Development 126, 3715-3723 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV7718

Heparan sulfate proteoglycans are essential for FGF receptor signaling during *Drosophila* embryonic development

Xinhua Lin[1], Eugene M. Buff[2,*], Norbert Perrimon[1] and Alan M. Michelson[2,‡]

[1]Department of Genetics and Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115, USA
[2]Division of Genetics, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School and Howard Hughes Medical Institute, Boston, MA 02115, USA
*Present address: Massachusetts General Hospital Cancer Center, Building 149, 13th Street, Charlestown, MA 02129, USA
‡Author for correspondence (e-mail: michelson@rascal.med.harvard.edu)

Accepted 16 June; published on WWW 5 August 1999

SUMMARY

The *Drosophila sugarless* and *sulfateless* genes encode enzymes required for the biosynthesis of heparan sulfate glycosaminoglycans. Biochemical studies have shown that heparan sulfate glycosaminoglycans are involved in signaling by fibroblast growth factor receptors, but evidence for such a requirement in an intact organism has not been available. We now demonstrate that *sugarless* and *sulfateless* mutant embryos have phenotypes similar to those lacking the functions of two *Drosophila* fibroblast growth factor receptors, Heartless and Breathless. Moreover, both Heartless- and Breathless-dependent MAPK activation is significantly reduced in embryos which fail to synthesize heparan sulfate glycosaminoglycans. Consistent with an involvement of Sulfateless and Sugarless in fibroblast growth factor receptor signaling, a constitutively activated form of Heartless partially rescues *sugarless* and *sulfateless* mutants, and dosage-sensitive interactions occur between *heartless* and the heparan sulfate glycosaminoglycan biosynthetic enzyme genes. We also find that overexpression of Branchless, the Breathless ligand, can partially overcome the requirement of Sugarless and Sulfateless for Breathless activity. These results provide the first genetic evidence that heparan sulfate glycosaminoglycans are essential for fibroblast growth factor receptor signaling in a well defined developmental context, and support a model in which heparan sulfate glycosaminoglycans facilitate fibroblast growth factor ligand and/or ligand-receptor oligomerization.

Key words: *Drosophila*, FGF, Heparan sulfate glycosaminoglycan, Mesoderm, Trachea, Cell migration

INTRODUCTION

The fibroblast growth factors (FGFs) constitute a large family of proteins that act as key intercellular signals in a wide range of developmental and pathological processes (Hanahan and Folkman, 1996; Martin, 1998; Wilkie et al., 1995; Yamaguchi and Rossant, 1995). The biological functions of FGFs include the regulation of cell proliferation, differentiation, survival, motility and tissue patterning. Both biochemical and genetic studies demonstrate that FGFs relay their signals through high affinity transmembrane protein tyrosine kinase receptors (Mason, 1994; Wilkie et al., 1995). By binding to the extracellular domains of these receptor tyrosine kinases (RTKs), FGFs induce the formation of receptor homo- or heterodimers. FGF receptor (FGFR) oligomerization results in RTK transphosphorylation followed by the activation of a Ras-dependent intracellular signal transduction pathway (Fantl et al., 1993).

In addition to its high affinity receptor, biochemical studies indicate that heparin/heparan sulfate proteoglycans (HSPGs) act as low affinity FGF co-receptors that facilitate FGF signal transduction (Mason, 1994; Ornitz et al., 1992; Rapraeger et al., 1991; Schlessinger et al., 1995; Spivak-Kroizman et al., 1994; Yayon et al., 1991). HSPGs are ubiquitous macromolecules that are associated with the cell surface and with the extracellular matrix (Bernfield et al., 1992; David, 1993; Kjellén and Lindahl, 1991; Yanagishita and Hascall, 1992). HSPGs consist of a protein core to which heparin/heparan sulfate glycosaminoglycan (HS GAG) is attached. The function of HSPGs in FGF signaling is mediated by HS GAG chains which are usually highly sulfated and negatively charged. While the precise mechanism by which HS GAGs participate in FGFR activation remains unclear, a variety of biochemical studies suggest that HS GAGs may stabilize or induce the formation of FGF dimers or a ternary complex composed of ligand plus high and low affinity receptors (DiGabriele et al., 1998; Faham et al., 1996; Herr et al., 1997; Ornitz et al., 1992, 1995; Spivak-Kroizman et al., 1994; Venkataraman et al., 1996; Zhu et al., 1993). Although there is strong in vitro evidence implicating HS GAGs in FGFR signaling, there is as yet no in vivo genetic support for this hypothesis.

Two FGFRs, Heartless (Htl) and Breathless (Btl), have been characterized in *Drosophila*. Genetic analyses have established

that each of these receptors has distinct expression patterns and developmental functions during embryogenesis. Htl is expressed uniformly in the early embryonic mesoderm where it is required for the normal dorsolateral migration of mesodermal cells following gastrulation (Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998b; Shishido et al., 1993, 1997). Htl expression is modulated after mesoderm migration is complete, and its continued activity is essential for the specification of particular cardiac and muscle cell fates (Carmena et al., 1998; Michelson et al., 1998b). Btl is expressed in the tracheal system as well as in a subset of cells in the CNS midline (Klämbt et al., 1992; Shishido et al., 1993). Both of these cell types depend on Btl for their specific patterns of migration. For example, in *btl* mutant embryos, the primary specification of tracheal cells is normal but these cells fail to migrate properly, leading to profound defects in the formation of the tracheal tree (Klämbt et al., 1992). Btl also is required for the determination of specialized cells at the ends of primary tracheal branches that initiate the formation of higher order branching (Lee et al., 1996; Reichman-Fried and Shilo, 1995). While the ligand for Htl has not yet been identified, the Btl ligand is encoded by *branchless* (*bnl*; Sutherland et al., 1996). Mutations in *bnl* are associated with defects in tracheal morphogenesis that are virtually identical to those seen in *btl* mutants. Bnl is expressed dynamically in clusters of cells that are positioned so as to guide the outgrowth and subsequent branching of neighboring tracheal cells. Ectopic expression of Bnl can redirect tracheal cell outgrowth and branch formation, substantiating the hypothesis that spatially localized activation of the Btl receptor is necessary for normal tracheal morphogenesis (Lee et al., 1996; Sutherland et al., 1996). The characterization of FGFRs and their ligands by a combination of genetic as well as molecular approaches in *Drosophila* provides a very useful paradigm for studying the biological functions of FGFs and for identifying other genetic components involved in FGF signaling.

We have recently identified and characterized two *Drosophila* mutations, *sugarless* (*sgl*, also known as *kiwi* and *suppenkasper*; Binari et al., 1997; Häcker et al., 1997; Haerry et al., 1997) and *sulfateless* (*sfl*; Lin and Perrimon, 1999) which encode the homologs of UDP-D-glucose dehydrogenase and heparin/heparan sulfate N-deacetylase/N-sulfotransferase, respectively. These enzymes are critical for the biosynthesis and modification of HS GAGs, and the corresponding mutants provide an in vivo model for examining the involvement of these molecules in FGFR signaling. In particular, we have used these mutants to test the hypothesis that HSPGs act as FGF co-receptors by determining whether *sfl* or *sgl* null embryos exhibit phenotypes characteristic of the high affinity FGFR mutants, *htl* and *btl*, whether *sfl* and *sgl* interact genetically with *htl*, and whether Htl- and Btl-dependent signaling pathways are activated in *sfl* and *sgl* null embryos. In this report, we demonstrate that the complete loss of both the maternal and zygotic components of either *sfl* or *sgl* expression is associated with profound defects in mesoderm and tracheal cell migration. Moreover, both the Htl- and Btl-dependent activation of MAPK that is seen normally in migrating mesodermal and tracheal cells is undetectable in *sfl* and *sgl* mutant embryos. These findings provide the first genetic evidence that HS GAGs are essential for signaling by FGFRs during embryonic development.

MATERIALS AND METHODS

Drosophila strains

The following *Drosophila* strains were employed: htl^{AB42}, htl^{YY262} (Gisselbrecht et al., 1996; Michelson et al., 1998a), $sfl^{l(3)03844}$ (Lin and Perrimon, 1999), $sgl^{l(3)08310}$ (Häcker et al., 1997), btl^{LG19} (Klämbt et al., 1992), bnl^{P1} (Sutherland et al., 1996), wg^{CX4} (Bejsovec and Wieschaus, 1993; van den Heuvel et al., 1993), *69B*-Gal4 (Brand and Perrimon 1993), *twi*-Gal4 (Greig and Akam, 1993), UAS-Bnl (Sutherland et al., 1996), UAS-Htl^{Act} (Michelson et al., 1998a) and *trh-lacZ* (Wilk et al., 1996). htl^{AB42}, $sfl^{l(3)03844}$, $sgl^{l(3)08310}$, btl^{LG19}, bnl^{P1} and wg^{CX4} are all null alleles by genetic and/or molecular criteria. *Oregon R* was used as a wild-type reference strain.

Generation of females with *sfl* and *sgl* germline clones

Females with germline clones were generated using the autosomal 'FLP-DFS' technique (Chou and Perrimon, 1996). Briefly, virgin females of the genotype *sfl* (or *sgl*) FRT^{2A}/*TM3, Sb* were mated with males of the genotype *y w* FLP^{22}/+, FRT^{2A} $P[ovo^{D1}]$/*TM3, Sb*. The resulting progeny were heat shocked at 37°C for 2 hours during the larval stages of development, and *y w* FLP^{22}/+; *sfl* (or *sgl*) FRT^{2A}/FRT^{2A} $P[ovo^{D1}]$ females carrying *sfl* (or *sgl*) homozygous germline clones were selected. Such females were mated to *sfl* (or *sgl*)/*TM3-ftzlacZ* males, and maternal/zygotic null embryos were identified by the absence of *lacZ* expression.

Antibody staining

Fixation of embryos, antibody staining and embryo sectioning were performed as described by Gisselbrecht et al. (1996); Michelson et al. (1998a); Patel (1994). Anti-tracheal lumen antibody mAb2A12 was obtained from the Developmental Studies Hybridoma Bank, anti-β-galactosidase antibody from Cappel and Promega, and anti-diphospho-MAPK antibody from Sigma.

Ectopic expression experiments

Targeted ectopic expression was accomplished using the Gal4/UAS system (Brand and Perrimon 1993). Chromosomes bearing Gal4 and UAS insertions were combined with appropriate mutations using standard genetic crosses. Mesodermal and ectodermal expression were achieved with *twi*-Gal4 and *69B*-Gal4 lines, respectively, in both wild-type and mutant genetic backgrounds.

Quantitation of mesodermal phenotypic severity

The hypomorphic htl^{YY262} mutation was recombined with null alleles of *sfl* and *sgl*. Embryos from stocks containing these recombinant chromosomes maintained over a *lacZ*-marked balancer were collected and double stained with antibodies against both Eve and β-galactosidase. Embryos homozygous for the zygotic loss of both genes were identified as lacking *lacZ* expression. Identical experiments were undertaken with each of the single zygotic mutants, and Eve expression in dorsal mesodermal cells was quantitated for each genotype, as previously described (Michelson et al., 1998a,b). The statistical significance of the difference in Eve expression for each pairwise comparison was calculated using both one-tailed *z*- and *t*-tests.

RESULTS

The genetics of HSPG biosynthesis in *Drosophila*

In a screen to characterize the maternal effects of zygotic lethal mutations (Perrimon et al., 1996), two mutants, *sfl* and *sgl*, were isolated on the basis of their abnormal embryonic segmentation phenotypes. Embryos lacking maternal germline-derived *sfl* or *sgl* activity, as well as the paternally

derived zygotic activity of these genes (referred to hereafter as *sfl* or *sgl* null embryos), exhibit a cuticle phenotype similar to that of the *wingless* (*wg*) segment polarity mutant (Binari et al., 1997; Häcker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999). *sgl* encodes a homolog of bovine UDP-glucose dehydrogenase (Hempel et al., 1994) which catalyzes the conversion of UDP-D-glucose to UDP-D-glucuronic acid, an essential substrate for GAG biosynthesis. Consistent with a critical role for Sgl in the synthesis of proteoglycans in *Drosophila*, prior biochemical experiments have established that Syndecan and Dally/Glypican lack GAG chains in *sgl* null embryos (Haerry et al., 1997) or homozygous *sgl* zygotic mutant third instar larvae (Tsuda et al., 1999), respectively. Moreover, injection of either UDP-glucuronic acid or heparan sulfate into *sgl* null embryos rescues the *wg*-like segment polarity defect, and injection of heparinases I and III (but not chondroitinase ABC) into wild-type embryos phenocopies loss of *sgl* function (Binari et al., 1997). These findings provide strong evidence that Sgl is essential for the biosynthesis of HS GAG chains which, in turn, are required for Wg signaling.

Drosophila Sfl (Lin and Perrimon, 1999) has 51% and 53% amino acid identity to rat and mouse heparin/heparan sulfate N-deacetylase/N-sulfotransferase, respectively (Eriksson et al., 1994; Hashimoto et al., 1992; Orellana et al., 1994). This enzyme catalyzes the N-deacetylation and N-sulfation of polymerized heparan (as opposed to chondroitin or deramatan) GAGs, the key step that initiates further GAG modifications (for review see Kjellén and Lindahl, 1991). Since the sulfation and epimerization of HS GAGs provide structural identity as well as the negative charge that is critical for their interaction with proteins, loss of Sfl activity will result in the generation of unmodified HS GAGs, thereby impairing normal HSPG functions. Indeed, similar to the case in *sgl* mutants (Tsuda et al., 1999), the HS GAG-modified Dally protein is markedly reduced in *sfl* zygotic mutant larvae, indicating that, in the absence of Sfl activity, HS GAG biosynthesis is abnormal (Lin and Perrimon, 1999). Together with the strong *wg*-like segment polarity phenotype associated with loss of *sfl* function, these results provide compelling evidence that Sfl is essential for the production of proteoglycans involved in Wg signaling (Lin and Perrimon, 1999). Moreover, the specificity of Sfl for heparan-containing GAGs distinguishes the type of GAG chain that participates in this signaling pathway. In summary, phenotypic analyses of *sfl* and *sgl* mutants can be used to study the roles of HSPGs in normal developmental processes.

sfl and *sgl* mutants phenocopy the mesoderm migration defect associated with loss of *htl* function

After invaginating through the ventral furrow at gastrulation, Twist- (Twi) expressing mesodermal cells migrate along the ectoderm in a dorsolateral direction. By late stage 9, the mesoderm is composed of an inner monolayer of cells that extends from the ventral midline to the dorsal edge of the ectoderm (Bate, 1993; Leptin, 1995; Fig. 1A,E). In *htl* mutant embryos, gastrulation is normal but mesoderm migration fails to occur properly, resulting in an irregular dorsal margin of Twi-positive cells and a relative accumulation of these cells in ventral and lateral positions (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997; Michelson et al., 1998b; Fig. 1B,F). A similar phenotype occurs in *sfl* and *sgl* null embryos (Fig. 1C,D,G,H). Of note, mesoderm migration is normal in *wg* mutant embryos (data not shown), suggesting that this effect of *sfl* and *sgl* is not due to an influence on Wg signaling, as is the case for the segmentation effects of these genes (Binari et

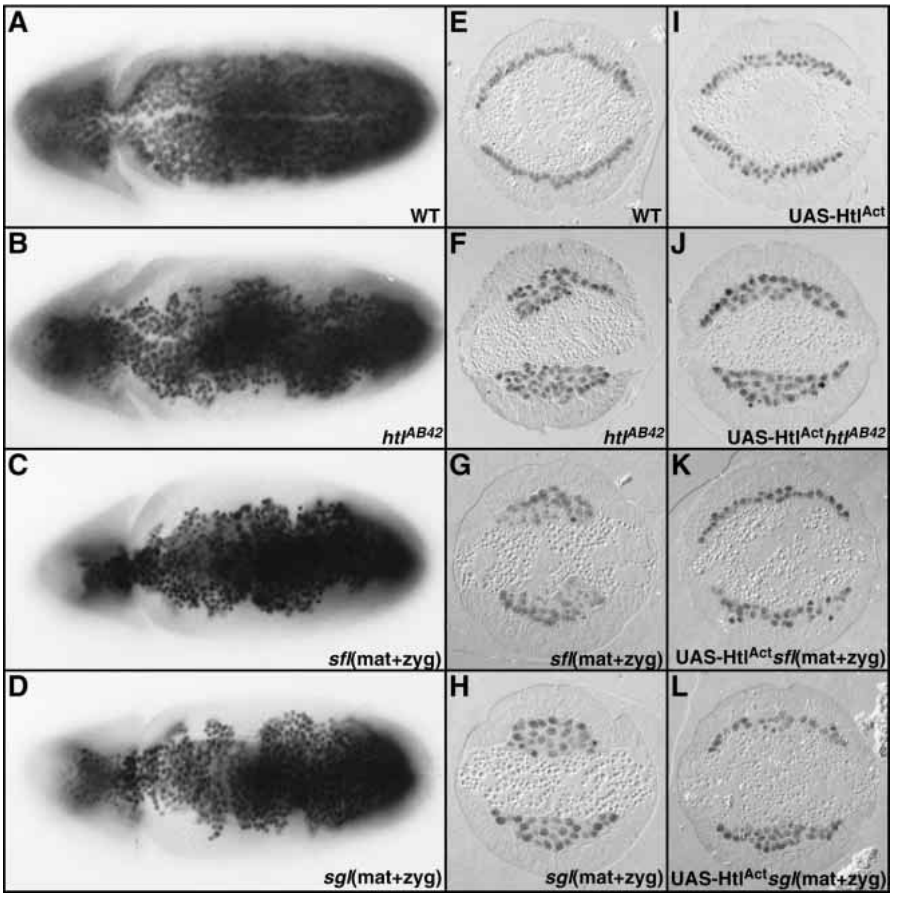

Fig. 1. *sfl* and *sgl* are required for the dorsolateral migration of the embryonic mesoderm, a requirement that is partially bypassed by constitutive Htl signaling. (A-D) Ventral views of late stage 9 embryos of the indicated genotypes immunostained for Twi expression. mat+zyg: an embryo from a germline clone female that has also inherited a mutant paternal chromosome. These embryos therefore lack both the maternal and zygotic functions of *sfl* or *sgl*. (E-H) Transverse sections of Twi-stained early stage 10 embryos of the indicated genotypes. Whereas Twi-positive mesodermal cells have reached the dorsal ectoderm in wild type, mesoderm migration fails to occur properly in null *htl* as well as in maternal/zygotic null *sfl* and *sgl* embryos. (I-L) *twi*-Gal4-mediated ectopic expression of a constitutively activated form of Htl partially rescues the mesoderm migration defects of null *htl* and maternal/zygotic null *sfl* and *sgl* embryos, but has no effect on migration in an otherwise wild-type genetic background (I).

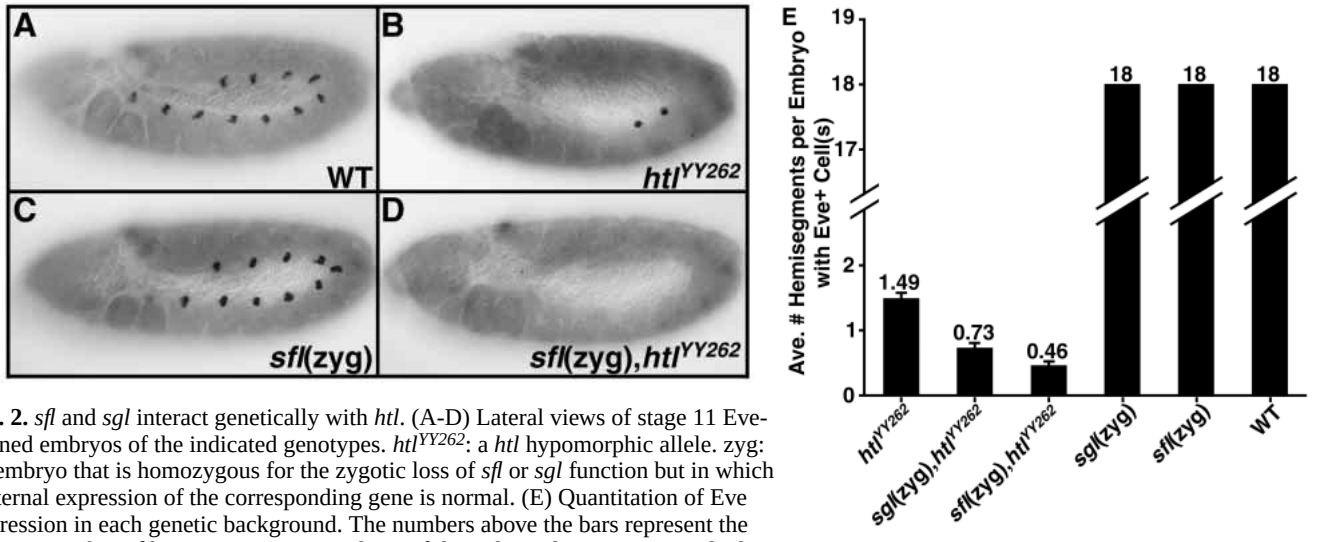

Fig. 2. *sfl* and *sgl* interact genetically with *htl*. (A-D) Lateral views of stage 11 Eve-stained embryos of the indicated genotypes. htl^{YY262}: a *htl* hypomorphic allele. zyg: an embryo that is homozygous for the zygotic loss of *sfl* or *sgl* function but in which maternal expression of the corresponding gene is normal. (E) Quantitation of Eve expression in each genetic background. The numbers above the bars represent the average number of hemisegments per embryo of the indicated genotypes in which Eve-positive cells develop. Each error bar indicates the standard error of the mean for that genotype. Eve expression was scored in the second and third thoracic and first seven abdominal segments on both sides of each embryo. At least 80 embryos of each genotype were scored. The Eve pattern is unaltered by zygotic loss of either *sfl* or *sgl* due to the normal maternal expression of these genes. In a *htl* hypomorph, only a partial reduction in Eve expression occurs, a phenotype that is significantly ($P<10^{-5}$) enhanced by the zygotic loss of either *sfl* or *sgl*.

al., 1997; Häcker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999).

The finding that mesoderm migration depends on *sfl* and *sgl*, as well as on *htl*, raises the possibility that HS GAG biosynthesis is required for signaling by the Htl FGFR. If, as has been suggested, HS GAGs participate in the activation of FGFRs (Ornitz et al., 1992; Rapraeger et al., 1991; Schlessinger et al., 1995; Yayon et al., 1991), then a constitutively active form of Htl should bypass the requirement of *sfl* and *sgl* for mesoderm migration. We tested this hypothesis by targeting the expression of activated Htl to the mesoderm of *sfl* or *sgl* null embryos using the Gal4/UAS system (Brand and Perrimon, 1993). We previously reported the construction of such an activated receptor in which the extracellular domain of wild-type Htl is replaced by the dimerization domain of the bacteriophage λ cI repressor (Michelson et al., 1998a). As with other RTKs, this manipulation generates constitutive, ligand-independent receptor activity (Lee et al., 1996; Michelson et al., 1998a; Queenan et al., 1997). In otherwise wild-type embryos, *twi*-Gal4-mediated ectopic expression of activated Htl has no significant effect on the migration of mesodermal cells (Michelson et al., 1998a; Fig. 1I). However, activated Htl is able to weakly restore mesoderm migration in a null *htl* mutant (Michelson et al., 1998a; Fig. 1J). Similarly, activated Htl partially rescues the migration defect of *sfl* and *sgl* null embryos (Fig. 1K,L). Only partial rescue is seen in all mutant backgrounds due to the relatively weak constitutive activation of Htl that is achieved by spontaneous dimerization (Michelson et al., 1998a). In addition, constitutive Htl does not reproduce the graded activity of this receptor that occurs during normal mesoderm migration (Gabay et al., 1997b; Michelson et al., 1998a). The timing of constitutive Htl expression induced by *twi*-Gal4 is unlikely to be a contributing factor since the equivalent expression of wild-type Htl completely rescues a null *htl* allele (Michelson et al., 1998b). In summary, these genetic epistasis experiments suggest that Htl acts downstream of Sfl and Sgl in migrating mesodermal cells.

If HS GAGs participate in the activation of Htl, then *sfl* and *sgl* should exhibit dosage-sensitive genetic interactions with *htl*. We investigated this possibility using a quantitative assay that is based on the expression of *even skipped* (*eve*) in dorsal mesodermal progenitor cells (Buff et al., 1998; Carmena et al., 1998; Frasch et al., 1987; Michelson et al., 1998a,b). The development of these Eve-expressing cardiac and somatic muscle cells depends on Htl for both mesoderm migration and cell fate specification. In wild-type embryos, Eve is found in segmentally repeated groups of cells that are confined to the dorsal mesoderm (Fig. 2A). All such cells are missing in a null *htl* mutant (Gisselbrecht et al., 1996). However, in a *htl* hypomorph, mesoderm migration and subsequent cell fate specification are only partially disrupted, permitting some dorsal Eve expression to occur (Fig. 2B; Michelson et al., 1998b). In embryos in which only the zygotic activity of *sfl* or *sgl* is absent, Eve expression is entirely normal, reflecting the strong maternal contribution of these genes (Fig. 2C and data not shown). However, when complete zygotic loss of *sfl* or *sgl* is combined with homozygosity for the *htl* hypomorphic allele, the severity of the Eve phenotype is enhanced (Fig. 2D). Quantitation of the number of Eve-positive hemisegments in *htl*, *sfl*, *sgl*, *htl sfl* and *htl sgl* embryos demonstrated that the interactions between *htl* and both *sfl* and *sgl* are highly significant (Fig. 2E; $P<10^{-5}$). These results, combined with the above findings that *htl* is epistatic to *sfl* and *sgl*, are consistent with the hypothesis that HS GAGs are essential for Htl activation during mesoderm migration in the *Drosophila* embryo.

sfl and *sgl* are required for Btl-dependent tracheal cell migration

Given the genetic evidence that Sfl and Sgl are required for Htl FGFR signaling in the mesoderm, we next determined whether

HS GAG biosynthesis is also involved in signaling by Bnl and Btl during trachea development. Since Wg function is compromised in *sgl* and *sfl* null mutant embryos, and *wg* loss-of-function itself leads to abnormal tracheal morphogenesis secondary to an accompanying segmentation defect (see below), it is difficult to solely correlate the tracheal cell migration phenotype of *sgl* and *sfl* null mutants with Bnl/Btl signaling. We therefore analyzed tracheal cell migration in embryos deficient only in the zygotic functions of *sfl* and *sgl*. Segmentation and the expression of Engrailed are normal in such embryos, indicating that Wg signaling is unaffected (Perrimon et al., 1996; Häcker et al., 1997; Lin and Perrimon, 1999).

The tracheal system of the *Drosophila* embryo forms by a sequential series of branching steps that can be visualized by following the expression of an enhancer trap in the *trachealess* (*trh*) gene (Isaac and Andrew, 1996; Wilk et al., 1996; Fig. 3A). In stage 13 embryos homozygous for loss of the zygotic functions of either *sfl* or *sgl*, the early steps in tracheal branching are significantly perturbed (Fig. 3B,C). By late stage 15, wild-type embryos have developed an extensive tracheal network in which the dorsal and lateral trunk branches have fused and additional primary and secondary branches have formed (Samakovlis et al., 1996; Fig. 3D). In either *sfl* or *sgl* zygotic mutants, tracheal branch formation is incomplete, as revealed by the presence of large gaps in the dorsal and lateral trunks, as well as stalled ganglionic branches (Fig. 3E,F). The penetrance of this phenotype is incomplete and the expressivity is variable in both *sfl* and *sgl* zygotic mutants; 16% of *sfl* (n=245) and 7% of *sgl* (n=198) zygotic mutant embryos exhibit some degree of abnormal tracheal morphogenesis, ranging from one to all segments having breaks in the dorsal trunk. In contrast, in *btl* and *bnl* null mutant embryos, tracheal cell migration is more severely affected and virtually no branches form from the initial tracheal invaginations (Klämbt et al., 1992; Sutherland et al., 1996; Fig. 3G). The partial disruption of the tracheal tree seen with zygotic loss of *sfl* and *sgl* is reminiscent of the defects observed in hypomorphic mutants of *btl*, *bnl* and *heartbroken* (*hbr*)/*downstream of FGF receptor* (*dof*)/*stumps*, a gene that encodes a specific effector of FGF receptor signaling (Klämbt et al., 1992; Michelson et al., 1998a; Sutherland et al., 1996; Vincent et al., 1998; Imam et al., 1999). The relatively weak zygotic *sfl* and *sgl* tracheal phenotypes are most likely due to partial rescue by the maternal expression of these genes, as previously noted for their mesodermal activities. The more severe tracheal defects that occur in *sfl* and *sgl* null embryos are consistent with this suggestion (see below). In summary, the present findings implicate Sfl- and Sgl-dependent HS GAG biosynthesis in signaling by the Btl FGFR.

Htl- and Btl-dependent MAPK activation depends on *sfl* and *sgl*

Htl and Btl are RTKs that transduce their intracellular signals by the conserved Ras/MAPK cascade (Cobb and Goldsmith, 1995; Seger and Krebs, 1995). As a result, RTK activity can be visualized in developing tissues with an antibody specific for the diphosphorylated, activated form of MAPK (Gabay et al., 1997a,b). Using this approach, diphospho-MAPK is observed at the leading edge of the migrating mesoderm in wild-type embryos (Gabay et al., 1997b; Michelson et al., 1998a; Vincent et al., 1998; Fig. 4A). In either *htl* or *hbr/dof/stumps* mutants, this expression of diphospho-MAPK is undetectable (Michelson et al., 1998a; Vincent et al., 1998; Fig. 4B). Similarly, the Htl-dependent mesodermal localization of diphospho-MAPK is below the level of detection in both *sfl* and *sgl* null embryos (Fig. 4C,D). In contrast, epidermal growth factor receptor (EGFR)-dependent MAPK activation in the ventral epidermis and amnioserosa does not require either *sfl* or *sgl*, consistent with the specific involvement of these genes in FGFR signaling.

During stage 11, MAPK is activated by Btl in the tracheal pits (Gabay et al., 1997b; Fig. 4E). As expected, this expression of diphospho-MAPK is markedly reduced in *btl* and *bnl* mutant embryos (Fig. 4F,G). The FGFR-specific signal transducer Hbr/Dof/Stumps also is required for MAPK activation in the tracheal pits (Michelson et al., 1998a; Vincent et al., 1998). In contrast, in *wg* mutants MAPK activation by Btl is unaffected, although the normal spacing between the tracheal pits is reduced due to the associated segmentation defect (Fig. 4H). As with *btl*, *bnl* and *hbr/dof/stumps*, Btl-dependent MAPK activation is significantly decreased in *sfl* or *sgl* null embryos (Fig. 4I,J), whereas the earlier EGFR-dependent expression of

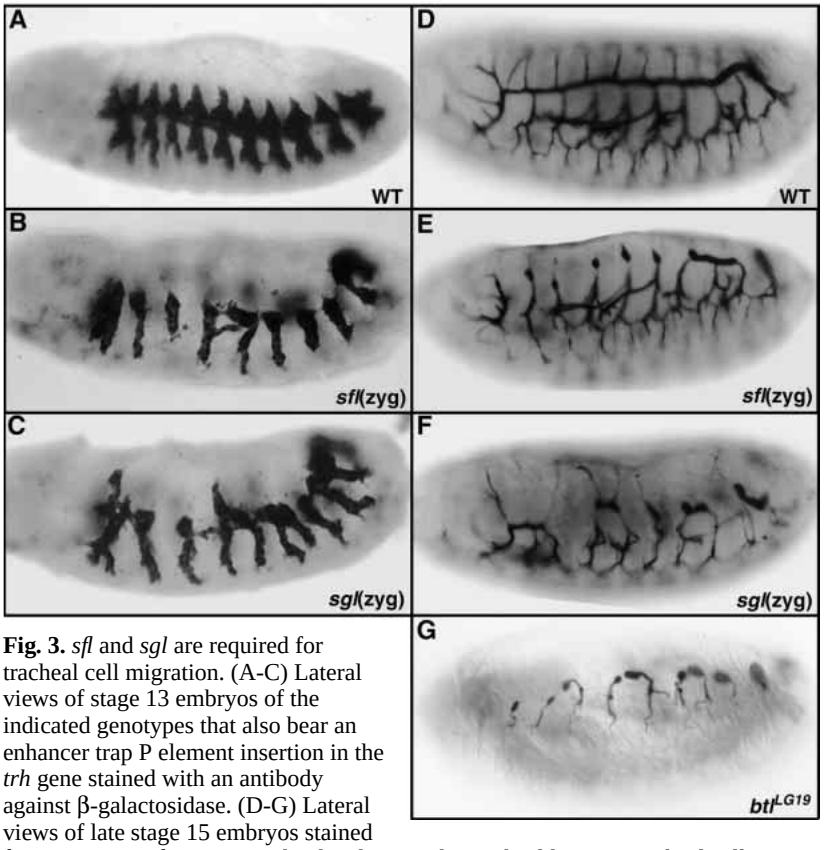

Fig. 3. *sfl* and *sgl* are required for tracheal cell migration. (A-C) Lateral views of stage 13 embryos of the indicated genotypes that also bear an enhancer trap P element insertion in the *trh* gene stained with an antibody against β-galactosidase. (D-G) Lateral views of late stage 15 embryos stained for expression of an antigen that localizes to the tracheal lumen. Tracheal cell migration is abnormal in embryos deficient in the zygotic functions of *sfl* and *sgl*. This phenotype is incompletely penetrant and its expressivity is variable (see text for details). The embryos shown in panels B,C,E and F represent phenotypes of intermediate severity.

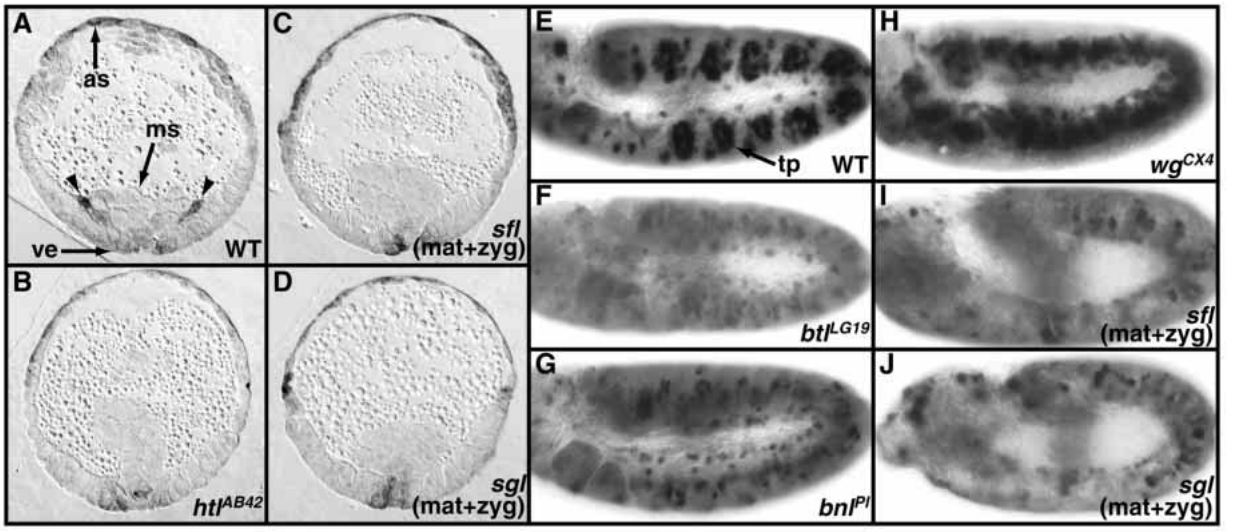

Fig. 4. *sfl* and *sgl* are essential for Htl- and Btl-dependent MAPK activation. (A-D) Transverse sections of stage 8 embryos immunostained with an antibody specific for the activated or diphosphorylated form of MAPK. In wild type, diphospho-MAPK expression is localized to the leading edge (arrowheads) of the migrating mesoderm (ms). This expression is below the level of detection in null *htl* as well as in maternal/zygotic null *sfl* and *sgl* embyros. However, EGFR-dependent diphospho-MAPK expression in the amnioserosa (as) and ventral epidermis (ve) is not affected in any of these mutants. (E-J) Lateral views of stage 11 embryos stained with the diphospho-MAPK-specific antibody. The strong expression that is observed in wild-type tracheal pits (tp) is markedly reduced in the corresponding positions in null *btl* and *bnl* as well as in maternal/zygotic null *sfl* and *sgl* embryos. In contrast, diphospho-MAPK is normally expressed in the tracheal pits of *wg* mutant embryos, although these structures are spaced more closely secondary to the *wg* segment polarity phenotype.

diphospho-MAPK in the tracheal placodes is unaffected in each of these mutants (Michelson et al., 1998a; data not shown). These in situ patterns of MAPK activation provide direct evidence that Sfl and Sgl are required for signaling by the two *Drosophila* FGFRs, Htl and Btl, independent of the requirement for HS GAGs in Wg function.

Overexpression of Bnl partially overcomes the requirement of *sfl* and *sgl* for tracheal cell migration

It has been suggested that monomeric FGF molecules are capable of self-assembling into dimers and higher order oligomers but, at physiological concentrations, require a HS GAG to stabilize this interaction (Sasisekharan et al., 1997; Venkataraman et al., 1996). If this is the case, then elevated levels of the growth factor may at least partially overcome the need for the HS GAG to generate a biological response. We tested this possibility by assessing the effects on tracheal cell migration of overexpressing Bnl, the Btl ligand, in *sfl* and *sgl* null embryos.

In an otherwise wild-type genetic background, high level ectopic ectodermal expression of Bnl hyperactivates Btl, leading to an inhibition of primary tracheal branching, as well as the overproduction of secondary and terminal branches (Sutherland et al., 1996; Michelson et al., 1998a; Fig. 5A,B). These effects of ectopic Bnl are weakly suppressed in the absence of zygotic *sfl* and *sgl* functions (Fig. 5C,D). In embryos lacking both the zygotic and maternal components of *sfl* and *sgl* expression, virtually no tracheal branches are observed (Fig. 5E,F). This correlates very well with the marked reduction in diphospho-MAPK expression that is seen at earlier stages in *sfl* and *sgl* null embryos (Fig. 4I,J). However, in *sfl* and *sgl* null embryos in which Bnl is ectopically expressed, there is a partial recovery of tracheal branching (Fig. 5G,H). Although the tracheal phenotype of *sfl* and *sgl* null embryos reflects the combined loss of Wg and Btl signaling, it is noteworthy that tracheal morphogenesis is not completely inhibited in *wg* as it is in null *sfl* and *sgl* mutants (compare Fig. 5E,F and I). Moreover, ectopic Bnl in the absence of *wg* function leads to a very marked increase in fine tracheal branching, very similar to the effect of ectopic Bnl in wild-type embryos (compare Fig. 5B and J). Thus, the severe tracheal phenotype associated with complete loss of *sfl* or *sgl* function primarily is attributable to an involvement of HS GAGs in Btl rather than in Wg signaling. The ability of Bnl overexpression to partially bypass the requirement for Sfl and Sgl is therefore consistent with a role for HS GAGs in stabilizing or facilitating FGF self-association (Zhu et al., 1993; Ornitz et al., 1995; Faham et al., 1996; Venkataraman et al., 1996; Sasisekharan et al., 1997; DiGabriele et al., 1998). Such a HS GAG-FGF dimer complex would, in turn, facilitate the dimerization of high affinity FGFRs, a prerequisite for receptor activation and the transmission of intracellular signals.

DISCUSSION

We have shown that two enzymes involved in the biosynthesis of HS GAGs are essential for signaling by both of the known *Drosophila* FGFRs, Htl and Btl. Loss of either *sfl* or *sgl* function leads to defects in the migration of mesodermal and tracheal cells during embryogenesis. In addition, Htl- and Btl-dependent activation of MAPK is markedly reduced in *sfl* and *sgl* null embryos. Taken together, these findings provide the first genetic evidence that HSPGs play a central role in FGFR signaling in a well-defined developmental context. A similar genetic approach has been used to establish that HSPGs are critical components of Wg signaling in *Drosophila* (Binari et al., 1997; Häcker et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999).

Several mechanisms have been proposed for how HSPGs participate in FGFR signaling. In one model, the binding of

FGF to abundant but low affinity HS GAGs on the cell surface limits the free diffusion of the ligand, thereby increasing its local concentration and the probability that it will interact with less abundant, high affinity signaling receptors (Schlessinger et al., 1995). Other studies have identified distinct HS GAG binding sites on both FGF (Blaber et al., 1996; Eriksson et al., 1991; Zhang et al., 1991) and high affinity FGFRs (Kan et al., 1993; Pantoliano et al., 1994), suggesting that the latter two components form a ternary complex with a proteoglycan. Direct interaction between the proteoglycan and FGFR, in addition to growth factor binding, may therefore be required for maximal receptor activation. HS GAGs also may promote the formation of FGF dimers or higher order oligomers, thereby facilitating FGFR dimerization and activation (Zhu et al., 1993; Spivak-Kroizman et al., 1994; Ornitz et al., 1995; Faham et al., 1996; Digabriele et al., 1998).

A variation of the last model proposes that FGF monomers are capable of self-associating, a process that is stabilized by HS GAGs (Venkataraman et al., 1996; Herr et al., 1997; Sasisekharan et al., 1997). This hypothesis predicts that elevated levels of FGF should compensate at least in part for a loss of dimer stabilization mediated by HS GAGs. The ability of Bnl overexpression to induce some tracheal branching in the complete absence of Sfl and Sgl activities is consistent with this last possibility, although it does not rule out an additional involvement of HS GAGs or the core protein of a proteoglycan in some other aspect of Btl activation. In the case of human FGF-2, self-association in the absence of HS GAGs has been observed by mass spectrometry and biochemical assays at physiological concentrations of ligand. In addition, elevated FGF-2 levels exert biological effects on tissue culture cells that fail to synthesize HS GAGs (Davis et al., 1999). Our Bnl overexpression experiments are in agreement with the latter data for FGF-2. Together, these findings support the proposal that HS GAGs function to stabilize the FGF dimers or higher order oligomers that are formed by a self-association mechanism (Venkataraman et al., 1996; Sasisekharan et al., 1997). Interestingly, ectopic expression of ligand also is able to overcome the requirement of HS GAGs in Wg signaling, although in this case the HS GAGs may be more important for increasing the local concentration of growth factor at the cell surface than for facilitating ligand dimerization (Häcker et al., 1997; Lin and Perrimon, 1999).

Under normal conditions, the concentrations of FGFs may be limiting, necessitating the presence of HS GAGs to augment or stabilize ligand dimerization and subsequent FGFR activation. This is of particular significance for Btl signaling since *bnl*, which encodes its ligand, is known to be haploinsufficient (Sutherland et al., 1996), and overexpression of Bnl partially bypasses the requirement for *sfl* and *sgl* in the promotion of tracheal branching. Thus, HS GAGs may ensure that a requisite FGFR activation threshold is surpassed when the amount of available ligand is normally low. Such a mechanism could additionally expand the sensitivity or spectrum of responses that can be achieved by small localized

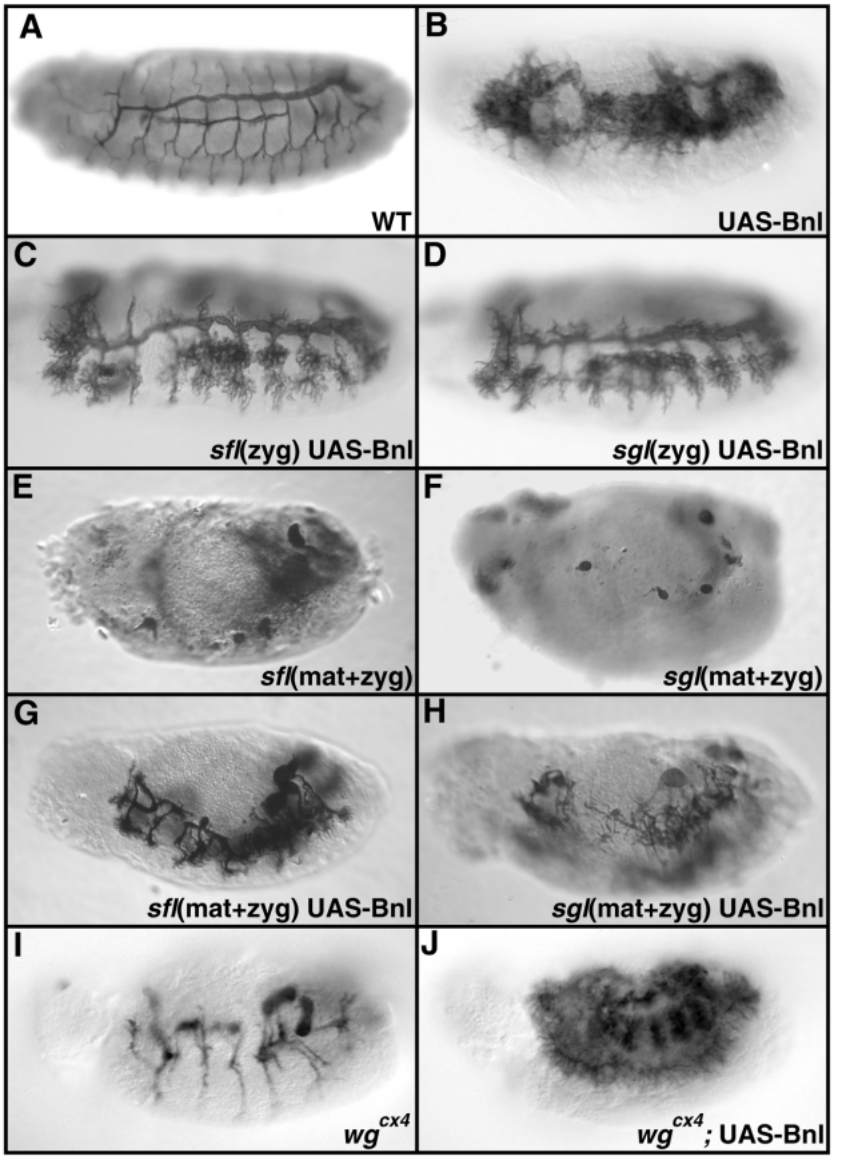

Fig. 5. Overexpression of Bnl partially rescues tracheal cell migration in *sfl* and *sgl* maternal/zygotic null mutant embryos. Lateral views of late stage 15 embryos immunostained with an antibody against a tracheal lumenal antigen. (A) Wild-type tracheal pattern. In B,C,D,G,H and J Bnl was ectopically expressed throughout the ectoderm of embryos of the indicated genotypes using the *69B*-Gal4 driver. (B) Tracheal branching is markedly perturbed by ectopic Bnl in an otherwise wild-type embryo. Primary branching is suppressed and an overabundance of secondary and terminal branches is induced. (C,D) In embryos lacking only the zygotic component of *sfl* or *sgl*, the effects of ectopic Bnl are weakly blocked. (E,F) Virtually no tracheal branching occurs in maternal/zygotic null *sfl* and *sgl* embryos. (G,H) Some tracheal branching is recovered in maternal/zygotic null *sfl* and *sgl* embryos in which Bnl is ectopically expressed at high levels. (I) *wg* mutants develop an extensive tracheal network which has an abnormal pattern due to the associated segmentation defect. (J) Ectopic Bnl in a *wg* mutant background leads to an overproduction of fine tracheal branches, much as occurs in wild type (compare with B).

differences in growth factor concentrations. This is particularly relevant to Bnl which is expressed in a highly dynamic pattern during normal tracheal morphogenesis, a pattern that, when perturbed, leads to severe defects in tracheal outgrowth (Sutherland et al., 1996). Local differences in Btl activity also dictate the sites at which secondary branches normally form, a process to which HSPG regulation might contribute by generating a zone of cells that are highly responsive to Bnl in the vicinity of the ligand signaling center (Hacohen et al., 1998). Similarly, HSPG-mediated ligand dimerization could play a role in the graded activation of Htl that occurs during embryonic mesoderm migration (Gabay et al., 1997b). Modulation of RTK signaling strength also has been implicated in the generation of mesodermal progenitor identities (Buff et al., 1998).

There are two well characterized HSPGs in *Drosophila*, Dally, a Glypican-like cell surface molecule that has been implicated in both Decapentaplegic and Wg signaling (Nakato et al., 1995; Lin and Perrimon, 1999; Tsuda et al., 1999), and a transmembrane proteoglycan related to the vertebrate Syndecan family (Spring et al., 1994). It has been suggested that syndecans particpate in signaling by vertebrate FGFRs (Bernfield et al., 1992; Dealy et al., 1997; Steinfeld et al., 1996), although other HSPGs may also be involved in this process (Aviezer et al., 1994, 1997; Sherman et al., 1998; Steinfeld et al., 1996). It is also possible that different HSPGs could be specific for particular FGF ligand-receptor combinations in individual tissues or at distinct developmental stages. Genetic analysis in *Drosophila* should provide a useful approach for addressing these important questions.

The present findings provide new insight into the mechanisms that regulate FGFR signaling in vivo. A more complete understanding of the function of HSPGs in Htl and Btl activation must await definitive identification of the specific proteoglycans involved, as well as structural and biochemical studies of the complexes formed between the high and low affinity receptors together with the corresponding ligands.

We thank Benny Shilo, Mark Krasnow, and the Bloomington *Drosophila* Stock Center for fly stocks, Siegfried Roth and Manfred Frasch for antibodies, and Ram Sasisekharan and Scott Selleck for communicating results in advance of publication. Stephen Gisselbrecht provided invaluable technical assistance and helpful comments on the manuscript. This work was supported by a Breast Cancer Fellowship from the US Army to X. L. A. M. M. is an Assistant Investigator and N. P. is an Investigator of the Howard Hughes Medical Institute.

REFERENCES

Aviezer, D., Hecht, D., Safran, M., Eisinger, M., David, G. and Yayon, A. (1994). Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor-receptor binding, mitogenesis, and angiogenesis. *Cell* **79**, 1005-1013.

Aviezer, D., Renato, I. V., Noonan, D. M. and Yayon, A. (1997). Suppresion of autocrine and paracrine functions of basic fibroblast growth factor by stable expression of perlecan antisense cDNA. *Mol. Cell. Biol.* **17**, 1938-1946.

Bate, M. (1993). The mesoderm and its derivatives. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez Arias), pp. 1013-1090: Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Beiman, M., Shilo, B. Z. and Volk, T. (1996). Heartless, a *Drosophila* FGF receptor homolog, is essential for cell migration and establishment of several mesodermal lineages. *Genes Dev.* **10**, 2993-3002.

Bejsovec, A. and Wieschaus, E. (1993). Segment polarity gene interactions modulate epidermal patterning in *Drosophila* embryos. *Development* **119**, 501-517.

Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L. and Lose, E. J. (1992). Biology of the syndecans: A family of transmembrane heparan sulfate proteoglycans. *Annu. Rev. Cell Biol.* **8**, 365-393.

Binari, R. C., Staveley, B. E., Johnson, W. A., Godavarti, R., Sasisekharan, R. and Manoukian, A. S. (1997). Genetic evidence that heparin-like glycosaminoglycans are involved in *wingless* signaling. *Development* **124**, 2623-2632.

Blaber, M., DiSalvo, J. and Thomas, K. A. (1996). X-ray crystal structure of human acidic fibroblast growth factor. *Biochemistry* **35**, 2086-2094.

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

Buff, E., Carmena, A., Gisselbrecht, S., Jiménez, F. and Michelson, A. M. (1998). Signalling by the *Drosophila* epidermal growth factor receptor is required for the specification and diversification of embryonic muscle progenitors. *Development* **125**, 2075-2086.

Carmena, A., Gisselbrecht, S., Harrison, J., Jiménez, F. and Michelson, A. M. (1998). Combinatorial signaling codes for the progressive determination of cell fates in the *Drosophila* embryonic mesoderm. *Genes Dev.* **12**, 3910-3922.

Chou, T.-b. and Perrimon, N. (1996). The autosomal FLP-DFS technique for generating germline mosaics in *Drosophila melanogaster*. *Genetics* **144**, 1673-1679.

Cobb, M. H. and Goldsmith, E. J. (1995). How MAP kinases are regulated. *J. Biol. Chem.* **270**, 14843-14846.

David, G. (1993). Integral membrane heparan sulfate proteoglycans. *FASEB J.* **7**, 1023-1030.

Davis, J., Venkataraman, G., Shriver, Z., Perithamby, A. and Sasisekharan, R. (1999). Oligomeric self-association of fibroblast growth factor-2 in the absence of heparin-like glycosaminoglycans. *Biochem. J.* (in press).

Dealy, C. N., Seghatoleslami, M. R., Ferrari, D. and Kosher, R. A. (1997). FGF-stimulated outgrowth and proliferation of limb mesoderm is dependent on syndecan-3. *Dev. Biol.* **184**, 343-350.

DiGabriele, A. D., Lax, I., Chen, D. I., Svahn, C. M., Jaye, M., Schlessinger, J. and Hendrickson, W. A. (1998). Structure of a heparin-linked biologically active dimer of fibroblast growth factor. *Nature* **393**, 812-817.

Eriksson, A. E., Cousens, L. S., Weaver, L. H. and Matthews, B. W. (1991). Three-dimensional structure of human basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA* **88**, 3441-3445.

Eriksson, I., Sandback, D., Lindahl, U. and Kjellén, L. (1994). cDNA cloning and sequencing of mouse mastocytoma glucosaminyl *N*-deacetylase/*N*-sulfotransferase, an enzyme involved in the biosynthesis of heparin. *J. Biol. Chem.* **269**, 10438-10443.

Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J. and Rees, D. C. (1996). Heparin structure and interactions with basic fibroblast growth factor. *Science* **271**, 1116-1120.

Fantl, W. J., Johnson, D. E. and Williams, L. T. (1993). Signalling by receptor tryrosine kinases. *Annu. Rev. Biochem.* **62**, 453-481.

Frasch, M., Hoey, T., Rushlow, C., Doyle, H. and Levine, M. (1987). Characterization and localization of the *even-skipped* protein of *Drosophila*. *EMBO J.* **6**, 749-759.

Gabay, L., Seger, R. and Shilo, B.-Z. (1997a). In situ activation pattern of *Drosophila* EGF receptor pathway during development. *Science* **277**, 1103-1106.

Gabay, L., Seger, R. and Shilo, B.-Z. (1997b). MAP kinase in situ activation atlas during *Drosophila* embryogenesis. *Development* **124**, 3535-3541.

Gisselbrecht, S., Skeath, J. B., Doe, C. Q. and Michelson, A. M. (1996). Heartless encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the *Drosophila* embryo. *Genes Dev.* **10**, 3003-3017.

Greig, S. and Akam, M. (1993). Homeotic genes autonomously specify one aspect of pattern in the *Drosophila* mesoderm. *Nature* **362**, 630-632.

Häcker, U., Lin, X. and Perrimon, N. (1997). The *Drosophila sugarless* gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis. *Development* **124**, 3565-3523.

Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y. and Krasnow, M. A. (1998). *sprouty* encodes a novel antagonist of FGF signaling that patterns apical branching of the *Drosophila* airways. *Cell* **92**, 253-263.

Haerry, T. E., Heslip, T. R., Marsh, J. L. and O'Connor, M. B. (1997). Defects in glucuronate biosynthesis disrupt Wingless signaling in *Drosophila*. *Development* **124**, 3055-3064.

Hanahan, D. and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353-364.

Hashimoto, Y., Orellana, A., Gil, G. and Hirschberg, C. B. (1992). Molecular cloning and expression of rat liver *N*-heparan sulfate sulfotransferase. *J. Biol. Chem.* **267**, 15744-15750.

Hempel, J., Perozich, J., Romavacek, H., Hinich, A., Kuo, I. and Feingold, D. S. (1994). UDP-glucose dehydrogenase from bovine liver: primary structure and relationship to other dehydrogenases. *Protein Sci.* **3**, 1074-1080.

Herr, A. B., Ornitz, D. M., Sasisekharan, R., Venkataraman, G. and Waksman, G. (1997). Heparin-induced self-association of fibroblast growth factor-2. *J. Biol. Chem.* **272**, 16382-16389.

Imam, F., Sutherland, D., Huang, W. and Krasnow, M. A. (1999). *stumps*, a *Drosophila* gene required for fibroblast growth (FGF)-directed migrations of tracheal and mesodermal cells. *Genetics* **152**, 307-318.

Isaac, D. D. and Andrew, D. J. (1996). Tubulogenesis in *Drosophila*: a requirement for the *trachealess* gene product. *Genes Dev.* **10**, 103-117.

Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J. and McKeehan, W. L. (1993). An essential heparin-binding domain in the fibroblast growth factor receptor kinase. *Science* **259**, 1918-1921.

Kjellén, L. and Lindahl, U. (1991). Proteoglycans: structures and interactions. *Annu. Rev. Biochem.* **60**, 443-475.

Klämbt, C., Glazer, L. and Shilo, B.-Z. (1992). *breathless*, a *Drosophila* FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. *Genes Dev.* **6**, 1668-1678.

Lee, T., Hacohen, N., Krasnow, M. and Montell, D. J. (1996). Regulated Breathless receptor tyrosine kinase activity required to pattern cell migration and branching in the *Drosophila* tracheal system. *Genes Dev.* **10**, 2912-2921.

Leptin, M. (1995). *Drosophila* gastrulation: from pattern formation to morphogenesis. *Annu. Rev. Cell Dev. Biol.* **11**, 189-212.

Lin, X. and Perrimon, N. (1999) Dally cooperates with *Drosophila* Frizzled 2 to transduce Wingless signalling. *Nature* (in press).

Martin, G. (1998). The roles of FGFs in the early development of vertebrate limbs. *Genes Dev.* **12**, 1571-1586.

Mason, I. J. (1994). The ins and outs of fibroblast growth factors. *Cell* **78**, 547-552.

Michelson, A. M., Gisselbrecht, S., Buff, E. and Skeath, J. B. (1998a). Heartbroken is a specific downstream mediator of FGF receptor signalling in *Drosophila*. *Development* **125**, 4379-4389.

Michelson, A. M., Gisselbrecht, S., Zhou, Y., Baek, K.-H. and Buff, E. M. (1998b). Dual functions of the Heartless fibroblast growth factor receptor in development of the *Drosophila* embryonic mesoderm. *Dev. Genet.* **22**, 212-229.

Nakato, H., Futch, T. A. and Selleck, S. B. (1995). The *division abnormally delayed (dally)* gene: a putative integral membrane proteoglycan required for cell division patterning during postembryonic development of the nervous system in *Drosophila*. *Development* **121**, 3687-3702.

Orellana, A., Hirschberg, C. B., Wei, Z., Swiedler, S. J. and Ishihara, M. (1994). Molecular cloning and expression of a glycosaminoglycan *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase from a heparin-producing cell line. *J. Biol. Chem.* **269**, 2270-2276.

Ornitz, D. M., Herr, A. B., Nilsson, M., Westman, J., Svahn, C.-M. and Waksman, G. (1995). FGF binding and FGF receptor activation by synthetic heparan-derived di- and triaccharides. *Science* **268**, 432-436.

Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E. and Leder, P. (1992). Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. *Mol. Cell. Biol.* **12**, 240-247.

Pantoliano, M. W., Horlick, R. A., Springer, B. A., Van Dyke, D. E., Tobery, T., Wetmore, D. R., Lear, J. D., Nahapetian, A. T., Bradley, J. D. and Sisk, W. P. (1994). Multivalent ligand-receptor binding interactions in the fibroblast growth factor system produce a cooperative growth factor and heparin mechanism for receptor dimerization. *Biochemistry* **33**, 10229-10248.

Patel, N. H. (1994). Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. In *Meths. Cell Biol.*, vol. 44 (ed. L. S. B. Goldstein and E. A. Fyrberg), pp. 445-487. New York: Academic Press.

Perrimon, N., Lanjuin, A., Arnold, C. and Noll, E. (1996). Zygotic lethal mutations with maternal effect phenotypes in *Drosophila melanogaster*. II. Loci on the second and third chromosomes identified by P-element-induced mutations. *Genetics* **144**, 1681-1692.

Queenan, A. M., Ghabrial, A. and Schüpbach, T. (1997). Ectopic activation of *torpedo*/*Egfr*, a *Drosophila* receptor tyrosine kinase, dorsalizes both the eggshell and the embryo. *Development* **124**, 3871-3880.

Rapraeger, A. C., Krufka, A. and Olwin, B. B. (1991). Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* **252**, 1705-1708.

Reichman-Fried, M. and Shilo, B.-Z. (1995). Breathless, a *Drosophila* FGF receptor homolog, is required for the onset of tracheal cell migration and tracheole formation. *Mechs. Dev.* **52**, 265-273.

Samakovlis, C., Hacohen, N., Manning, G., Sutherland, D. C., Guillemin, K. and Krasnow, M. A. (1996). Development of the *Drosophila* tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. *Development* **122**, 1395-1407.

Sasisekharan, R., Ernst, S. and Venkataraman, G. (1997). On the regulation of fibroblast growth factor activity by heparin-like glycosaminoglycans. *Angiogenesis* **1**, 45-54.

Schlessinger, J., Lax, I. and Lemmon, M. (1995). Regulation of growth factor activation by proteoglycans: what is the role of the low affinity receptors? *Cell* **83**, 357-360.

Seger, R. and Krebs, E. G. (1995). The MAPK signaling cascade. *FASEB J.* **9**, 726-735.

Sherman, L., Wainwright, D., Ponta, H. and Herrlich, P. (1998). A splice variant of CD44 expressed in the apical ectodermal ridge presents fibroblast growth factors to limb mesenchyme and is required for limb outgrowth. *Genes Dev.* **12**, 1058-1071.

Shishido, E., Higashijima, S., Emori, Y. and Saigo, K. (1993). Two FGF-receptor homologues of *Drosophila* – one is expressed in mesodermal primordium in early embryos. *Development* **117**, 751-761.

Shishido, E., Ono, N., Kojima, T. and Saigo, K. (1997). Requirements of DFR1/Heartless, a mesoderm-specific *Drosophila* FGF-receptor, for the formation of heart, visceral and somatic muscles, and ensheathing of longitudinal axon tracts in CNS. *Development* **124**, 2119-2128.

Spivak-Kroizman, T., Lemmon, M. A., Dikic, I., Ladburt, J. E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J. and Lax, I. (1994). Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. *Cell* **79**, 1015-1024.

Spring, J., Paine-Saunders, S. E., Hynes, R. O. and Bernfield, M. (1994). *Drosophila* syndecan: Conservation of a cell-surface heparan sulfate proteoglycan. *Proc. Natl. Acad. Sci. USA* **91**, 3334-3338.

Steinfeld, R., Van Den Berghe, H. and David, G. (1996). Stimulation of fibroblast growth factor receptor-1 occupancy and signaling by cell surface-assocated syndecans and glypican. *J. Cell Biol.* **133**, 405-416.

Sutherland, D., Samakovlis, C. and Krasnow, M. (1996). *branchless* encodes a *Drosophila* FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell* **87**, 1091-1101.

Tsuda, M., Kamimura, K., Nakato, H., Archer, M., Staatz, W., Leggin, B., Humphrey, M., Olson, S., Futch, T., Siegfried, E., Stam, L. and Selleck, S. B. (1999). A cell surface proteoglycan, Dally, regulates Wingless signaling in *Drosophila*. *Nature* (in press).

van den Heuvel, M., Harryman-Samos, C., Klingensmith, J., Perrimon, N. and Nusse, R. (1993). Mutations in the segment polarity genes *wingless* and *porcupine* impair secretion or the Wingless protein. *EMBO J.* **12**, 5293-5302.

Venkataraman, G., Sasisekharan, V., Herr, A. B., Ornitz, D. M., Waksman, G., Cooney, C. L., Langer, R. and Sasisekharan, R. (1996). Preferential self-association of basic fibroblast growth factor is stabilized by heparin during receptor dimerization and activation. *Proc. Natl. Acad. Sci. USA* ***93***, 845-850.

Vincent, S., Wilson, R., Coelho, C., Affolter, M. and Leptin, M. (1998). The *Drosophila* protein Dof is specifically required for FGF signaling. *Mol. Cell* **2**, 515-525.

Wilk, R., Weizman, I. and Shilo, B.-Z. (1996). *trachealess* encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in *Drosophila*. *Genes Dev.* **10**, 93-102.

Wilkie, A. O. M., Morriss-Kay, G. M., Jones, E. Y. and Heath, J. K. (1995). Functions of fibroblast growth factors and their receptors. *Curr. Biol.* **5**, 500-507.

Yamaguchi, T. P. and Rossant, J. (1995). Fibroblast growth factors in mammalian development. *Curr. Opin. Genet. Dev.* **5**, 485-491.

Yanagishita, M. and Hascall, V. C. (1992). Cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* **267**, 9451-9454.

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P. and Ornitz, D. M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64**, 841-848.

Zhang, J., Cousens, L. S., Barr, P. J. and Sprang, S. R. (1991). Three-dimensional structure of human basic fibroblast growth factor, a structural homolog of interleukin 1β. *Proc. Natl. Acad. Sci. USA* **88**, 3446-3450.

Zhu, X., Hsu, B. T. and Rees, D. C. (1993). Structural studies of the binding of the anti-ulcer drug sucrose octasulfate to acidic fibroblast growth factor. *Structure* **1**, 27-34.