation, which indicates that the *rho* gene expression is regulated at transcriptional level by the *S* and *spi* genes.

The *vn* gene belongs to the final EGFR signaling target genes that are activated by *pnt*

To elucidate the role of the *vn* gene in the fate determination of the ventral neuroectoderm, the expression of *vn* was examined in the *spitz* class and *Egfr* signaling mutants. *vn* is expressed in the two longitudinal stripes of the ventral-most ectodermal cells on either side of the CNS midline cells at stage 11 (Fig. 5A). The expression of *vn* in the ventral-most neuroectoderm is abolished in the *Egfr* signaling and *spitz* class mutants (Fig. 5 B–F). On the other hand, *vn* expression in the ventral-most neuroectoderm expands in the *aos*, *hs-Draf*, *hs-S* mutants and when *pnt P1*, which is expressed in the ventral neuroectoderm, was overexpressed in the mesectoderm and ventral-most ectoderm by *twi-Gal4* lines (Fig. 5 G–J). These results indicate that the *otd* and *vn* genes are positively regulated by the EGFR signaling pathway involving the *spitz* class genes and are the lowest downstream targets of *spitz* class genes. In order to investigate the role of the *vn* gene in patterning of the ventral neuroectoderm, an epidermal marker, BP28, was employed. β -galactosidase expression of the wild-type BP28 at stage 15 (Fig. 5K) is absent in the lateral ectodermal cells and is occasionally disrupted in the ventral-most ectodermal cells of *vn* embryos (Fig. 5L). This result indicates that *vn* plays a more important role in the proper patterning of the lateral ectodermal cells than in patterning of the ventral-most cells.

***sim* induces ventral neuroectodermal fate by activation of the EGFR signaling pathway**

In order to demonstrate clearly that *sim* activates the EGFR signaling pathway which involves the *spitz* class and *Egfr* signaling genes to induce the ventral neuroectodermal fate, *otd* expression was examined after *spi* and *Egfr* mutants were crossed with the *hs-sim* mutant. In wild-type embryos at stage 11, *otd* expression in 1–2 stripes of the ventral-most ectodermal cells (Fig. 6A) is abolished in 23% of the *Egfr^{flb} hs-sim* and *spi hs-sim* embryos (Fig. 6 C,D), which is the similar phenotype to that of the *sim* mutant (Fig. 6B). This result indicates that the Spi/EGFR signaling pathway is required to activate the *otd* expression in the ventral-most neuroectodermal cells by the CNS midline cells. To confirm that activated EGFR signaling, triggered by the CNS midline cells, is sufficient to induce the ventral neuroectodermal fate, *spi* and *Draf* were overexpressed in the *sim* mutant background. *otd* expression is recovered in all the *sim* mutant embryos that harbor the *hs-spi* (Fig. 6 E,F) or *hs-Draf* (Fig. 6 G,H), which can be identified by the absence of Sim protein with anti-Sim antibody staining. Finally, active MAPK was detected by the anti-diphospho-MAPK antibody to demonstrate whether the CNS midline cells activate the MAPK in the EGFR signaling pathway. Active MAPK in the ventral-most neuroectoderm of wild-type embryos at stage 11 (Fig. 6I) is absent in *sim* embryos, although active MAPK remains in the trachea (Fig. 6J).

Taken together, these results demonstrate clearly that *sim* activates the Spi ligand to trigger the EGFR signaling pathway in the ventral neuroectoderm, and that the activated *Draf* carries out the downstream events such as the activation of the MAPK to induce the ventral-most ectodermal fate.

***sim* induces ectopic expression of downstream target genes by EGFR signaling**

To demonstrate that the CNS midline cells are able to provide sufficient positional information for the induction of the ventral-

most ectodermal fate by activating the EGFR signaling pathway, *sim* was ectopically expressed in the *en*-expressing cells by crossing the *en-Gal4* line with the *UAS-sim* line. In the *en-Gal4/UAS-sim* embryos at stage 10, *otd* is expressed both in the *en*-positive cells and in the neighboring cells by activation of the EGFR signaling pathway (Fig. 7A). *rho* is ectopically expressed in the *en*-expressing cells and in the midline cells (Fig. 7B). *pnt* and *vn* are ectopically expressed in the ectodermal cells, which are located next to the *en*-expressing cells (Fig. 7 C,D). *aos* is also ectopically expressed in the ectodermal cells which is located in each segment (data not shown). This result indicates that the CNS midline cells provide sufficient positional information to activate the expression of *Egfr* signaling genes that are essential for the establishment of the ventral ectodermal fate.

Discussion

This study aims to elucidate not only the roles of the CNS midline cells in the proper patterning of the ventral neuroectoderm by analyzing the spatial and temporal pattern of expression in the loss-of- and gain-of-function mutants, but also the hierarchical relationship among the *sim*, *spitz* class and *Egfr* signaling genes. This analysis showed that the *sim* gene activates the EGFR signaling pathway to establish the ventral neuroectodermal cell fate by acting upstream of all the *spitz* class and *Egfr* signaling genes. *sim* activates *spi* expression in the midline cells and ventral neuroectoderm, and *spi* and *S* regulate the expression of *rho* in the ventral neuroectoderm. Ectopic expression of *sim* induces the downstream target genes, indicating that *sim* provides sufficient positional information to derive the ventral neuroectodermal fate. Thus, it is concluded that the CNS midline cells integrate the EGFR signaling pathway with the correct cell fate determination and proper patterning of the ventral neuroectoderm.

CNS midline cells control proper patterning of the ventral neuroectoderm by activating the EGFR signaling pathway

These analyses showed that the expression of all the *spitz* class and *Egfr* signaling genes such as *aos*, *otd*, *pnt*, *rho*, *spi*, and *vn* is abolished in the *sim* and is expanded in the *hs-sim* mutants (Fig. 2). This result indicates that *sim* is a master regulator of cell fate determination and proper patterning of the ventral neuroectoderm as well as of the CNS midline cells. The present study demonstrated that *sim* regulates the expression of *spi* gene in the midline cells and ventral neuroectoderm (Fig. 3), which provides a supporting evidence to a possibility that the CNS midline cells control mRNA level of *spi* gene itself in order to promote the generation of active ligand for *Egfr* or the enhancement of EGFR signaling in the neighboring ventral neuroectoderm. In addition, it was demonstrated that the expression of *rho* gene in the ventral neuroectoderm is reduced and discontinuous in *spi* and *S* mutants and is expanded along the anteroposterior axis in the *sca-Gal4/UAS-S* and *sca-Gal4/UAS-spi* embryos (Fig. 4 E,F,K,L), which indicates that *rho* expression in the ventral neuroectoderm and midline cells is regulated by *S* and *spi* genes. This finding suggests that the *Egfr* signaling, activated by the CNS midline cells, controls *rho* expression which is known to be involved in the processing of Spi ligand. This suggestion is in contrast to the proposition that Rho and S regulate the processing of membrane-bound Spi to an active secreted form, based on the genetic analysis which demonstrated that the effect of the secreted Spi on

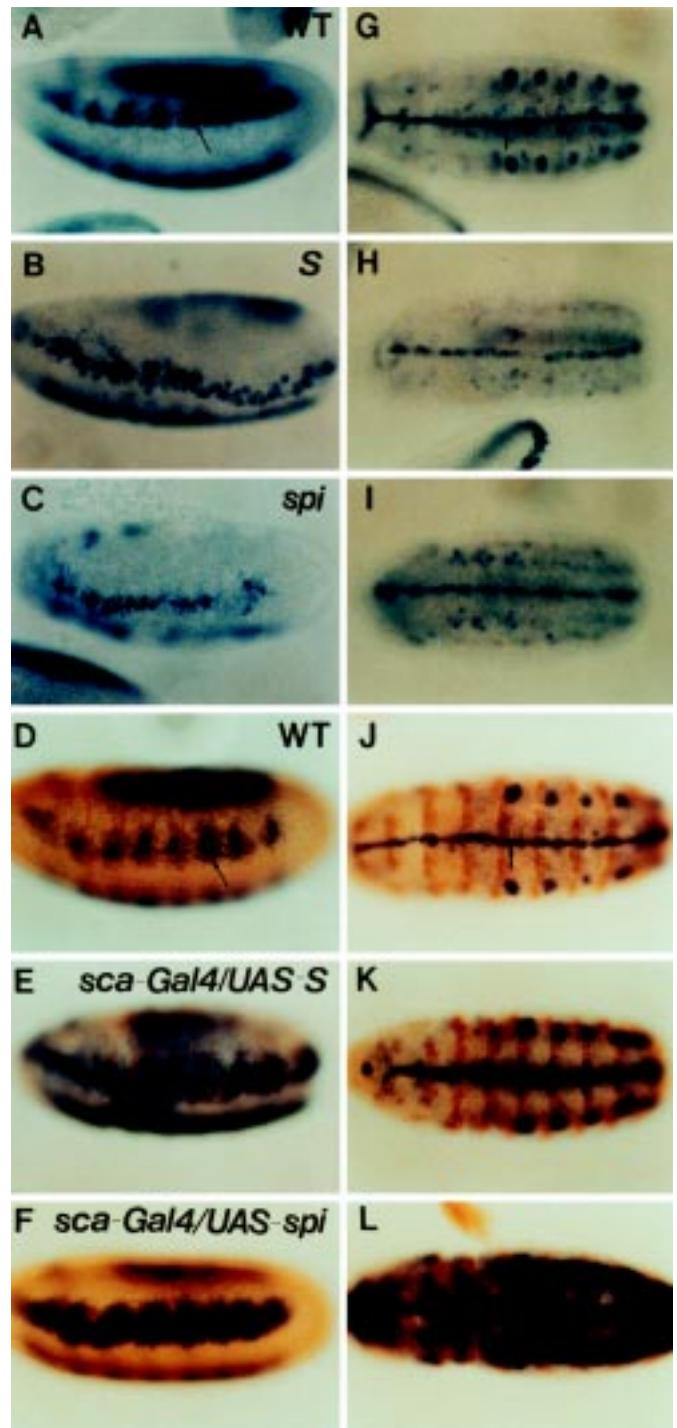


Fig. 4. *rho* expression in the ventral neuroectoderm is regulated by the *spitz* class genes. *rho* expression was analyzed in the wild-type (A,D,G,J), *S* (B,H), *spi* (C,I), *sca-Gal4/UAS-S* (E,K) and *sca-Gal4/UAS-spi* (F,L) mutants. *rho* expression in wild-type embryos at stage 5 is detected in the ventrolateral neuroectoderm with a periodically repeated pattern along the anteroposterior axis (long arrows; A,D) and is restricted to the midline cells and trachea at stage 11 (short arrows; G,J). *rho* expression in the ventrolateral neuroectoderm and midline is reduced and discontinuous in the *S* (B,H) and *spi* (C,I) mutants, while it expands at stage 5 and becomes widened at stage 11 in *sca-Gal4/UAS-S* (E,K) and *sca-Gal4/UAS-spi* (F,L) embryos. (A-F) and (G,L) show the ventrolateral and ventral views of embryos, respectively, and anterior is to the left.

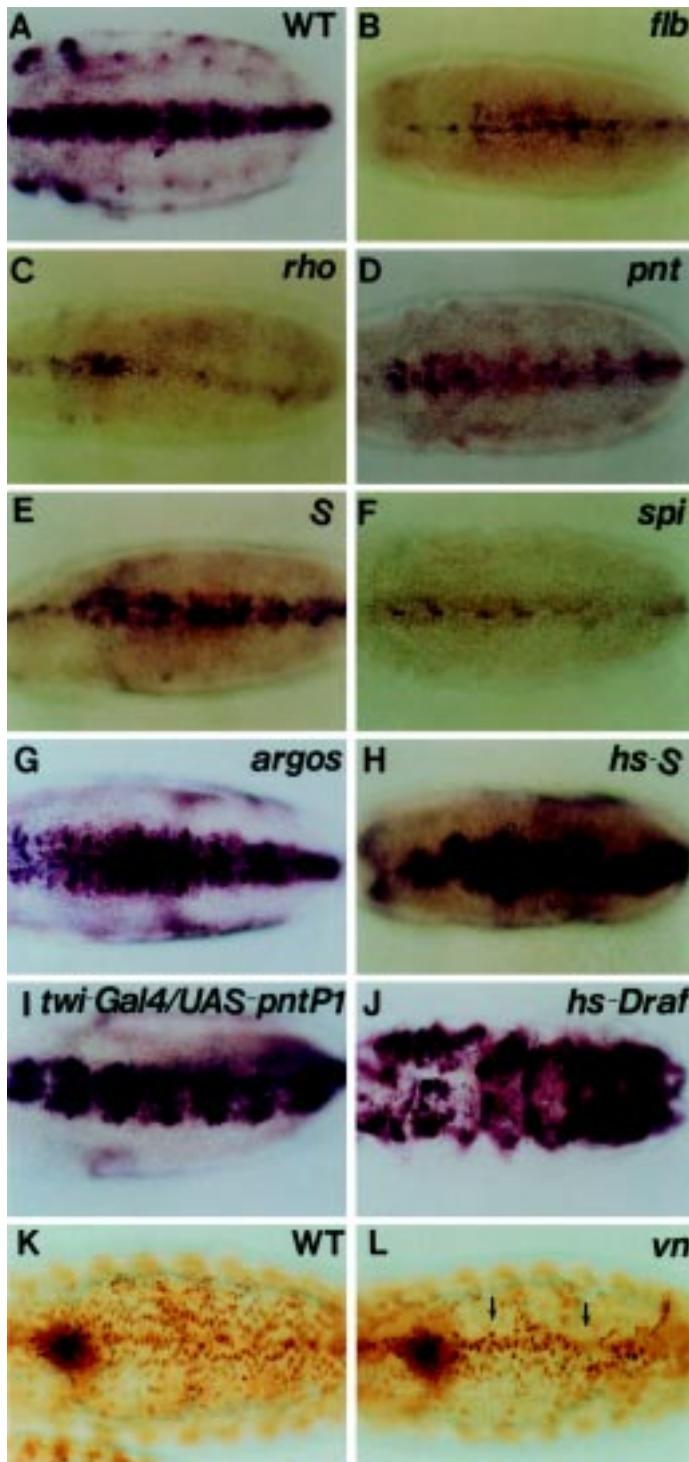


Fig. 5. *vn* is also activated by the *spitz* class and *Egfr* signaling genes. (A–G) *vn* expression in two stripes of the ventral-most ectodermal cells on either side of the midline of the wild-type embryos at early stage 11 (A) is absent in *Egfr^{flb}* (B), *rho* (C), *pnt* (D), *S* (E), *spi* (F), and *aos* (G) embryos. (H–J) *vn* expression is expanded in the *hs-S* (H), *twi-Gal4/UAS-pnt P1* (I) and *hs-Draf* (J) mutants. β -galactosidase expression in the ventral ectodermal cells of wild-type BP28 enhancer trap line at stage 15 (K) is absent in many parts of the lateral ectoderm and is occasionally disrupted in the ventral-most ectodermal cells (arrows) in *vn* embryos (L). All panels show the ventral view of embryos, and anterior is to the left.

ventralization is epistatic to the phenotypes of the *rho* and *S* mutants (Golembo *et al.*, 1996). Recent molecular study, which employed the *Xenopus* animal cap assay system, supports this proposal (Bang and Kintner, 2000). However, several studies support the proposal that Rho and S regulate receptor function by promoting the aggregation of receptor and ligand, or receptor dimer formation (Stemerdink and Jacobs, 1997; Guichard *et al.*, 1999). It remains to be determined whether the reduction of *rho* expression in *spi* and *S* mutants is dependent on the EGFR signaling. In addition, further biochemical and molecular genetic studies would also be necessary for determination of the precise roles of *rho*, *S*, and *spi* in the activation of EGFR signaling.

It has not been well established how the *vn* gene play a role in the fate determination of the ventral neuroectoderm. This study demonstrated that *vn* plays a more important role in the proper patterning of the lateral ectodermal cells than in patterning of the ventral-most cells. Once initial fate of the ventral-most ectodermal cells is established, diffusible Aos and Vn, induced by EGFR signaling via Pnt, modulate EGFR activity in the lateral neuroectoderm to determine the fate of neighboring lateral cells at the late stage. Vn may be required to ensure the establishment of the lateral neuroectodermal fate in the presence of the antagonistic Aos against Spi, whose level may not be sufficient to induce the lateral ectodermal fate. Thus, it is plausible that the intricate balance in the protein level between Aos and Vn is critical for establishing the lateral ectodermal fate. *vn* expression in the ventral-most ectoderm may consolidate the EGFR activity gradient in the ventral-most region in the presence of the inhibitory Aos and contribute to the establishment of the lateral ectodermal fate.

It was shown that overexpression of *sim* by heat shock cannot activate the expression of *otd*, a ventral-most ectodermal marker, in the *spi* and *Egfr^{flb}* mutants, and that overexpression of *spi* or *Draf* in the *sim* mutant is sufficient for activating *otd* expression. Ectopic expression of *sim* can induce the ventral neuroectodermal fate in the *en*-expressing cells in each segment. These results clearly demonstrate that the CNS midline cells induce the ventral-most ectodermal fate by activating the EGFR signaling pathway. It is plausible that the activated EGFR signaling controls cell division cycle to provide sufficient number of the neuroectodermal cells and to determine the unique identity of each individual neuroectodermal cells that express an individual set of genes within each NB, depending on their own developmental history (Chang *et al.*, 2000). Together with the recent findings that the CNS midline cells are required for the identity determination and differentiation of the ventral neuroblasts and neurons (Menne *et al.*, 1997; Lee *et al.*, 1999; Yu *et al.*, 1999; Chang *et al.*, 2000), this study demonstrates that the CNS midline cells play central roles in correct cell fate determination and proper patterning of the ventral neuroectoderm by integrating the positional information with the patterning through the EGFR signaling pathway.

Considering the observation that the *sim* mutant shows the most severe defects in the ventral neuroectoderm, extensive effects of the CNS midline cells on the expression of many downstream genes are consistent with this observation. Especially, influence of *sim* on the expression of *aos*, *rho*, *spi*, and *vn* genes may be very critical for establishing the initial EGFR signaling event by generating the effective graded concentration of the signaling ligands to subdivide the ventral neuroectoderm into medial, intermediate, and lateral regions. It will be important to elucidate how *sim* regulates *rho*, *S* and *spi* to establish the initial

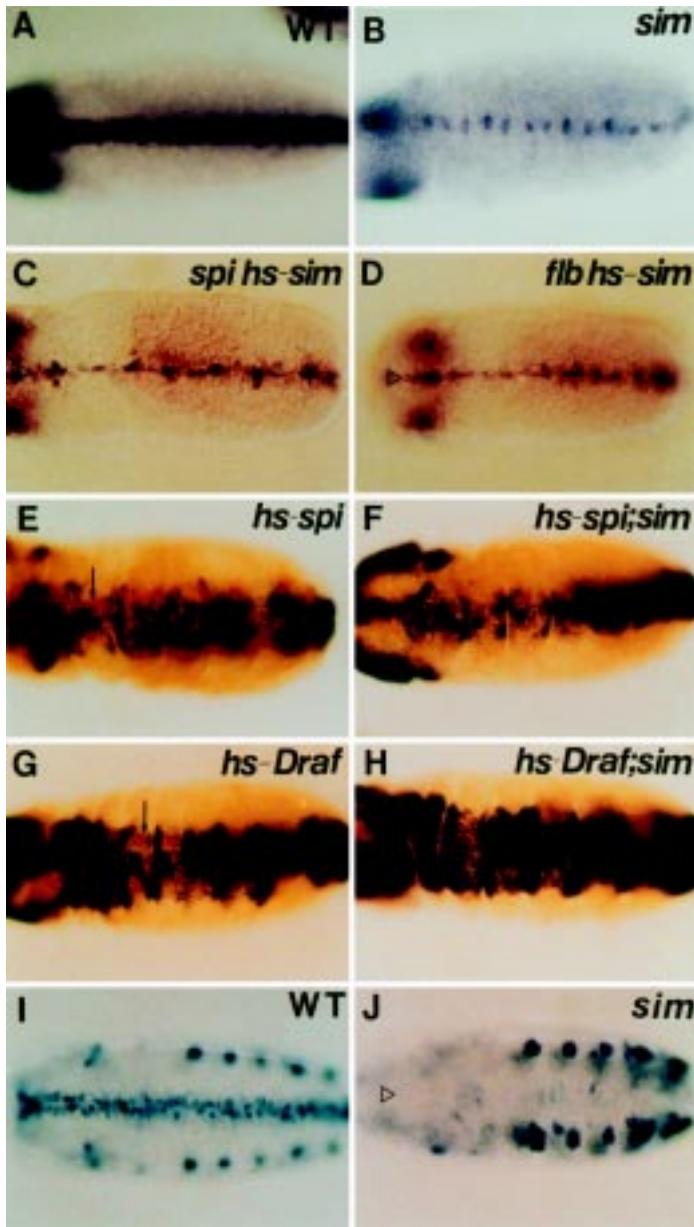


Fig. 6. *sim* induces the ventral ectodermal fate by activating the EGFR signaling pathway. The expression of a ventral-most ectodermal marker, *otd*, is abolished in *sim* (B), *spi hs-sim* (C) and *Egfr^{flb} hs-sim* (D) embryos at stage 11. Overexpression of *otd* in *hs-spi* (E) and *hs-Draf* (G) embryos can be retained even in *hs-spi; sim* (F) and *hs-Draf; sim* (H), indicating that *sim* acts through the *Spi/EGFR* signaling pathway. In *sim* embryos, the *sim*-expressing midline and mesodermal cells are not detected with the anti-*Sim* antibody, compared to wild-type embryo (arrows). Active MAPK, detected in 1-2 longitudinal stripes of the ventral-most ectodermal cells of wild-type embryos (I), is abolished in *sim* mutant embryos (J) at stage 11, whereas active MAPK expression is not altered in trachea. All panels show the ventral view with the ventral midline marked with an open arrowhead, and anterior is to the left.

signal triggering event, and *aos* and *vn* to consolidate and refine initial signals for the subdivisions of the ventral neuroectoderm. It could be proceeded either by generating the graded distribution of signaling ligand(s) or by setting up the relay of cell-cell interactions between neuroectodermal cells.

A model for proper patterning of the ventral neuroectoderm by the spitz class and Egfr signaling genes

On the basis of the present and previous studies, the following model of patterning of the ventral neuroectoderm is proposed (Fig. 8). *sim* controls the expression of *spi* in the midline cells at transcriptional level and the production of *Spi* ligand for *Egfr*. *spi* and *S* regulate *rho* expression in the ventral neuroectoderm, which acts as signal mediators to facilitate the EGFR signaling pathway in the ventral neuroectodermal cells, in addition to the processing of the membrane-bound *Spi* precursor into the secreted form. This process activates EGFR signaling and EGFR signaling pathway acts through the MAPK cascade inside the cytoplasm of the ventral-most neuroectodermal cells, and then the signal is relayed to the nucleus by transcription factors such as *Otd* and *Pnt*. The activation of *Egfr* also induces an inhibitor of EGFR signaling, *Aos*, by the activation of *Pnt*. Diffusible *Aos* in the neighboring cells inhibits EGFR signaling to form differential EGFR activity in the lateral neuroectoderm. *Vn* may be involved in consolidating the initial signaling event by increasing the concentration of ligand(s), which acts in the ventral neuroectoderm and by competing with the repressive effect of *Aos* in the ventral-most neuroectoderm.

In conclusion, the *sim* gene is a master regulator in the cell fate determination of both the CNS midline cells and ventral neuroectoderm. It controls the expression of many genes that act as positive ligands (*spi*, *vn*), as a negative ligand (*aos*), as transmembrane molecules (*rho*, *S*), and transcription factors (*otd*, *pnt*) in the EGFR signaling pathway to establish correct cell fate of the ventral neuroectoderm through cell-cell interaction by the CNS midline cells. It remains to be investigated how *sim* activates the EGFR signaling pathway, by elucidating the molecular interactions among *rho*, *S* and *spi* to activate the EGFR signaling pathway, and by understanding the molecular mechanism of positive and negative regulation of the

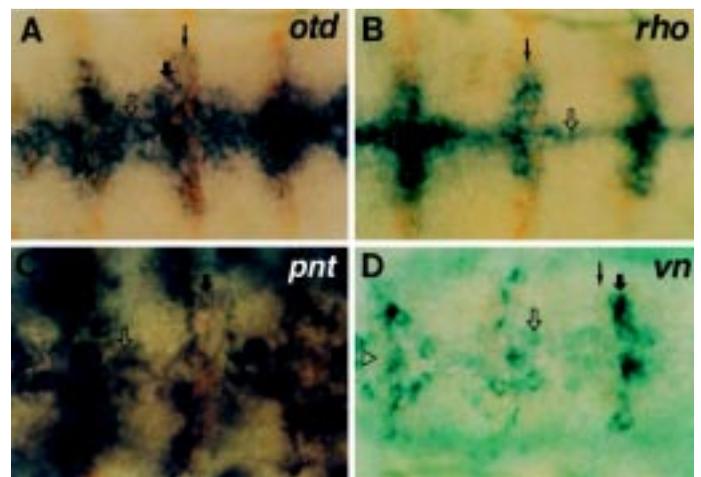


Fig. 7. *sim* induces ectopic expression of the target genes of the Egfr signaling pathway. Ectopic expression of *sim* in the *en-Gal4/UAS-sim* embryos induces *otd* (A) and *vn* (D) expression in the *en*-positive (arrows) and neighboring (thick arrows) cells and in the ventral-most neuroectodermal cells (empty arrows). (B) *rho* expression is induced in the *en*-positive cells (arrow) and in the midline cells (empty arrow). (C) *pnt* expression is induced in the neighboring cells (thick arrow) next to the *en*-positive cells (arrow) in the ventral-most neuroectodermal cells (empty arrow). All panels show the ventral view with the ventral midline marked with an open arrowhead, and anterior is to the left.

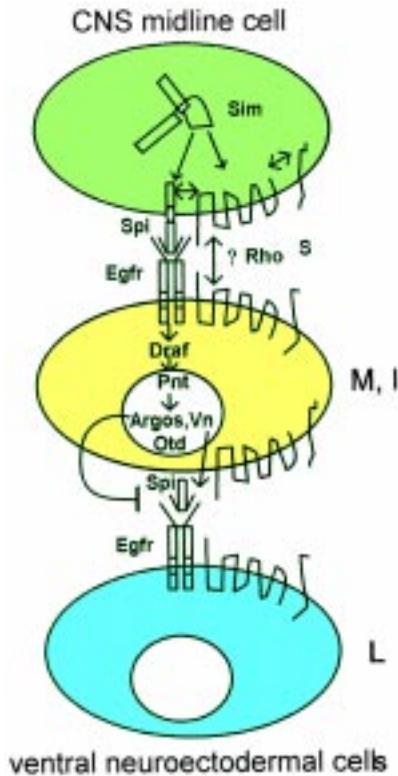


Fig. 8. Hierarchical relationship of the spitz class and Egfr signaling genes. In CNS midline cells, *spi* expression and the production of the secreted Spi may be regulated by *sim*. Since the *sim* gene is required for rho gene expression in CNS midline cells, *sim* may control the initiation of EGFR signaling event by regulating the expression of rho and S. Rho and S, which are located at the membrane of both sides of CNS midline cells and neuroectodermal cells, may be involved in the processing of the membrane-bound Spi ligand or may increase the specificity and affinity of ligand, Spi to the Egfr. The secreted Spi ligand binds and activates the EGFR signaling pathway in the medial (M) and intermediate (I) columns of the ventral neuroectoderm. This signaling pathway acts through the MAPK cascade inside the cytoplasm, and then the signal is relayed to the nucleus by transcription factors such as Otd and Pnt. Egfr activity is regulated by a negative ligand, AOS, and a positive ligand, Vn, to generate the Egfr activity gradient which is required for the establishment of lateral (L) cell fate in the ventral ectoderm.

EGFR signaling pathway. The molecular genetic and biochemical analyses are also required to reveal how the positive ligand, Vn, and negative ligand, AOS, modulate the activity of Spi, and how they interact each other to refine initial EGFR signaling pathway. This study may help understand the basic knowledge on the mechanisms of cell-cell interaction which is required for the cell fate determination during development and devise ways to treat many types of cancers by manipulating the conserved components in the receptor tyrosine kinase signaling pathway.

Materials and Methods

Fly strains

The following mutants were used as the loss-of-function mutants: *aos*^{Δ7}, *Egfr*^{r2}, *Egfr*^{E3}, *otd*⁹, *pnt*¹, *rho*⁴, *S*^{IN}, *sim*², *spi*¹, and *vn*^{RY506} mutants. The *vn*^{RY506} mutant strain was kindly provided by Dr. A. Simcox. *hs-argos* (Freeman et al., 1996), *hs-sim* (Nambu et al., 1991), *hs-S* (Kolodkin et al.,

1994), *hs-rho* (Sturtevant et al., 1993), *hs-spi* (N. Perrimon, personal communication), and *hs-Draf* (Brand and Perrimon, 1994), kindly provided by Drs. M. Freeman, S. T. Crews, U. Banerjee, E. Bier, and N. Perrimon, respectively, were used as the gain-of-function mutants. The UAS/GAL4 system was employed as another kind of the gain-of-function mutant. *UAS-sim* (Xiao et al., 1996) was kindly provided by Dr. J. Nambu, *UAS-S* and *UAS-spi* (Gabay et al., 1996) by Dr. C. Klämbt, and *engrailed (en)-Gal4* (Scholz et al., 1997) and *scabrous (sca)-Gal4* (Kleas et al., 1994) were kindly provided by Dr. S. DiNardo. *UAS-pnt P1* and *twi-2PE-Gal4* (Golembo et al., 1996) which drives expression in the mesoderm and ventral-most ectoderm were obtained from the Bloomington Stock Center. The BP28 enhancer trap line (Kim and Crews, 1993) was used as the ventral epidermal markers.

Overexpression of spitz class genes

To test whether the expression of a gene is sufficient to regulate the other downstream target genes, two different methods were used for ectopic expression of the gene of interest in this study: heat shock induction and the UAS/GAL4 system (Brand et al., 1994). Heat shock was carried out by incubating the embryos at 38°C in water bath for 30-60 min; *hs-sim* and *hs-rho* lines were incubated for 60 min, and the others for 30 min. Heat-shocked embryos were incubated for additional 2-7 hrs at 25°C to recover to normal development, and then embryos were collected. For the ectopic expression of *sim*, *S*, *spi*, and *pnt P1*, *UAS-sim*, *UAS-S*, *UAS-spi*, and *UAS-pnt P1* flies were crossed with the *en-*, *sca-*, and *twi-2PE-Gal4* flies, respectively.

Immunohistochemistry and whole mount in situ hybridization

Anti-diphospho-MAPK antibody (New England Biolab) was used at 1:200 dilution, detected by horseradish peroxidase immunohistochemistry according to Nambu et al. (1991) and signal was amplified by using the biotylated tyramide (NEN) according to the manufacturer's protocol. *aos* (Freeman et al., 1992), *otd* (Finkelstein et al., 1990), *pnt P1* (Klambt, 1993), *rho* (Bier et al., 1990), *S* (Kolodkin et al., 1994), *sim* (Nambu et al., 1990), *spi* (Rutledge et al., 1992), *vn* (Schnepp et al., 1996) cDNAs were used to analyze the respective gene expression. Whole-mount *in situ* hybridization with the digoxigenin-labeled DNA and RNA probes was performed as described by Tautz and Pfeifle (1989) and O'Neill and Bier (1994), respectively. Stained embryos were viewed and photographed using an Olympus microscope with Normaski optics.

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