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Tip cell-derived RTK signaling initiates cell movements in the *Drosophila* stomatogastric nervous system anlage

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The stomatogastric nervous system (SNS) of *Drosophila* is a simply organized neural circuitry that innervates the anterior enteric system. Unlike the central and the peripheral nervous systems, the SNS derives from a compact epithelial anlage in which three invagination centers, each giving rise to an invagination fold headed by a tip cell, are generated. Tip cell selection involves lateral inhibition, a process in which Wingless (Wg) activity adjusts the range of *Notch* signaling. Here we show that RTK signaling mediated by the *Drosophila* homolog of the epidermal growth factor receptor, DER, plays a key role in two consecutive steps during early SNS development. Like Wg, DER signaling participates in adjusting the range of *Notch*-dependent lateral inhibition during tip cell selection. Subsequently, tip cells secrete the DER ligand Spitz and trigger local RTK signaling, which initiates morphogenetic movements resulting in the tip cell-directed invaginations within the SNS anlage.

INTRODUCTION

The stomatogastric nervous system (SNS) of *Drosophila* derives through the formation of a compact epithelial anlage at the roof of the stomodeum (González-Gaitán and Jäckle, 1995; Hartenstein, 1997) in response to maternal Torso activity in the terminal region of the embryo (González-Gaitán and Jäckle, 1995). Torso is a receptor tyrosine kinase (RTK) (Sprenger *et al.*, 1989). Upon activation, it causes local Ras/Raf signaling that leads to the spatially restricted expression of *Krüppel*, *wingless* (*wg*) and proneural genes of the *Achaete-Scute Complex* (*AS-C*), including *achaetae* (*ac*), covering the SNS anlage (Hartenstein *et al.*, 1994; González-Gaitán and Jäckle, 1995; Hartenstein, 1997). Once the SNS anlage is formed at the roof of the stomodeum, *AS-C* gene expression becomes restricted to three cells (González-Gaitán and Jäckle, 1995). These cells, termed tip cells, define invagination centers from which three invagination folds, each headed by a single *ac*-expressing tip cell, are generated (González-Gaitán and Jäckle, 1995). Subsequently, the invagination folds pinch off from the stomodeal epithelium and form separate vesicles that give rise to a stereotyped pattern of four SNS ganglia, termed frontal ganglion, esophagial ganglion 1 and 2 and proventricular ganglion, including their nerve tracts (Hartenstein *et al.*, 1994; González-Gaitán and Jäckle, 1995; Hartenstein, 1997).

Singling out of the three tip cells involves *Notch*-dependent lateral inhibition in which *wg* activity adjusts the range of *Notch* signaling (González-Gaitán and Jäckle, 1995). In the absence of *wg* or components of the Wg pathway, such as *dishevelled* or *armadillo*, only one *achaete*-expressing tip cell is selected and it gives rise to only a single invagination fold in the center of the SNS anlage (González-Gaitán and Jäckle, 1995). These findings show that *wg* activity adjusts the range of *Notch*-dependent lateral inhibition required for the singling out of the three tip cells, whereas morphogenetic movements underlying the invagination process are independent of *wg* activity. Here we show that RTK signaling, which depends on the *Drosophila* homolog of the epidermal growth factor receptor (DER), plays a key role in two consecutive steps of early SNS development. The results demonstrate that RTK signaling functions in parallel or in concert with *wg* to adjust properly the range of *Notch*-dependent lateral inhibition within the SNS anlage and that DER signaling initiates morphogenetic movements. The DER ligand Spitz (Rutledge *et al.*, 1992) emanates from the tip cells and triggers RTK signaling in the neighboring cells that participate in the tip cell-dependent invagination process.

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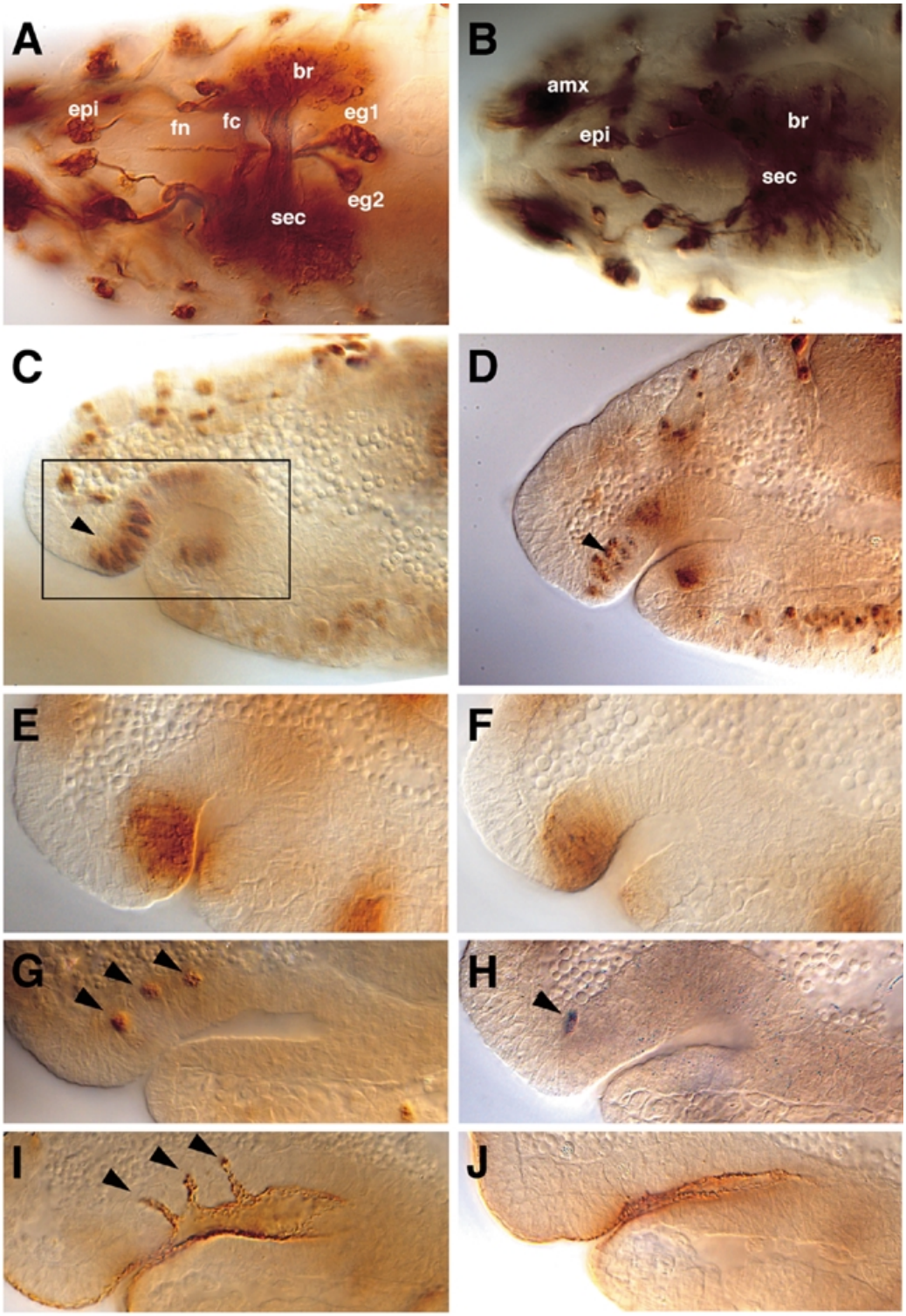

Fig. 1. *spitz*^{IIT} mutant SNS phenotype. (A and B) Mab22c10 immunostaining showing the wild-type SNS (**A**) and the disrupted SNS of a *spitz*^{IIT} mutant embryo (**B**) at stage 17 (dorsal view). Abbreviations: epi, epiphysis; amx, antennomaxillary complex; br, brain hemisphere; sec; supraesophageal commissure; fn, frontal nerve; fc, frontal commissure; eg1 and 2, esophageal ganglia 1 and 2. Note that PNS (epi, amx) and CNS (sec, br) structures are present in *spitz* mutants, whereas SNS ganglia and nerves are absent. (C and D) Krüppel immunostaining showing the SNS anlage (arrowheads) in wild-type (**C**) and *spitz* mutant (**D**) embryos at stage 10. The box in (C) corresponding to the stomodeal invagination within the cephalic region shows the enlarged area shown in (E–J). (E and F) Wingless immunostaining to show the SNS primordium of a wild-type (**E**) and a *spitz* mutant embryo (**F**) at stage 10. (G and H) Achaete immunostaining showing the three singled-out cells (arrowheads) in wild-type (**G**) and only one singled-out cell in *spitz* mutant embryos (**H**) at stage 10. (I and J) Crumbs immunostaining showing three invaginations (arrowheads) within the stomodeum of wild-type (**I**) and their absence in *spitz* mutant embryos (**J**) at stage 11 (lateral views). Orientation: anterior is left, dorsal is up. Staging of embryos was according to Campos-Ortega and Hartenstein (1997).

RESULTS AND DISCUSSION

A recent study aimed at the identification of genes required for SNS development suggested that Ras/Raf signaling is not only required to establish the SNS anlage (González-Gaitán and Jäckle, 1995), but may also participate in later aspects of SNS formation (Forjanic *et al.*, 1997). In order to investigate this role of RTK signaling, we examined SNS development in lack-of-function mutants of the DER ligand Spitz (Rutledge *et al.*, 1992). In *spitz* mutants, the formation of the four SNS ganglia is strongly impaired (Figure 1A and B). The SNS anlage, however, forms normally (Figure 1C and D). In addition, the expression domain of *wg* (Figure 1E and F) and proneural AS-C genes (not shown) was indistinguishable from a wild-type SNS anlage. At the stage when the three *ac*-expressing cells were singled-out within the wild-type SNS anlage (Figure 1G), we found only one *ac* positive cell in *spitz* mutants (Figure 1H). The same phenotype had been observed in *wg* mutants or mutants lacking an integral component of the *wg* pathway (González-Gaitán and Jäckle, 1995). Since no altered *wg* pattern was found in the *spitz* mutant SNS anlage, Spitz-dependent RTK signaling may act in parallel or in combination with *wg* to adjust the proper range of *Notch*-dependent lateral inhibition. In contrast to *wg* mutants, however, no invagination fold is observed (compare Figure 1I and J). This observation indicates that the singled-out *ac*-expressing cell of *spitz* mutants (Figure 1H) has lost the ability to function as a tip cell and possibly fails to induce morphogenetic movements within the SNS anlage.

spitz, like other genes encoding components of the DER signaling pathway such as DER, Ras, Raf and the cascade of MAP kinases, is ubiquitously expressed (Rutledge *et al.*, 1992). Local activation of DER signaling requires the transmembrane protein Star, which is necessary for the secretion of Spitz (Schweitzer *et al.*, 1995; Golembo *et al.*, 1996; Pickup and Banerjee, 1999; Bang and Kintner, 2000). Star is expressed in restricted patterns corresponding to the Spitz secreting cells (Kolodkin *et al.*, 1994). In the SNS anlage, we noted that Star becomes restricted to the three tip cells and is maintained in these cells when invagination takes place (Figure 2A–C). As in *spitz* mutants, the *Star* mutant SNS anlage is established normally (Figure 2D–E), only one *ac*-expressing cell is selected (Figure 2F) and no invagination occurred (Figure 2G). Consistently, *Star* mutants fail to develop the proper set of SNS ganglia and the associated nerves (Figure 2H). These observations suggest that tip cells are a Star-dependent source of Spitz activity that triggers DER-dependent RTK signaling in the neighboring cells within the SNS anlage. This conclusion is supported by the finding that phosphorylated MAPK, a cellular marker for RTK signaling activity (Gabay *et al.*, 1997), is indeed activated in cells of the invagination folds (Figure 2I), whereas phosphorylated MAPK did not appear in the Star mutant (Figure 2J) or in the *spitz* mutant SNS anlage (not shown).

To examine whether activated Spitz is sufficient to induce cell movements within the SNS anlage, we made use of the GAL4/UAS system (Brand and Perrimon, 1993) to misexpress secreted Spitz in an ectopic pattern. This was achieved through the expression of activated Spitz from a UAS promotor driven transgene (Schweitzer *et al.*, 1995) that was activated by Gal4 under the control of the actin promotor. Under the conditions applied (see Methods), we observed scattered UAS-dependent transgene expression throughout the early embryo, including the SNS anlage (Figure 3A). When activated Spitz was expressed in such a pattern, a variable number of supernumerary infoldings within the SNS anlagen were observed (Figure 3B–F), indicating that activated Spitz is sufficient to initiate cell movements. This result, in conjunction with the observation that the invaginated cells express phosphorylated MAPK (Figure 2I), provides evidence that tip cell-derived activated Spitz triggers RTK signaling to initiate the invagination process. We tested this proposal by blocking DER signaling in the anterior most region of the SNS anlage that gives rise to the first invagination fold. For this, we used a GAL4 driver (SNS1–Gal4; see Methods) that causes UAS-dependent gene expression in the corresponding region of the SNS anlage (Figure 3G). SNS1–Gal4-mediated expression of a dominant-negative DER mutant form (Buff *et al.*, 1998) from a UAS-controlled transgene causes a specific suppression of the anterior most invagination fold without affecting the others (Figure 3H).

The results demonstrate that RTK signaling participates in the selection of tip-cell-dependent invagination centers in the SNS anlage and is subsequently required to initiate morphogenetic movements resulting in invagination folds. We have not focussed on how RTK signaling ties into the *wg*-modulated *Notch* signaling process previously shown to be necessary for the selection of the three SNS invagination centers (González-Gaitán and Jäckle, 1995). Our data indicate, however, that RTK signaling acts either in parallel or in combination with *wg* signaling to adjust the proper range of *Notch*-dependent lateral inhibition. Although in both *wg* and DER signaling mutants, only one *ac*-expressing cell is singled-out, the selected cells differ with respect to whether they function as tip cells or not. In *wg* mutants, the single cell causes an invagination, whereas in DER signaling mutants, the selected cell fails to provide this feature of SNS invagination centers. The results, therefore, consistently argue that tip cell-derived Spitz triggers local RTK signaling and thereby initiates the formation of invagination folds each headed by the Spitz-secreting tip cell. Thus, DER-dependent RTK signaling in *Drosophila* does not only participate in cell fate decisions (Sprenger *et al.*, 1989; Perrimon, 1993; Raz and Shilo, 1993; Schweitzer *et al.*, 1995; Freeman, 1996; Golembo *et al.*, 1996; Tio and Moses, 1997), cell migration (Klämbt *et al.*, 1992; Beiman *et al.*, 1996; Gisselbrecht *et al.*, 1996) and cell proliferation (Díaz-Benjumea and García-Bellido, 1990; Simcox, 1997; Nagaraj *et al.*, 1999), but also triggers morphogenetic movements within an epithelium, as has been recently demonstrated for fibroblast growth factor (FGF) signaling (Glazer and Shilo, 1991). It will be interesting to see whether the role of the EGF pathway in cell migration differs at the cellular level from cell migration events triggered by activated FGF receptors.

METHODS

Mutant strains, immunostainings and *in situ* hybridization. Mutant flies were maintained using CyO, hb-lacZ blue balancer as described (González-Gaitán and Jäckle, 1995). *l(2)05671* is a P-element insertion in the *Star* gene (Freeman, 1994; Forjanic *et al.*, 1997), and spi^{llT} and S^{54} are strong lack-of-function mutants (Lindsley and Zimm, 1992). SNS1–Gal4 is an enhancer-trap P-element insertion from G. Technau's laboratory collection (Ito *et al.*, 1995) (Mz798hII:gal4) carrying *Gal4* as a reporter gene.

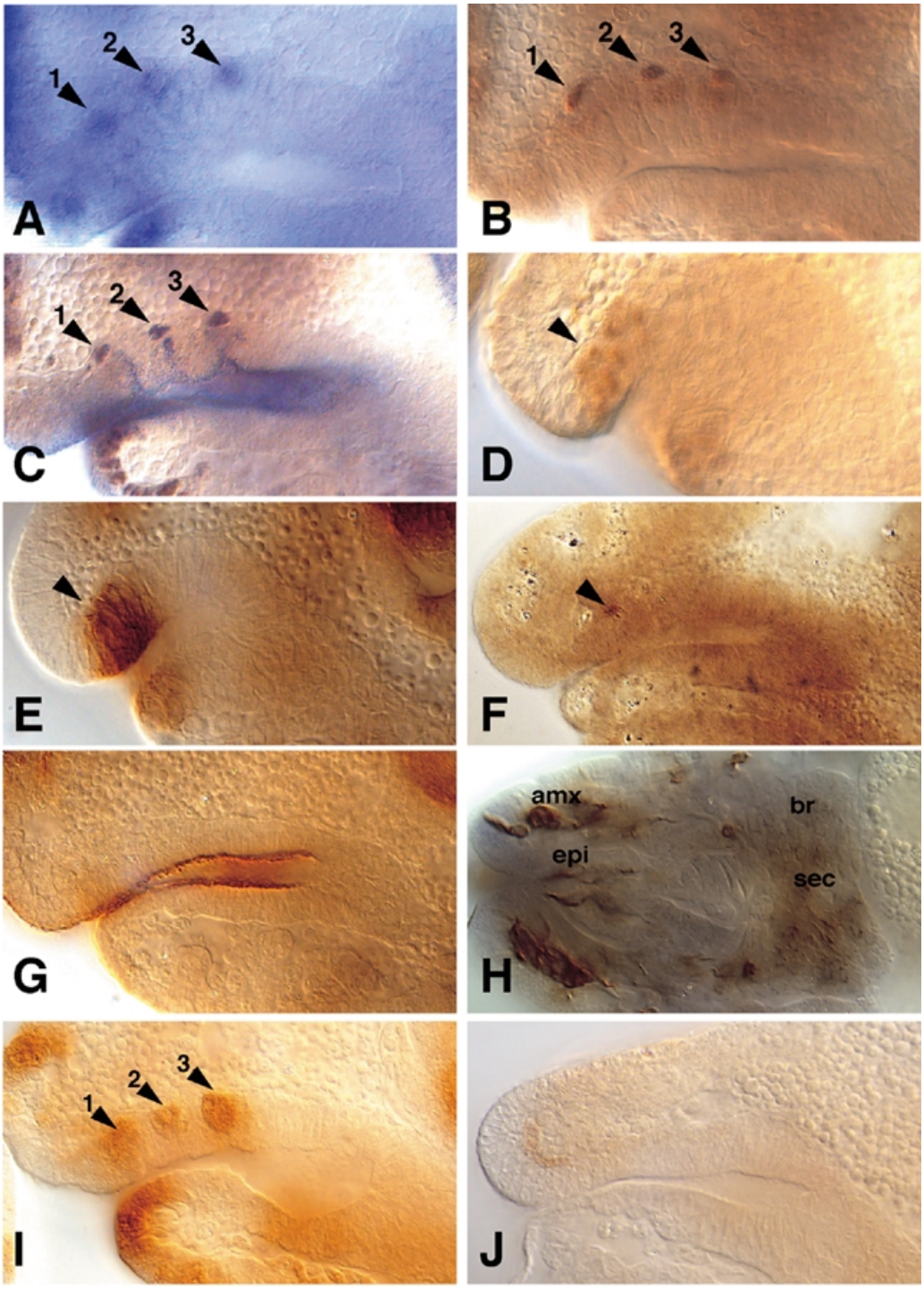

Fig. 2. *Star* SNS expression and S^{54} mutant SNS primordium. (**A**) *Star* RNA *in situ* hybridization to show expression of Star in the three single cells of the SNS primordium (1,2,3). (**B**) β-galactosidase immunostaining to show reporter gene expression in an embryo heterozygous for a P-element [l(2)05671] insertion at the Star locus (1,2,3). (**C**) Double immunostaining showing Star-expressing tip cells labelled by β-galactosidase (brown) and the three SNS invaginations (blue) labelled by Crumbs in a l(2)05671/+ embryo. (**D**) Krüppel immunostaining showing the SNS primordium (arrowhead) in an S^{54} mutant embryo. (E–H) Immunostainings of S^{54} mutant embryos with Wingless (**E**), Achaete (**F**), Crumbs (**G**) and Mab22c10 antibodies (**H**). In Star mutant embryos the SNS anlage is established normally as monitored by early Wg expression (E; arrowhead). A single Achaete-expressing cell (F; arrowhead) is unable to drive SNS invagination within the stomodeum (G), leading to a lack of SNS ganglia and nerves (H). (I and J) Immunostaining showing phosphorylated MAPK (1,2,3) within the wild-type (**I**) and an S^{54} mutant (**J**) SNS anlage. (A–G, I and J) Lateral views (dorsal is up). (H) Dorsal view. (D and E) Stage 10; (A–C, F, G, I and J) stage 11, (H) stage 17. For staging, orientation and position of the enlarged area see legend of Figure 1.

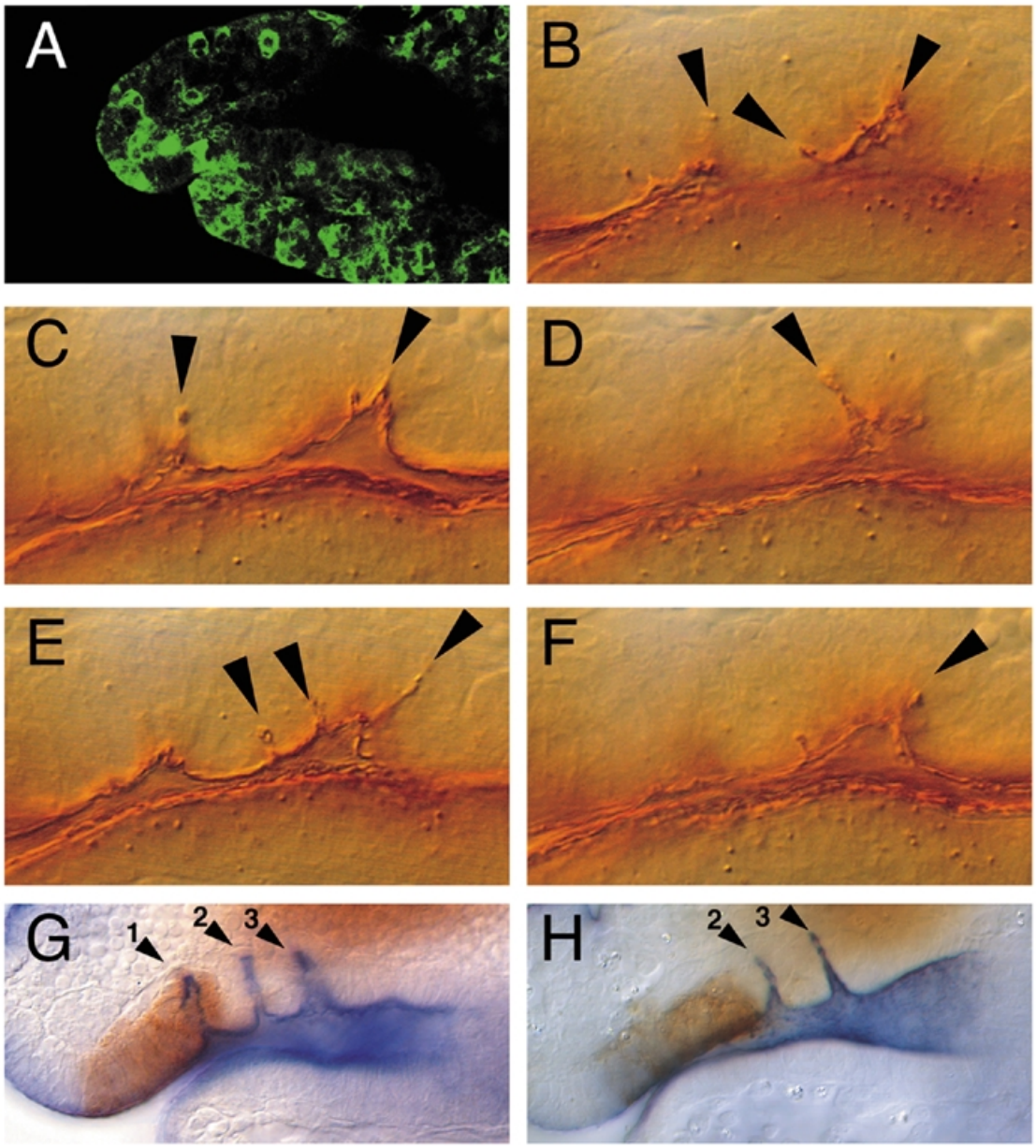

Fig. 3. Expression of secreted Spitz and DERDN at the invaginating SNS. (**A**) GFP immunolabeling to show embryonic expression of GFP under the control of an actin promotor during stage 11. Genotype *Actin-Gal4/UAS-GFP*. At this stage, the actin promotor in the Gal4 driver activates scattered embryonic expression of the reporter gene. (**B**–**F**) Crumbs immunolabeling of an embryo (genotype: *actin-Gal4/UAS-sspi*) in which secreted Spitz was expressed under the control of the actin promotor. The different focal planes (five consecutive Nomarski optical sections) through the SNS anlage show the supernumerary SNS invaginations (arrowheads). (**G**) Double immunolabeling showing GFP reporter gene expression (brown) covering the first of the three SNS invaginations (1,2,3) stained with anti-Crumbs antibodies (blue) in a transgene containing wild-type embryo (genotype: *SNS1–Gal4 UAS-GFP*). (**H**) GFP and Crumbs double immunolabeling in embryos expressing a dominant-negative version of DER in the area of the first invagination (genotype: *SNS1–Gal4 UAS-GFP/UAS-DERDN*). Note that the first invagination is absent, whereas the second and third (2,3) are formed normally. Lateral views of stage 11 embryos (dorsal is up). For staging, orientation and position of the enlarged area see legend of Figure 1.

Insertion is on the second chromosome and reproduces the embryonic pattern of expression of *goosecoid*, leaving open if the P-element is inserted in this gene. UAS–sspi (Schweitzer *et al.*, 1995) and UAS–DERDN (Buff *et al.*, 1998) are UAS constructs driving activated *spitz* and dominant-negative *DER*, respectively. For Gal4 driven expression, embryos developed at 25°C. Under these conditions actin–Gal4 drives embryonic expression in a scattered pattern (Figure 3A). Other mutants, Gal4 and UAS flies are described in Lindsley and Zimm (1992) and flybase.

Immunostainings and *in situ* hybridization were performed as previously described (González-Gaitán and Jäckle, 1995, 1996). Primary antibodies were used at the following dilutions: Mab22C10 (Fujita *et al.*, 1982) (Hybridoma bank), 1:50; rabbit anti-Krüppel (Gaul and Jäckle, 1987), 1:500; mouse anti-Achaete (Skeath and Carroll, 1992), 1:3; mouse anti-Crumbs

(Tepass and Knust, 1993), 1:20; mouse anti-Wingless (Brook and Cohen, 1996) (Hybridoma), 1:5; mouse anti-MAPK-P (Gabay *et al.*, 1997) (Sigma), 1:40; rabbit anti-GFP (Brock *et al.*, 1999), 1:3. Star *in situ* was performed using a full-length *Star* cDNA as an RNA probe.

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