stumps, a Drosophila Gene Required for Fibroblast Growth Factor (FGF)-directed Migrations of Tracheal and Mesodermal Cells

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ABSTRACT

Fibroblast growth factors (FGFs) bind to FGF receptors, transmembrane tyrosine kinases that activate mitogenic, motogenic, and differentiative responses in different tissues. While there has been substantial progress in elucidating the Ras-MAP kinase pathway that mediates the differentiative responses, the signal transduction pathways that lead to directed cell migrations are not well defined. Here we describe a Drosophila gene called *stumps* that is required for FGF-dependent migrations of tracheal and mesodermal cells. These migrations are controlled by different FGF ligands and receptors, and they occur by different cellular mechanisms: the tracheal migrations occur as part of an epithelium whereas the mesodermal migrations are fibroblast-like. In the *stumps* mutant, tracheal cells fail to move out from the epithelial sacs, and only rudimentary tracheal branches form. Mesodermal cells fail in their dorsal migrations after gastrulation. The *stumps* mutation does not block all FGF signaling effects in these tissues: both random cell migrations and Ras-MAP kinase-mediated induction of FGF-specific effector genes occurred upon ectopic expression of the ligand or upon expression of a constitutively activated Ras protein in the migrating cells. The results suggest that *stumps* function promotes FGF-directed cell migrations, either by potentiating the FGF signaling process or by coupling the signal to the cellular machinery required for directed cell movement.

DIRECTED cell migration is required during the development of many tissues and is essential for proper immune system function and wound healing. Inappropriate cell migrations contribute to spreading of cancer cells throughout the body. Cell migrations occur by different cellular mechanisms, ranging from the crawling of isolated fibroblasts in culture to the coordinated movements of entire epithelial sheets during organ morphogenesis (Bray 1992), and they are controlled by different types of signaling molecules. Fibroblast growth factors (FGFs) are a large family of secreted signaling molecules that have been implicated in the control of cell migration in a variety of contexts *in vitro* and *in vivo* (Terranova *et al.* 1985; Klambt *et al.* 1992; DeVore *et al.* 1995; Beiman *et al.* 1996; Gisselbrecht *et al.* 1996; Sutherland *et al.* 1996). However, like many other signaling molecules and their receptors, FGF pathways regulate other cellular processes besides cell migration, notably mitogenic and differentiative responses (Johnson and Williams 1993; Thomas 1993; Martin 1998). While there has been substantial progress in elucidating the Ras-MAP kinase signal transduction pathway that mediates the differentiative responses of FGF receptors and other receptor tyrosine kinases,

the intracellular signaling pathways that mediate directed cell migration are still poorly understood. In this article, we describe a gene required for two distinct FGF-dependent cell migrations in *Drosophila melanogaster.*

One FGF gene and two FGF receptor genes are known in *D. melanogaster.* The Branchless FGF is a ligand for the Breathless FGF receptor (Klambt *et al.* 1992; Sutherland *et al.* 1996). A ligand for the other FGF receptor, Heartless, has not yet been identified (Beiman *et al.* 1996; Gisselbrecht *et al.* 1996). These FGF pathways are active in different tissues and play different roles in development. The Heartless FGF receptor is expressed in the developing mesoderm and plays an important role in mesodermal development. It is required for the dorsal-directed mesodermal cell migrations that are necessary to form dorsal mesodermal derivatives including the heart and dorsal somatic muscles. The Branchless-Breathless pathway plays a critical role in tracheal (respiratory) system development, as described below. Both pathways also have additional roles in the development of specific glial cells (Klambt *et al.* 1992; Shishido *et al.* 1997).

The Drosophila tracheal system is a ramified network of epithelial tubes that delivers oxygen throughout the body (Manning and Krasnow 1993). It arises at mid-embryogenesis from 20 clusters of ectodermal cells, 10 on each side of the embryo. Each cluster of ∼80 cells invaginates and forms an epithelial sac that gives rise to one tracheal hemi-segment by a series of cell migrations and tubulogenesis events (Samakovlis *et al.* 1996).

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Initially, six primary branches bud from specific positions in the sac. These branches are formed by groups of 3–20 cells that migrate out from the sac in specific directions, organizing into tubular structures as they move. Individual cells near the ends of growing primary branches then elongate and form unicellular secondary branches. Most secondary branch cells go on to extend long cytoplasmic processes that form vast arrays of fine terminal branches that transport oxygen directly to the larval tissues. Different sets of genes are expressed in or near the cells that form each type of branch, and studies of these genes have begun to elucidate the pathways that control the outgrowth and branching pattern.

The Branchless FGF controls the early tracheal branching events (Sutherland *et al.* 1996). *bnl* is required for branching and is expressed dynamically in discrete clusters of cells surrounding the developing tracheal sacs at each position where a new branch will bud. The secreted growth factor activates the Breathless FGF receptor, which is expressed on all tracheal cells and guides the tracheal cell migrations during primary branch outgrowth (Glazer and Shilo 1991; Klambt *et al.* 1992; Lee *et al.* 1996b; Sutherland *et al.* 1996). The Branchless-Breathless pathway also has another important role in branch patterning: it induces the later programs of branching in cells near the ends of the growing primary branches and thereby specifies the positions of secondary branch sprouting (Sutherland *et al.* 1996; Hacohen *et al.* 1998). This function appears to be transduced by a Ras-MAP kinase cascade that culminates in the activation of MAP kinase and the induction of downstream effector genes including *pointed*, an ETS domain transcription factor (Klambt 1993), *sprouty*, an antagonist of FGF signaling (Hacohen *et al.* 1998), and *blistered (pruned)*, which encodes the Drosophila homologue of mammalian Serum Response Factor (DSRF; Affolter *et al.* 1994; Guillemin *et al.* 1996). The signal transduction pathways that mediate the early tracheal cell migrations have not been well studied, but Ras and Raf have been implicated (Reichman-Fried *et al.* 1994).

In a screen for mutations that affect tracheal development, a mutation called *stumps* was discovered that caused a striking arrest in tracheal cell migration, resulting in a tracheal phenotype resembling that of null mutations in *breathless* (*btl*) or *branchless* (*bnl*; Sutherland *et al.* 1996). Here we present the genetic characterization of the gene. The gene was named *stumps* because no tracheal branches form in the mutant, except for an occasional short stump. *stumps* is also required for the Heartless-dependent migrations of mesodermal cells. The *stumps* mutation, however, does not prevent all FGF signaling effects, because both random cell migrations and Ras-MAP kinase-mediated induction of FGF-specific effector genes can occur upon ectopic expression of the FGF ligand. The results suggest that *stumps* function promotes FGF-directed cell migrations, either by po-

tentiating the FGF signaling process or by coupling the signal to the cellular machinery required for directed cell movement.

MATERIALS AND METHODS

Fly strains and genetics: The *stumps¹* mutation was identified by Dr. Christos Samakovlis in enhancer trap line l(3)9904 generated by A. Spradling and colleagues (Spradling *et al.* 1995). The $P[ry^+, lacZ]$ insertion at 82F8-9 and an independent lethal mutation present on the original *stumps* chromosome were recombined away from the *stumps¹* mutation at 88D using the multiply marked *rucuca* chromosome (Lindsley and Zimm 1992) to generate the *stumps¹* mutant chromosome used here, *th st stumps*.

btl^{LG18} and *btl^{LG19}* (Klambt *et al.* 1992), *bnl^{P1}* (Sutherland *et al.* 1996), and *htl^{AB42}* (Gisselbrecht *et al.* 1996) are null or strong loss-of-function alleles. The *lacZ* enhancer trap markers used to monitor *trachealess* (1-eve-1), *breathless* (T1), *pointed* [l(3)7825], and *sprouty* [l(3)9143] expression have been described (Samakovlis *et al.* 1996). The following chromosomal deficiencies were used: *wrl^{hc4Rγ2}* (referred to as Df(3R)γ2; M. Fuller, personal communication), Df(3R)*ry^{506-85C}* [derived from Tp(3;Y)*ry^{506-85C}* (Flybase; http://flybase.bio.indiana.edu)], Df(3R)*red¹*, Df(3R)*ry^{85}*, and Df(3R)*red^{3L}* (Lindsley and Zimm 1992).

Genetic mapping and cytology: Meiotic recombination mapping crosses were carried out between the original *stumps* chromosome and the multiply marked *rucuca* chromosome, and between *stumps¹* and a *cv-c* $P[w^+, lacZ]0429$ chromosome constructed by Dr. Julie Brill. Recombinants were scored for visible adult markers on the *rucuca* or *cv-c* $P[w^+, lacZ]0429$ chromosomes and for the *stumps* tracheal phenotype. Recombination frequencies were analyzed in an F_1 cross of *w/w*; *stumps/cv-c* $P[w^+, lacZ]0429$ females to *w*; *cv-c e* males; the genotypes of 2617 F_2 male progeny were determined.

For fine scale recombination mapping, several restriction fragment length polymorphisms (RFLPs) were identified in the 88C,D region using genomic DNA probes derived from P1 phage DS02646 (Berkeley Drosophila Genome Project; http://www.fruitfly.org) or from λ EMBL3 genomic phage (from J. Tamkun) that hybridized to P1 phage DS02646 (RFLPs 20-RT and 20-LT) and from genomic DNA flanking the $P[w^+, lacZ]3263$ insertion (gift from J. Brill).

Preparation and orcein staining of salivary gland polytene chromosomes were carried out for *stumps* and Df(3R)γ2 as described (Ashburner 1989). The endpoints of the other chromosomal deficiencies shown in Figure 2C were taken from the references indicated above.

Immunohistochemistry and *in situ* hybridization: Embryo fixation and staining, antibodies against tracheal antigens, β-galactosidase, Engrailed, mAb 22C10, mAbBP102, and secondary antibodies have been described (Goldstein and Fyrberg 1994; Samakovlis *et al.* 1996). Other primary antibodies used were as follows: anti-Trachealess (Ward *et al.* 1998) at 1:1000 dilution, anti-diphospho-ERK (Gabay *et al.* 1997a) at 1:500, mAb2-161 (from M. Gilman) against DSRF at 1:300, mAbFMM5 (from D. Kiehart) against muscle myosin at 1:8, anti-dMEF2 (Bour *et al.* 1995) at 1:2000, anti-Even-skipped (Frasch *et al.* 1987) at 1:3000, and anti-Tinman (Frasch 1995) at 1:400. Whole-mount *in situ* hybridizations using a *bnl* probe were carried out as described (Sutherland *et al.* 1996).

Ectopic expression of *bnl* and *ras*: The Gal4/UAS expression system was used (Brand and Perrimon 1993). The following Gal4 drivers were used: *btl*Gal4 line on chromosome II (Shiga *et al.* 1996), which drives expression in tracheal cells from stage 11 onward; *twi*Gal4 (Greig and Akam 1995), which ex-
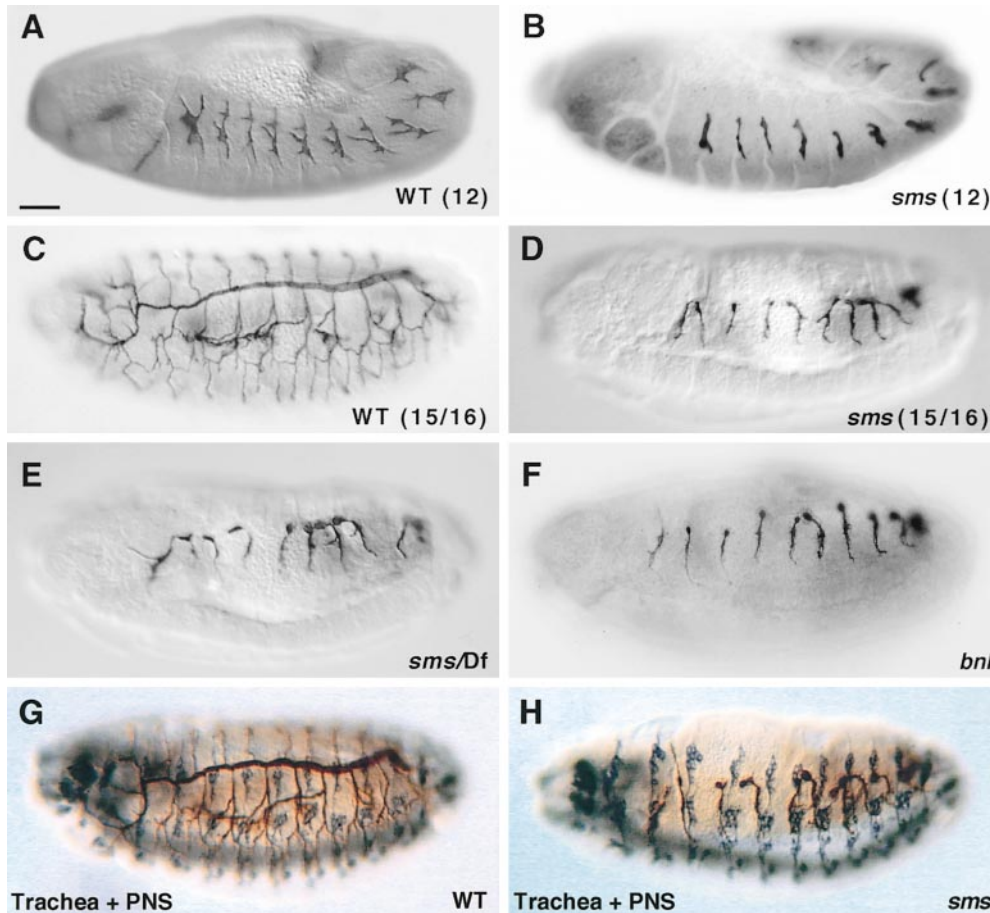
Figure 1.—Tracheal phenotype of *stumps*. (A) Stage 12 wild-type embryo stained with TL-1 antiserum to label the tracheal lumen. Primary branches have sprouted and are migrating out from the tracheal sacs. (B) Stage 12 *stumps¹* homozygote. There is little sprouting or outgrowth of primary branches. (C) Stage 15/16 wild-type embryo stained with mAb2A12 to label the tracheal lumen. (D) Stage 15/16 *stumps¹* mutant embryo. Note the almost complete absence of branches except for an occasional stump. (E) Stage 15/16 *stumps¹*/Df(3R)γ2 hemizygote. Note the almost complete absence of branches as in the *stumps¹* homozygote in D. Df(3R)γ2 homozygotes have a similar phenotype although they also show other embryonic defects that presumably result from deletion of other genes besides *stumps* (not shown). (F) Stage 15/16 *bnl^{P1}* homozygote. The *stumps* tracheal phenotype (D) is indistinguishable from that of the *bnl* mutant. (G) Stage 16 wild-type embryo double stained with mAb22C10 to show the peripheral nervous system (purple) and mAb2A12 to show trachea (brown). (H) Stage 16 *stumps¹* homozygote stained as in G. The PNS appears unaffected. Anterior is left and dorsal is up in all figures unless noted otherwise. Bar, 40 μm.

presses in the mesoderm from stage 6 on; and *hs*Gal4 (Brand and Perrimon 1993). The UAS lines used were UAS*Dras1^{V12}* on chromosome II (Lee *et al.* 1996a) and UAS*bnl*B4-2 on the X chromosome (Sutherland *et al.* 1996). Embryos from the following crosses were examined: (i) *twi*Gal4 (or *hs*Gal4)/+; *stumps*/+ × UAS*Ras¹*/+; *stumps*/+, (ii) *btl*Gal4/+; *stumps*/+ × UAS*Ras¹*/+; *stumps*/+, and (iii) *twi*Gal4 (or *btl*Gal4)/+; *stumps*/+ × UAS*bnl*B4-2/UAS*bnl*B4-2; *stumps*/+. The tracheal and/or mesodermal phenotypes of large numbers of embryos were scored; they fell into distinct phenotypic classes that were populated in the expected Mendelian ratios. *twi*Gal4 and *btl*Gal4 embryos used in the misexpression experiments were raised at 29° to maximize activity of the Gal4 driver. *hs*Gal4 embryos were pulsed at 37° for 20 min at 4 and 5 hr after egg lay (AEL).

RESULTS

Genetic characterization and mapping of *stumps*: In a screen of P[*lacZ*] enhancer trap lines, Christos Samakovlis discovered a line [l(3)9904] carrying a homozygous lethal mutation that almost completely blocked tracheal branching (Figure 1D), much like null (amorphic) mutations in the Breathless FGF receptor and Branchless FGF genes (Figure 1F). The mutation complemented *btl^{LG18}* and *bnl^{P1}* mutations for viability and mapped to a different chromosomal region, indicating

that it defined a separate gene (see below). The gene was named *stumps* (Sutherland *et al.* 1996) because even at late stages in embryonic development, no tracheal branches were seen except for an occasional, rudimentary stump of a branch (Figure 1D). The *stumps¹* mutation is recessive, as heterozygous embryos did not display a tracheal phenotype (see below). The phenotype of hemizygous embryos [*stumps¹*/Df(3R)γ2 or *stumps*/Df(3R)*ry^{506-85C}*; Figure 1E)] was indistinguishable from that of the homozygote (Figure 1D), implying that *stumps¹* is a strong loss-of-function or null (amorphic) allele. Several other tissues examined were not grossly affected in the mutant. The overall structure of the embryo was normal, the segment polarity gene *engrailed* was expressed in its usual segmental stripes, and the peripheral nervous system (PNS) visualized with mAb 22C10 was unaffected (Figure 1H and data not shown). There were, however, defects in the central nervous system visualized by staining with mAbBP102 including increased separation, irregularities, and occasional breaks of the longitudinal connectives and fusions and irregularities in the transverse connectives (not shown). There were also gross defects in mesodermal development that are described in detail below.

The original *stumps[1]* chromosome carried a $P[ry^+, lacZ]$ element at cytological position 82F8-9. However, chromosomal deficiencies that remove this region complemented the lethality and tracheal phenotype of *stumps[1]*, indicating that the *stumps* mutation was unlinked to the $P[ry^+, lacZ]$ insertion. The *stumps[1]* mutation was localized to cytological interval 88D3-4 to 88D8 on the right arm of chromosome III by complementa-
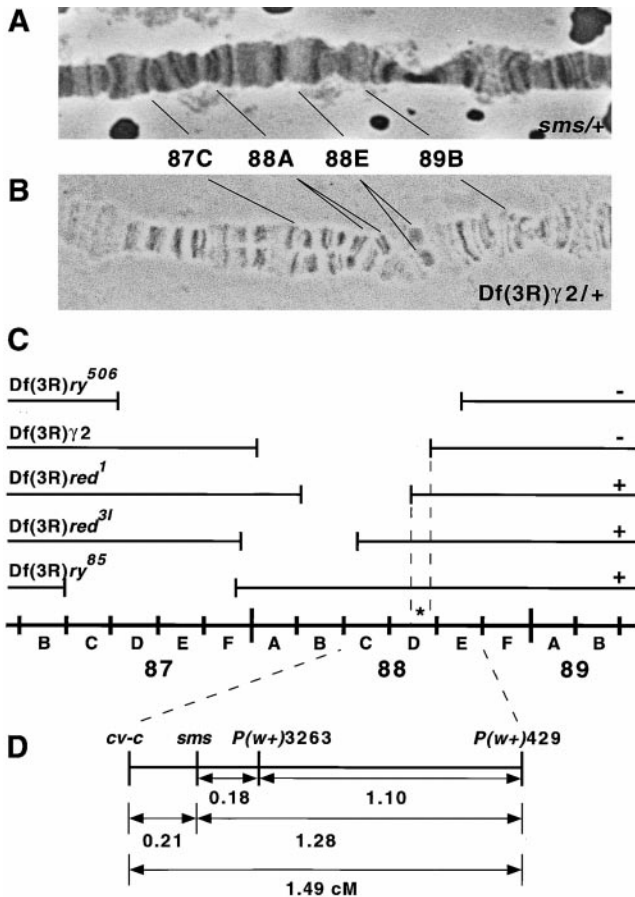

Figure 2.—Genetic mapping of *stumps*. (A) Cytology of a *stumps[1]*/+ polytene salivary gland chromosome showing the relevant region of chromosome 3. No abnormalities are visible. (B) Cytology of a Df(3R)γ2/+ polytene salivary gland chromosome. Region 88A3-12 to 88D3-8 is missing in the Df(3R)γ2 chromosome. (C) Map of the chromosomal deficiencies in the 87-88 region used to localize the *stumps* locus. (+) and (−) indicate complementation behavior of the deficiencies with *stumps[1]*. Df(3R)ry^506 (87D1-2; 88E5-6) and Df(3R)γ2 (88A3-12; 88D3-8) did not complement the *stumps[1]* tracheal phenotype or lethality, whereas Df(3R)red^3l (88B1; 88D3-4), Df(3R)ry^85 (87B15-C1; 87F10-11), and Df(3R)red^3l (87F12-14; 88C1-3) all complemented *stumps[1]*. This localizes *stumps* to the interval 88D3 to 88D8 (asterisk). (D) Recombination map showing the distances between *cv-c*, *stumps*, and $P[w^+, lacZ]0429$. The 1.28-cM interval between *stumps* and $P[w^+, lacZ]0429$ was further defined using a RFLP marker from $P[w^+, lacZ]3263$. Six of the seven crossovers analyzed occurred distal to the RFLP marker and one occurred proximally to the marker. The other RFLP markers tested (20RT and 20LT) mapped proximal to all of the recombination breakpoints and are not indicated in the figure.

tion tests with a set of chromosomal deficiencies that remove different regions of the chromosome (Figure 2, B and C). No cytological abnormalities were detected in this region of the *stumps[1]* chromosome (Figure 2A), indicating that the mutation might be a small deletion or point mutation. Meiotic recombination mapping was used to localize *stumps* with respect to other genes in the region and to several RFLP markers that we identified. This analysis placed *stumps* 0.21 cM distal to *crossveinless-c* (*cv-c*) and 0.18 cM proximal to $P[w^+, lacZ]3263$ (Figure 2D). For the rest of the experiments described here, we used a chromosome in which the $P[ry^+, lacZ]$ insertion and an unrelated lethal mutation had been recombined away from the *stumps[1]* mutation (see materials and methods).

stumps **is required for tracheal cell migration:** Cellular and molecular markers were used to characterize the tracheal defects in the *stumps[1]* mutant. The initial steps in tracheal development were normal in the mutant. The tracheal precursor cells invaginated to form the epithelial sacs at embryonic stage 11 (~6 hr AEL) and expressed the early tracheal markers *trachealess (trh)* and *breathless* normally (compare Figure 3, A and B, and data not shown). The initial activation of MAP kinase in the tracheal sacs by the EGF receptor pathway at stage 10 (Gabay *et al.* 1997a,b) was also unaffected in the mutant, as assayed by staining with antisera specific for the diphosphorylated form of MAP kinase (data not shown). *bnl* was also expressed normally in clusters of cells surrounding the sac, as shown by whole-mount embryo *in situ* hybridizations (data not shown). However, during stages 11–14 (6–11 hr AEL), when tracheal cells in the sac normally migrate toward these clusters and form primary branches (Figures 1A and 3, A and C), tracheal cells in the *stumps[1]* mutant moved very little (Figures 1B and 3, B and D).

During stages 13 and 14 (10–11 hr AEL), when secondary and terminal branch markers such as *pointed* (*pnt*), *sprouty* (*spry*), and DSRF have normally been induced by the Branchless FGF pathway in groups of cells at the ends of growing primary branches, only rare (*pnt*) or no (*spry*, DSRF) expressing cells were observed in *stumps[1]* mutant embryos (Figure 3H and data not shown). Similarly, only sporadic tracheal cells showed staining with the antiserum specific for activated MAP kinase during stages 11–12, indicating that the Branchless signaling pathway was inactive or very weakly active in the trachea at this stage (Figure 3F). During stages 15–16 (12–15 hr AEL), when primary and secondary branching are complete and the first terminal branches are forming in wild-type embryos (Figure 1C), the tracheal sacs in *stumps[1]* mutant embryos were still mostly unbranched with only an occasional rudimentary branch (Figure 1D).

stumps **interacts genetically with** *branchless***:** The tracheal phenotypes described above for the *stumps* mutant are very similar to those of null mutations in *bnl* and *btl*
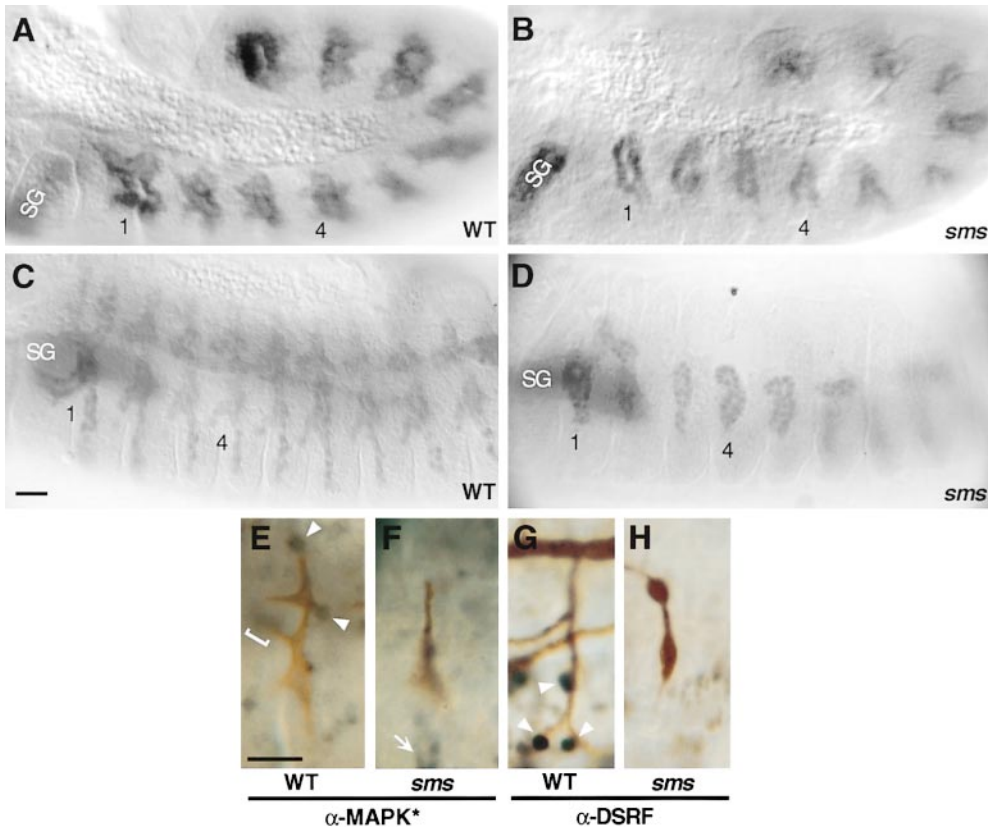
Figure 3.—Tracheal cellular and gene expression defects in the *stumps* mutant. (A) Stage 12 wild-type embryo stained for the Trachealess transcription factor expressed in the salivary gland (SG) and all tracheal cells (Tr1 and Tr4 tracheal hemi-segments are indicated). Small groups of tracheal cells have begun migrating away from the tracheal sacs to form primary branch buds. (B) Stage 12 *stumps^l* homozygote. Trachealess is expressed normally but there is little movement of tracheal cells out from the sacs. (C) Stage 14 wild-type embryo. The tracheal cells have migrated out and formed mature primary branches. (D) Stage 14 *stumps^l* homozygote. There is still very little tracheal cell migration or branch formation. Similar results were obtained with a *trachealess lacZ* enhancer trap marker that labels the cytoplasm of the tracheal cells; little cell migration and few cytoplasmic projections from the cells were seen in the mutant (not shown). (E) Close-up of a hemi-segment from a Stage 12/13 wild-type embryo double stained with an antiserum against the diphosphorylated form of ERK to show MAP kinase activation (purple) and TL-1 antiserum to show trachea (brown). Activated MAP kinase is seen in tracheal cells at the ends of the DB and DTp (arrowheads) and VB (bracket) primary branches. The VB is just deep to the plane of focus. (F) *stumps^l* homozygote at a similar stage stained as in E. No activated MAPK is seen in the trachea, although it is expressed normally in other tissues (arrow). In 23% of *stumps* mutant embryos (*n* = 17) at stages 12 or 13, weak expression was detected in an occasional tracheal cell. (G) Hemi-segment of a stage 16 wild-type embryo double stained with mAb2-161 against DSRF (purple) and mAb2A12 to the tracheal lumen (brown). Arrowheads, DSRF-expressing cells in the lateral tracheal trunk and transverse connective. (H) *stumps^l* homozygote at a similar stage stained as in G. No tracheal cells express DSRF. Bar, 20 μm.

(Klambt *et al.* 1992; Sutherland *et al.* 1996), suggesting that *stumps* might function in this FGF pathway. Because genes in the same signaling pathway commonly display dosage-sensitive interactions, we tested for an interaction between *stumps* and *bnl.* Null mutations in *bnl* are haploinsufficient, as heterozygous and hemizygous embryos show sporadic defects in primary branch outgrowth. This is manifest most significantly by the ganglionic tracheal branches, 9% of which fail to reach the CNS (Sutherland *et al.* 1996; Figure 4C). The *stumps^l* mutation is not haploinsufficient, as heterozygous embryos did not show an increase in ganglionic branch or other primary branch outgrowth defects above the low background seen in wild-type embryos (<3%; Figure 4, A and B). The *stumps* +/+ *bnl* double heterozygote, however, showed a dramatic increase in outgrowth defects, with nearly four times as many stalled ganglionic branches (33%; *n* = 460) as the *bnl* heterozygote alone (Figure 4D). In the most severe cases, all 20 ganglionic branches failed to migrate normally in the double heterozygote. The double heterozygotes also dis-

played increased dorsal branch outgrowth defects compared to *bnl* heterozygotes as well as rare dorsal trunk outgrowth defects which were never seen in *bnl* heterozygotes. Thus, *stumps* displays a dosage-sensitive genetic interaction with *bnl.* The interaction is very similar to the one previously described between *bnl* and *btl* (Sutherland *et al.* 1996), supporting the hypothesis that *stumps* functions in this FGF pathway.

Some downstream effects of Branchless FGF signaling can be activated in the *stumps* mutant: Like *bnl* and *btl* mutations, the *stumps* mutation blocks tracheal cell migration and causes failure of MAP kinase activation, loss of induction of secondary and terminal branch markers, and failure to form secondary and terminal branches. This could mean that the Branchless signaling pathway is completely inactivated by the *stumps* mutation. Alternatively, the secreted signal might fail to diffuse properly and reach the tracheal cells, or the tracheal cells might receive the initial signal but fail to migrate toward the signaling source and thus never receive enough signal to trigger the later gene inductive
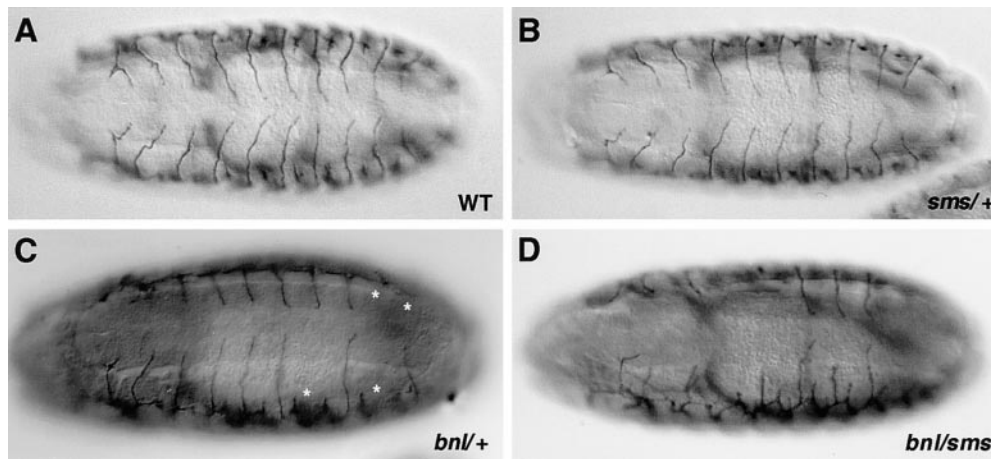
Figure 4.—Dosage-sensitive genetic interaction between *bnl* and *stumps.* (A) Ventral view of a stage 16 wild-type embryo stained with mAb2A12. The 10 pairs of ganglionic branches have grown out onto the ventral nerve cord. (B) *stumps¹/+* heterozygote. The ganglionic branches have grown out normally. (C) *bnl^{P1}/+* heterozygote. Outgrowth of 4 of the 20 ganglionic branches has stalled (asterisks). (D) A *stumps¹/bnl^{P1}* double heterozygote. Outgrowth of 14 ganglionic branches has stalled. *btl^{LG18}/+* heterozygote (Sutherland *et al.* 1996) and *stumps¹ +/+ btl^{LG18}* double heterozygotes did not show any tracheal defects (data not shown).

responses. To test whether any downstream signaling effects of the Branchless pathway were intact in the *stumps* mutant, we ectopically expressed the Branchless FGF near the tracheal sacs and monitored the tracheal response.

The Gal4/UAS system was used to express Branchless in either the mesoderm of *stumps¹* mutants beginning at stage 6 or the tracheal cells themselves beginning at stage 11. Both conditions produced similar results. All of the later FGF signaling functions assayed were strongly activated in the portions of the tracheal sacs near the ectopic Branchless-expressing cells. MAP kinase was phosphorylated in these cells (Figure 5B), DSRF expression was induced (Figure 5E), and there was significant cytoplasmic outgrowth and ramification of terminal branches (Figure 5G). Some erratic outgrowth of primary branches also occurred (Figure 5G). Similar effects of ectopic Branchless expression were observed in Df(3R)γ2 homozygotes (not shown). We also obtained very similar effects when a constitutively activated Ras protein (*Dras1^{V12}*) was expressed in the developing tracheal system of *stumps¹* mutants using a *btl*Gal4 or a *hs*Gal4 driver. Under these conditions too, downstream FGF signaling functions were activated in many or all tracheal cells (Figure 5, C, F, and H), and there was erratic outgrowth of primary branches (Figure 5H). Thus, many downstream signaling events in the Branchless FGF signaling pathway can be activated in the *stumps* mutant upon ectopic expression of the ligand or by expression of an activated Ras. Interestingly, expression of the activated Ras in wild-type embryos did not disrupt the primary branching pattern, resulting only in ectopic induction of downstream genes and in extra terminal branching (Figure 5I and data not shown).

stumps is required for mesodermal cell migration mediated by the Heartless FGF receptor: In addition to the tracheal defects, the *stumps¹* mutant displayed a severe

defect in mesodermal development. In wild-type embryos, mesodermal cells invaginate at the ventral midline at stage 6 (3 hr AEL) and migrate along the inner surface of the epidermis until reaching the dorsal margin of the epidermis two hr later at stage 10 (Michelson *et al.* 1998). The migrating cells turn on different muscle markers and differentiate into visceral, somatic, and cardiac muscles and other mesodermal derivatives. In the *stumps¹* mutant, mesodermal cells appeared to invaginate normally at the ventral midline but failed in their dorsal migrations. The migration defect was evident in stage 9 embryos where mesodermal cells stained for Tinman remained clustered near the ventral midline (Figure 6B). In wild-type embryos of the same age many of the cells have already moved out of the region (Figure 6A). In older embryos, the migration defect was manifest by the absence of the heart and most other dorsal mesoderm derivatives (Figure 6, D, F, and I). The mesodermal phenotype decreased in severity from dorsal to ventral positions (compare Figure 6I to 6H), implying that the mesodermal cells that fail to migrate can still undergo many aspects of the normal ventral muscle differentiation program.

Although the tracheal phenotype of *stumps* mutant embryos is nearly identical to those shown by *bnl* and *btl* mutants, null mutants in these genes do not display any obvious mesodermal defects. The other known FGF receptor in Drosophila, however, encoded by *heartless,* is known to be expressed in and function in the developing mesoderm. Null *htl* mutations cause a mesodermal phenotype that is indistinguishable from that of the *stumps¹* mutant in every aspect in which they have been compared. This includes all of the morphological effects described above (Beiman *et al.* 1996; Gisselbrecht *et al.* 1996; see also Figure 6, G and J) and all of the effects on marker expression that have been tested (see below). This suggests that *stumps* functions in the Heartless FGF

receptor pathway as well as the Branchless-Breathless pathway.

In the *stumps[1]* mutant, as in null *htl* mutants, mesodermal cells failed to activate MAP kinase (data not shown), the dorsal mesodermal marker Tinman was only sporadically induced (Figure 6D), and the Even-skipped marker was never induced (Figure 7B). In *htl* mutants,

the failure to induce the dorsal markers was shown to be a secondary consequence of the failure of most mesodermal cells to reach dorsal positions near the DPP inductive signal secreted by the dorsal-most ectodermal cells (Frasch 1995; Beiman *et al.* 1996; Gisselbrecht *et al.* 1996). The results described below imply a similar cause in the *stumps* mutant.

We tested whether the mesodermal defects in *stumps[1]* mutants could be ameliorated by expression of the constitutively activated Ras protein in the developing mesodermal cells using the same approach used to test for rescue of the tracheal defects. As in the tracheal system, expression of the activated Ras caused activation of MAP kinase and a general increase in motility, as well as induction of the dorsal muscle markers Even-skipped and Tinman (Figure 7D and data not shown). The effects of activated Ras in the *stumps* mutant were very similar to the reported effects of activated Ras in *htl* mutants (Gisselbrecht *et al.* 1996). We conclude that the *stumps* mutation causes severe defects in the Heartless-dependent migrations of mesodermal cells, and that some of these defects can be overcome by expression of an activated Ras.
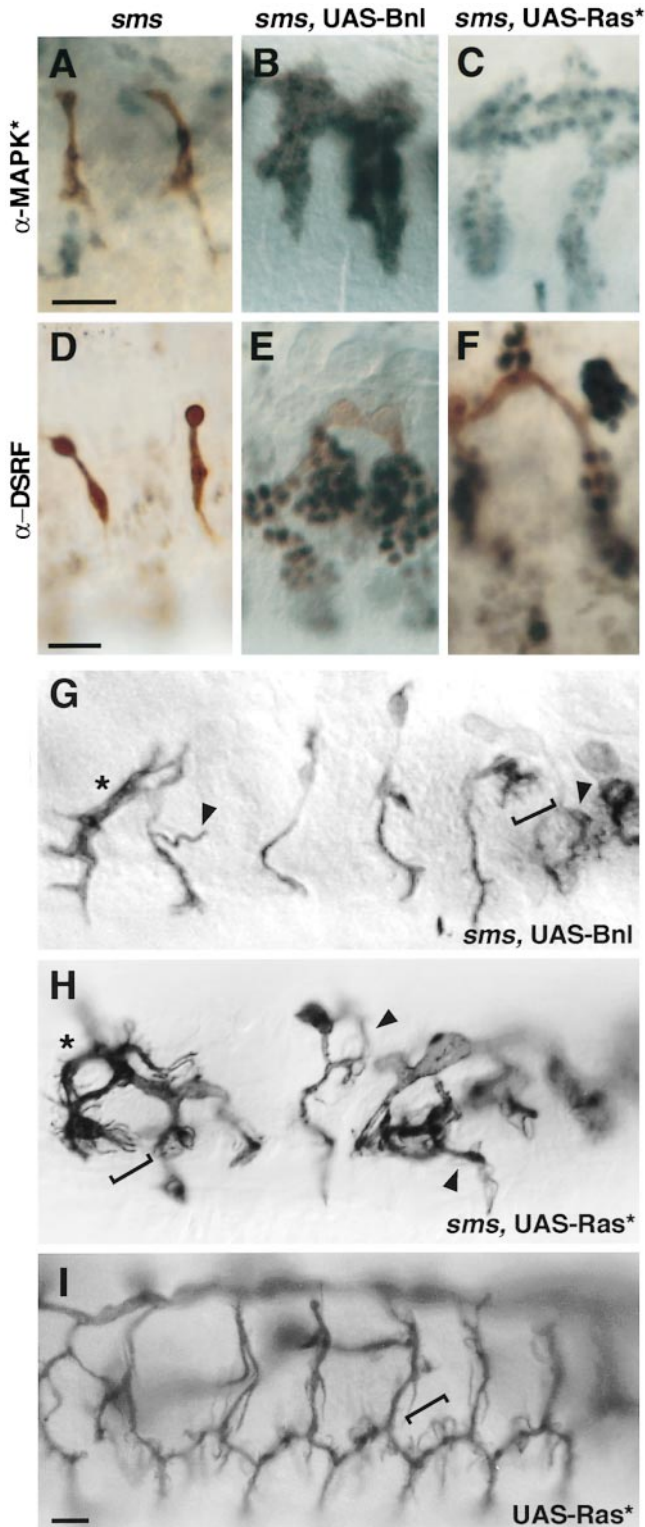
Figure 5.—Effects of ectopic expression of Branchless and an activated Ras on the trachea in the *stumps* mutant. (A) Stage 12/13 *stumps[1]/stumps[1]* embryo double stained with TL-1 for the tracheal lumen (brown) and the anti-dpERK antiserum to show MAP kinase activation (purple). No expression of activated MAP kinase is seen in the two tracheal hemi-segments shown although some can be seen in the tissues nearby. (B) UAS*bnl*B4-2/+; *twi*Gal4/+; *stumps[1]/stumps[1]* mutant embryo stained as in A. *twi*Gal4 drives UAS*bnl* expression throughout the mesoderm, which lies just deep to the developing tracheal system. Ectopic Branchless expression leads to MAP kinase activation in virtually every tracheal cell. (C) Similarly staged UAS-*Dras1[V12]*/*btl*Gal4; *stumps[1]/stumps[1]* mutant embryo stained as in A. *btl*Gal4 drives activated Ras expression throughout the developing tracheal system. Expression of the activated Ras leads to activation of MAP kinase in every tracheal cell. (D) Stage 16 *stumps[1]/stumps[1]* embryo double stained for DSRF (purple) and the mAb2A12 tracheal antigen (brown). No tracheal expression of DSRF is seen. (E) Similarly staged UAS*bnl*B4-2/+; *twi*Gal4/+; *stumps[1]/stumps[1]* embryo stained as in D. Ectopic *bnl* expression induces DSRF expression in most tracheal cells. (F) Similarly staged UAS-*Dras1[V12]*/*btl*Gal4; *stumps[1]/stumps[1]* embryo stained as in D. Expression of the activated Ras induces DSRF expression in most tracheal cells. (G) Stage 16 UAS*bnl*B4-2/+; *twi*Gal4/+; *stumps[1]/stumps[1]* embryo stained for the mAb2A12 tracheal antigen. The ectopic *bnl* expression causes erratic outgrowth of primary branches (arrowheads), occasional dorsal trunk fusion (asterisk), and sprouting of fine terminal branches (bracket). (H) Stage 16 UAS-*Dras1[V12]*/*btl*Gal4; *stumps[1]/stumps[1]* mutant embryo stained for mAb2A12 tracheal antigen. Expression of the activated Ras causes a similar constellation of effects to those caused by ectopic Branchless in G. (I) Stage 16 UAS-*Dras1[V12]*/*btl*Gal4 embryo. Expression of the activated Ras causes excessive sprouting of fine terminal branches throughout the tracheal system but does not disrupt the primary branch pattern. Bar, 20 μm.
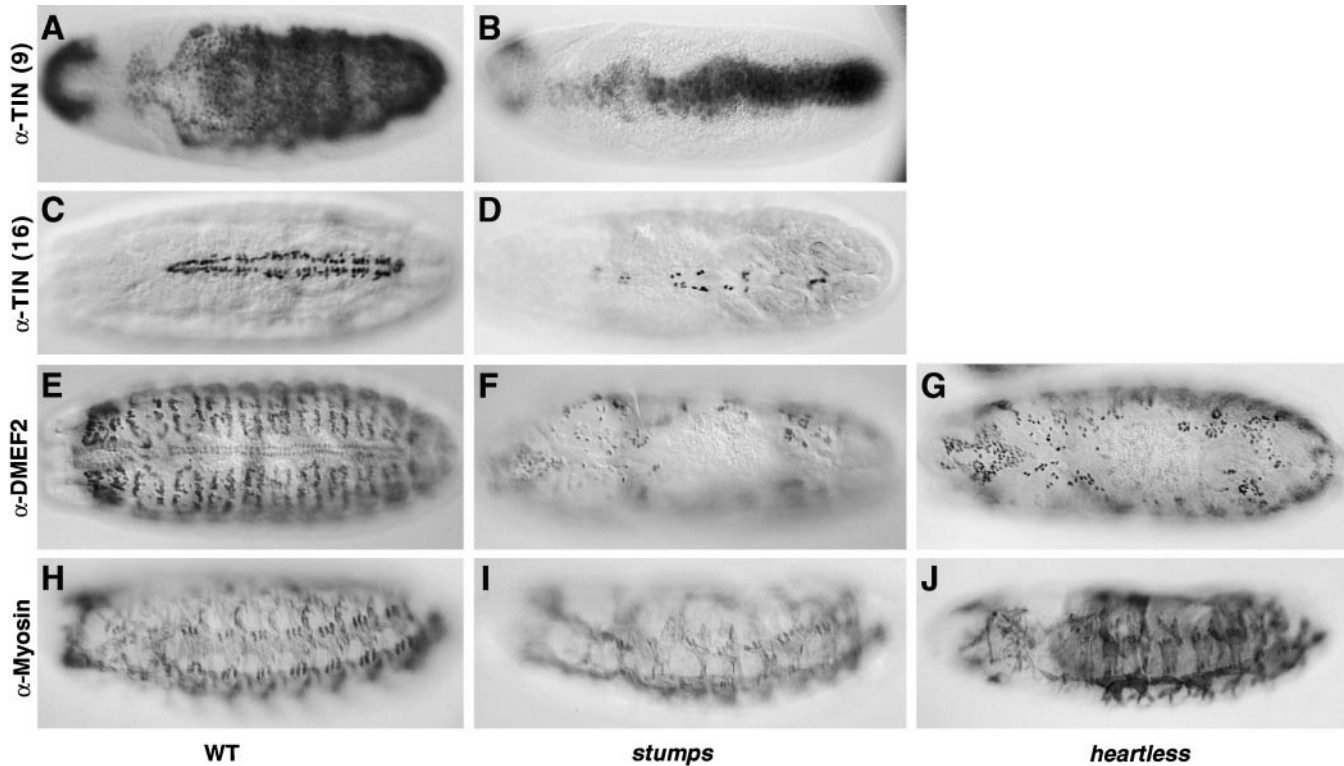
Figure 6.—Mesodermal phenotype of *stumps¹* mutant. A, C, E, and H are wild-type embryos; B, D, F, and I are *stumps¹* mutant embryos; G and J are *htl^{AB42}* mutant embryos. (A) Stage 9 wild-type embryo stained for Tinman, a mesodermal marker (ventral view). (B) Stage 9 *stumps¹* mutant stained as in A. Note the mesodermal cells are defective in their migrations out from the ventral midline. The same phenotype is seen for a null *heartless* mutation (Gisselbrecht *et al.* 1996). (C) Stage 16 wild-type embryo stained with Tinman (dorsal view). At this stage, Tinman expression is restricted to just the cardial cells of the heart at the dorsal midline. (D) Stage 16 *stumps¹* mutant stained as in C. Only very few Tinman-expressing cells are present at the dorsal midline. The same phenotype is seen for a null *htl* mutation (Beiman *et al.* 1996; Gisselbrecht *et al.* 1996). (E) Stage 16 wild-type embryo (dorsal view) stained with anti-DMEF2, a muscle cell transcription factor. The cardial cells are seen at the dorsal midline flanked by myocytes of the dorsal somatic muscles. (F) Stage 16 *stumps¹* mutant stained as in E. Most of the cardial and dorsal somatic muscle cells fail in their dorsal migrations, and only a small number of scattered DMEF2-expressing cells are seen. (G) Stage 16 *htl^{AB42}* mutant stained as in E. The null *htl* phenotype is the same as that of *stumps¹* (shown in F). (H) Stage 16 wild-type embryo (lateral view) stained for muscle myosin (mAbFMM5) to show the pattern of the muscles. (I) Stage 16 *stumps¹* mutant stained as in H. Note the almost complete absence of dorsal muscles but the presence of some of the ventral muscles. (J) Stage 16 *htl^{AB42}* mutant stained as in H. The null *htl* phenotype is virtually the same as that of *stumps¹* (see I). The *stumps¹* mutation also causes a visceral muscle phenotype that includes the absence of the second gut constriction (not shown) as does the null *htl* mutation (Gisselbrecht *et al.* 1996).

DISCUSSION

***stumps* is required for two distinct sets of embryonic cell migrations:** We have characterized the *stumps¹* mutant and shown that the gene is required for tracheal and mesodermal cell migrations. In the *stumps* mutant, tracheal precursor cells execute the initial steps of their morphogenetic program normally and invaginate to form epithelial sacs. However, the migrations of tracheal cells out from the sacs to form primary branches fail, and only an occasional stump ever develops. Similarly, the mesodermal precursor cells undergo the initial steps of their morphogenetic program normally, invaginating at the ventral midline to form the mesodermal primordium. The subsequent dorsal migrations of mesodermal cells fail, and the heart and other dorsal mesodermal derivatives are almost completely absent. Although many late tracheal and mesodermal processes fail in the *stumps* mutant, these may all be secondary to the migration failure because many of the later differentiative processes can be restored by ectopic expression of signaling pathway genes, as discussed further below. *stumps* does not appear to be a general factor required for cell migration because many other cell migrations, such as the endodermal migrations required to establish the gut, the neural migrations that form the PNS, and the spreading of the epidermal sheet during dorsal closure are all unaffected in the mutant. The two types of migrations that are affected do not have any notable properties in common with respect to migration mechanism, as the tracheal cell movements occur as part of an epithelium whereas the mesodermal migrations apparently occur by fibroblast-like movements. The only obvious shared feature of the two types of migrations affected by *stumps* is that both depend on FGF signaling pathways.

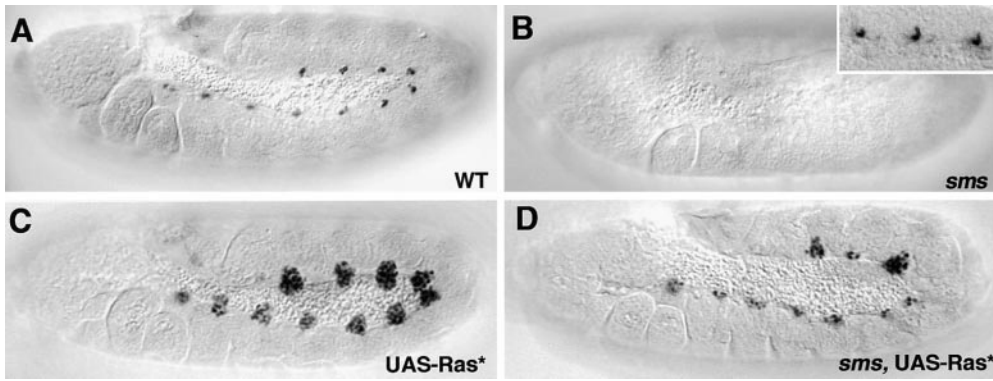***stumps* functions in FGF signaling pathways:** Three

Figure 7.—Effect of mesodermal expression of an activated Ras in the *stumps^l* mutant. (A) Stage 11 wild-type embryo stained for the Even-skipped homeodomain (EVE) transcription factor. EVE is expressed in segmentally repeated clusters of three cells. (B) *stumps^l* homozygote stained as in A. The EVE-staining mesodermal cells are absent, although normal EVE expression is detected in the CNS (inset shows CNS from same embryo). (C) UAS-*Dras1^{V12}*/*twi*Gal4 embryo stained as in A. Expression of activated Ras throughout the mesoderm increases the number of EVE-expressing cells in each mesodermal cluster (see also Gisselbrecht *et al.* 1996). (D) UAS-*Dras1^{V12}*/*twi*Gal4; *stumps^l*/*stumps^l* mutant stained as in A. Expression of activated Ras throughout the mesoderm restores some random mesodermal migrations and expression of EVE. A similar restoration in the mesoderm is seen when activated Ras is expressed in *htl* mutants (Gisselbrecht *et al.* 1996).

lines of evidence support the hypothesis that *stumps* functions in FGF signaling pathways. First, the only two processes that are known to be affected in *stumps* mutant embryos are both dependent on FGF signaling pathways. The tracheal cell migrations require the Branchless FGF, which binds and activates the Breathless FGF receptor on tracheal cells and guides their migrations. Although the ligand for the mesodermal migrations is not known, these migrations require the Heartless FGF receptor, which is expressed specifically on the migrating cells. The tracheal and mesodermal migration defects observed in the *stumps* mutant are a virtual composite of those seen in null mutants of *bnl* or *btl*, and *htl*. No mutations in any other known genes cause a phenotype that so closely mimics either the *bnl* and *btl* phenotype or that of *htl*. Second, we observed dosage-dependent genetic interactions between *bnl* and *stumps*, as are often seen for genes in the same signaling pathway. The *bnl* FGF gene is haploinsufficient as *bnl^{P1}* heterozygotes show mild tracheal outgrowth defects (Sutherland *et al.* 1996). Although *stumps^l* embryos did not show any defects when heterozygous, the *bnl^{P1}*/*stumps^l* double heterozygote showed a substantially increased severity of outgrowth defects compared to the *bnl^{P1}* heterozygote. Indeed, the genetic interaction between *bnl* and *stumps* was similar in strength to the interaction observed between *bnl* and the *btl* FGF receptor (Sutherland *et al.* 1996). Finally, we were able to obtain partial rescue of the *stumps* mutant phenotypes by expression of FGF pathway components. Some of the tracheal migration defects in the *stumps* mutant were ameliorated by ectopic expression of the Branchless FGF or by expression of a constitutively activated Ras in the tracheal cells. Similarly, expression of the activated Ras in mesodermal cells of the *stumps* mutant caused increased migration and rescue of dorsal mesodermal cell fates, just as the activated Ras partially rescues *htl* mutants (Gisselbrecht *et al.* 1996). Taken together, the results suggest that *stumps* functions as a critical component of both

the Branchless-Breathless and Heartless FGF signaling pathways.

Other cell migrations in Drosophila, such as glial cell migrations and border cell migrations in the ovary, also involve these two FGF signaling pathways (Klambt *et al.* 1992; Montell 1994; Murphy *et al.* 1995; Shishido *et al.* 1997). Aside from the involvement of FGF signaling, these other migration processes appear to share little in common with the tracheal or mesodermal migrations that require *stumps* function. The glial cell migrations are a complex set of cellular rearrangements that take place on developing CNS neurons, and the border cell migrations occur as small groups of cells that weave their way through a matrix of nurse cells to reach the oocyte. It will be interesting to determine whether *stumps* is required for all FGF-dependent cell migrations. Indeed, the CNS defects we observed in the *stumps* mutant are consistent with a role for *stumps* in both the *breathless*- and *heartless*-dependent glial cell migrations.

Models of *stumps* function in FGF signaling and cell migration: An important consideration with regard to the role of *stumps* in FGF-mediated tracheal migrations is that the gene does not appear to be required for all aspects of the tracheal FGF signaling pathway. All downstream effects of the Branchless FGF pathway we examined in addition to cell migration failed in the *stumps* mutant, including the activation of MAP kinase, the induction of secondary and terminal branching genes, and the formation of secondary and terminal branches. However, all of the gene inductive and differentiative effects could be restored by ectopic expression of the Branchless FGF or expression of the activated Ras. These treatments also induced some tracheal cell migration, although the migrations were limited in extent and did not follow the normal outgrowth pathways. Thus, the *stumps^l* mutant has the ability to generate an active FGF signal, receive the signal, and transduce it through MAP kinase and on to downstream target genes. The residual signaling capability is unlikely to

A Potentiator model   **B** Bifurcating pathway models
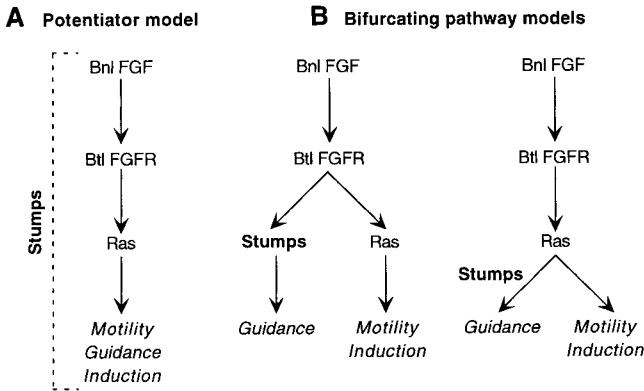

Figure 8.—Models of *stumps* function in the Branchless FGF pathway. (A) Potentiator models. *stumps* function is not absolutely required for any step in the pathway, but it serves to enhance the efficiency of one or more of the steps required for general motility, directional movement (guidance), and gene induction. (B) Bifurcating pathway models. The Branchless signaling pathway is a bifurcating pathway, and *stumps* function is required only for the branch required for directional movement. Gene induction and general motility can occur in the absence of *stumps*, but the cell movements would not be guided toward the signaling source and thus would secondarily diminish all downstream signaling events. The data do not distinguish between the two pathways shown which differ in the point of bifurcation relative to Ras.

result from leakiness of the *stumps¹* allele because *stumps¹* behaved as a null allele in genetic tests, and the same residual signaling capability was found in Df(3R)γ2 homozygotes that presumably lack the *stumps* locus.

Given that aspects of the FGF pathway are intact in the *stumps* mutant, including expression of the Branchless FGF and Breathless FGF receptor genes, we suggest two general models for how *stumps* may function in the tracheal FGF pathway. The first is that *stumps* acts as a potentiator in the pathway (Figure 8A). In this model, *stumps* is not absolutely required for any step in the process, but it is necessary to amplify or focus one or more steps. For example, it might facilitate transport or reception of the ligand, as heparan sulfate proteoglycans are believed to function in presenting the FGF ligand to FGF receptors in cultured mammalian cells (Spivak *et al.* 1994). Or, it might function like the LIN-2, LIN-7, and LIN-10 proteins in *C. elegans* to concentrate the receptor tyrosine kinase at the basal (signal receiving) side of the tracheal cells (Kaech *et al.* 1998). It could also function downstream as a scaffold that concentrates components of the signal transduction machinery, thereby amplifying the signal (Pawson and Scott 1997). If the potentiator model is correct, then our data do not allow us to more precisely place *stumps* on the FGF pathway, because overexpression of the ligand or introduction of the activated Ras could either force signaling through or bypass the signaling bottleneck caused by the absence of *stumps*.

The second general class of models for *stumps* function requires a bifurcation in the FGF signaling pathway (Figure 8B). In these models, *stumps* is required only for one fork in the pathway—the fork needed for directed cell migration (guidance). The other fork would remain intact in the *stumps* mutant. Because MAP kinase activation and downstream effector genes were able to be induced in the *stumps* mutant, FGF signal production, reception, and this fork of the signal transduction pathway would be independent of *stumps*. General cell motility would also not require *stumps*, as tracheal cells in the *stumps* mutant could be induced to move, albeit erratically, upon ectopic expression of the Branchless FGF or expression of an activated Ras. Ectopic expression of activated Ras in the wild type did not perturb directed cell migration, demonstrating that the cells continued to receive the normal FGF guidance cues under these conditions. According to the bifurcating pathway models, all of the other FGF signaling failures observed in the *stumps* mutant would be secondary consequences of the tracheal cells' inability to follow the FGF signal toward its source and to receive continuous high levels of signal. Thus, if the signal were artificially brought close to the tracheal cells, as in our ectopic expression experiments, the mutant cells could respond normally in many respects, except for proper directional migration, just as was observed.

In the bifurcating pathway models, *stumps* functions downstream of the receptor. It might serve, for example, to mark the highest point of signaling activity on the membrane of the receiving cell and couple the activated receptor to the cytoskeletal machinery required for directed cell movement. Our data, however, do not allow us to place the split in the pathway with respect to Ras (or MAP kinase), as indicated by the alternative models diagramed in Figure 8B.

Although our analysis of the mesodermal migration defects in the *stumps* mutant was more limited because the ligand for the mesodermal FGF pathway is not known, the available data also fit well with a bifurcating signal transduction pathway. Mesodermal cells in the *stumps* mutant failed in their directed migrations, although they were still able to undergo limited, apparently random migrations. Expression of activated Ras stimulated motility and restored some dorsal mesodermal development. If the bifurcation model is correct (Figure 8B), it would suggest that the Heartless and Breathless FGF receptors use related signal transduction machineries to mediate directed cell migrations. Indeed, the intracellular portion of the Heartless FGF receptor can partially rescue defective tracheal outgrowth in a Breathless FGF receptor mutant, implying common downstream effectors in migration (Reichman-Fried *et al.* 1994).

Many FGF receptors and other receptor tyrosine kinase pathways stimulate a variety of cellular effects including mitogenic, motogenic, and differentiative re-

sponses (Johnson and Williams 1993; Thomas 1993; Martin 1998). There is growing evidence that directed cell migrations are mediated by a special branch in RTK signal transduction pathways that is not shared by the mitogenic and differentiative responses (Lee *et al.* 1996a; Sundaram *et al.* 1996). Various intracellular signaling molecules including Ras, Rac, phospholipase C-γ, phosphoinositide-3′ kinase, and Src have been implicated in signal transduction processes associated with migration (Anand and Zetter 1997). Molecular characterization of the *stumps* gene and biochemical analysis of its products should help elucidate the FGF signal transduction pathways that control tracheal and mesodermal cell migrations and may provide insight into how these and other RTK pathways guide cell movements.

Note added in proof: Complementation tests demonstrate that two recently described genes, *Dof* (Vincent, S., R. Wilson, C. Coelho, M. Affolter and M. Leptin, 1998, The Drosophila protein Dof is specifically required for FGF signaling. Mol. Cell **2:** 515–525) and *heartbroken* (Michelson, A. M., S. Gisselbrecht, E. Buff and J. B. Skeath, 1998, Heartbroken is a specific downstream mediator of FGF receptor signalling in Drosophila. Development **125:** 4379–4389), are allelic to *stumps*.

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Communicating editor: R. S. Hawley