Heartbroken is a specific downstream mediator of FGF receptor signalling in

**Drosophila**

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**SUMMARY**

**Drosophila** possesses two FGF receptors which are encoded by the *heartless* and *breathless* genes. *HEARTLESS* is essential for early migration and patterning of the embryonic mesoderm, while *BREATHLESS* is required for proper branching of the tracheal system. We have identified a new gene, *heartbroken*, that participates in the signalling pathways of both FGF receptors. Mutations in *heartbroken* are associated with defects in the migration and later specification of mesodermal and tracheal cells. Genetic interaction and epistasis experiments indicate that *heartbroken* acts downstream of the two FGF receptors but either upstream of or parallel to RAS1. Furthermore, *heartbroken* is involved in both the HEARTLESS- and BREATHLESS-dependent activation of MAPK. In contrast, EGF receptor-dependent embryonic functions and MAPK activation are not perturbed in *heartbroken* mutant embryos. A strong *heartbroken* allele also suppresses the effects of hyperactivated FGF but not EGF receptors. Thus, *heartbroken* may contribute to the specificity of developmental responses elicited by FGF receptor signalling.

Key words: Receptor tyrosine kinase, Mesoderm, Trachea, Cell migration, *Drosophila*, FGF,

**INTRODUCTION**

Signalling by fibroblast growth factor receptors (FGFRs) has been implicated in a diversity of biological processes. These include the migration, specification, differentiation, proliferation and survival of cells during the development of numerous tissues in a wide variety of organisms (Mason, 1994; Yamaguchi and Rossant, 1995; Green et al., 1996). FGFRs are receptor tyrosine kinases (RTKs) that upon ligand binding are activated by dimerization and autophosphorylation (Schlessinger et al., 1995). This is followed by the receptor-mediated phosphorylation of additional protein substrates, leading to the stimulation of a conserved set of signal transduction molecules. The latter include the adapter protein GRB2/DRK, the guanine nucleotide exchange factor SOS, the small GTP-binding protein RAS, the serine-threonine kinase RAF and the mitogen-activated protein kinase (MAPK) that directly mediates the nuclear responses to receptor activation (Fantl et al., 1993; van der Geer et al., 1994). Many of the components of this intracellular signal transduction pathway are utilized by all RTKs, raising the question of how signal specificity is achieved for each receptor. A number of mechanisms can potentially contribute to RTK output specificity (Mason, 1994; Marshall, 1995; Songyang et al., 1995; Weiss et al., 1997). Since a biological response is determined by the combination of pathways transducing a signal from a cell surface receptor, the spectrum of signalling molecules present in a given cell and capable of interacting with a particular receptor will dictate how the cell will respond to the activating ligand. Thus, specificity can be achieved by a dedicated factor acting downstream of a receptor if that factor is able to recruit or activate novel combinations of additional signalling molecules. Such a function may be served by IRS1 and FRS2 for the mammalian insulin and FGF receptors, respectively, although even these adapters are not entirely specific to a single class of receptor (White, 1994; Kouhara et al., 1997). The activation of tissue-specific effectors can also determine the unique effects of a single RTK (Clandinin et al., 1998).

The mammalian nerve growth factor receptor activates MAPK in a novel manner that is independent of the well-characterized RAS-mediated mechanism (York et al., 1998). This pathway affects the duration and magnitude of MAPK activation which, in turn, determines whether the stimulated cell will differentiate or proliferate (Marshall, 1995). A RAS-independent process also operates downstream of at least one *Drosophila* RTK (Hou et al., 1995). Furthermore, effects of RAS that are independent of RAF have been described (D’Arcangelo and Halegoua, 1993; Lee et al., 1996a). Modulation of RTK signals by other classes of receptors can additionally affect cellular responses (Christian et al., 1992; LaBonne et al., 1995; Pan et al., 1995). Thus, novel elements of a common signal transduction apparatus, branching pathways unique to particular
receptors and interactions with other signalling systems can contribute to RTK response specificity.

Two FGFRs have been characterized in *Drosophila*. *heartless* (*htl*) encodes an FGFR that is expressed in the mesoderm and central nervous system where it is required for cell migration and for the determination of a subset of mesodermal cell fates (Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998; Shishido et al., 1993, 1997). In *htl* mutants, the early mesoderm fails to spread into the dorsal region of the embryo. This migration defect accounts for the absence from *htl* mutant embryos of the heart, visceral musculature and dorsal somatic muscles since the formation of these mesodermal derivatives requires induction by DECAPENTAPLEGIC (DPP), a growth factor produced by the dorsal ectoderm (Staehling-Hampton et al., 1994; Frasch, 1995). Ectopic expression of DPP activates the expression of dorsal mesodermal markers in *htl* mutant embryos, indicating that *htl* is not required for the capacity of these cells to respond to this growth factor (Beiman et al., 1996; Gisselbrecht et al., 1996). HTL expression persists in the mesoderm at later developmental times and inhibition of HTL activity after migration is complete blocks the specification of certain mesodermal cells (Beiman et al., 1996; Michelson et al., 1998). Thus, the HTL FGFR is involved not only in cell movement, but also has a direct role in cellular commitment.

A second FGFR is encoded by the *Drosophila* *breathless* (*btl*) gene. *btl* is expressed in embryonic trochal and midline glial cells, as well as in the border cells of the ovary (Glazer and Shilo, 1991; Klämbt et al., 1992; Murphy et al., 1995). BTL is essential for normal trochal and glial cell migration, and also participates in the movement of border cells. Ectopic expression of a constitutively active BTL receptor suggested that proper trochal branching depends on the spatially regulated activation of this RTK (Lee et al., 1996b). This hypothesis was confirmed with the identification of BRANCHLESS (BNL), the BTL ligand, which is expressed at sites surrounding the trochal system where new branches form (Sutherland et al., 1996). The dynamic pattern of BNL expression guides primary trochal branching and ectopic BNL is capable of redirecting normal trochal migration. In addition, BNL activates BTL in the determination of the trochal cells that give rise to secondary and terminal branches (Reichman-Fried and Shilo, 1995; Lee et al., 1996b; Sutherland et al., 1996). Thus, the *Drosophila* HTL and BTL FGFRs function in similar processes – the early directional migration of the cells in which they are expressed and the subsequent specification of particular mesodermal and trochal cell fates.

The structural and functional similarities between the two *Drosophila* FGFRs raise the possibility that these RTKs share a common signalling mechanism. It might also be expected that some components of this pathway will confer specificity to the related responses of these two receptors. RAS1 functions downstream of both HTL and BTL, but also is involved in signalling by other RTKs in *Drosophila* (Simon et al., 1991; Diaz-Benjumea and Hafen, 1994; Reichman-Fried et al., 1994; Hou et al., 1995; Gisselbrecht et al., 1996). Thus, unique FGFR outputs must be achieved by more specific components. Of further relevance to the signalling specificity of HTL and BTL is the fact that another RTK, the *Drosophila* epidermal growth factor receptor (EGFR or DER), functions in some of the same cells as these FGFRs (Wapner et al., 1997; Buff et al., 1998).

We now describe the identification of *heartbroken* (*hbr*), a novel gene that is involved in the migration of both mesodermal and tracheal cells. *hbr* interacts genetically with *htl* and *btl*, and reduction of *hbr* function is epistatic to the hyperactivation of both FGFRs. Moreover, *hbr* functions either upstream of RAS or on a parallel pathway leading to MAPK activation by HTL and BTL. In contrast, *hbr* does not participate in embryonic signalling by DER. These results indicate that *hbr* encodes a unique component of an RTK signalling pathway that may contribute to FGFR response specificity.

**MATERIALS AND METHODS**

**Drosophila strains and genetics**

The following *Drosophila* mutant strains were employed – *htl* AB42, *htl* YY252 (Gisselbrecht et al., 1996), *htl* G19, *htl* B2/L (Klämbt et al., 1992), *bnl* P1 (Sutherland et al., 1996) and *flb* JK55 (Clifford and Schüpbach, 1994). The *htl* YY252 allele was isolated in an ethylmethane sulfonate (EMS) mutagenesis screen for lethal mutations on the third chromosome that disrupt mesoderm and nervous system development (Gisselbrecht et al., 1996). Two additional *hbr* alleles, *hbr* em and *hbr* em2, were obtained in a separate EMS mutagenesis designed to isolate mutations that fail to complement *htl* YY252, *hbr* also fails to complement *tet* (3R)p 506-85C which was obtained from the Bloomington *Drosophila* Stock Center. Targeted expression of the following transgenes was accomplished using the GAL4/UAS system (Brand and Perrimon, 1993) – UAS-activated RAS1 (Gisselbrecht et al., 1996), UAS-DPP (Staehling-Hampton et al., 1994), UAS-BNL (Sutherland et al., 1996), UAS-activated DER (Queenan et al., 1997) and UAS-activated HTL (see below). Mesodermal expression was directed by tpi-GAL4 (Greig and Akam, 1993) and ectodermal expression by 69B-GAL4 (Brand and Perrimon, 1993). Combinations of mutations and GAL4 or UAS insertions were generated by standard genetic crosses. Balance chromosomes marked with lacZ expression constructs facilitated the identification of homozygous mutant embryos. *Oregon R* was used as a wild-type reference strain.

**Immunohistochemistry and in situ hybridization**

Standard methods were used for embryo fixation, immunohistochemical staining and in situ hybridization (Gisselbrecht et al., 1996). All antibodies and probes have been described previously (Gisselbrecht et al., 1996; Samakovlis et al., 1996; Gabay et al., 1997a,b). In the case of anti-diphospho-MAPK (Gabay et al., 1997a,b), peroxidase staining was enhanced using Tyramide Signal Amplification reagents (New England Nuclear) in combination with the Vectastain ABC Elite kit (Vector Laboratories). Embryos were sectioned and EVE expression in the dorsal mesoderm was quantitated, as described (Gisselbrecht et al., 1996; Michelson et al., 1998). The statistical significance of differences in EVE expression values was established using both one-tailed z- and t-tests.

**Construction of a constitutively activated form of HTL**

The dimerization domain of the bacteriophage λ c1 repressor was obtained as a 0.6 kb *Notl*-BglII restriction fragment from the plasmid, pH52b1 (Lee et al., 1996b). Using the polymerase chain reaction (PCR), a BglII restriction site was introduced into the *htl*-coding sequence at amino acids 279/280, just N-terminal to the HTL transmembrane domain (Shishido et al., 1993; Gisselbrecht et al., 1996), and a *Notl* site was simultaneously created in the *htl* 3′-untranslated region. The resulting 1.35 kb PCR product was cloned and sequenced in its entirety to verify that an intact *htl* coding region was obtained, flanked by the desired restriction sites. The λ repressor and *htl* Notl-BglII fragments were cloned into the *Notl* site of pUAST (Brand and Perrimon, 1993) to generate pUAS-Jhtl. DNA sequencing confirmed that an in-frame fusion had been generated at the BglII site between the λ repressor and *htl* coding regions.
Germline transformation

pUAS-htl was injected along with a helper plasmid encoding P transposase into embryos derived from a y w Drosophila strain (Spradling, 1986). w+ transgenic lines were selected and insertion sites were mapped by standard genetic crosses.

RESULTS

**hbr is required for the establishment of dorsal mesodermal cell fates**

We previously described the isolation of mutations in the Drosophila htl gene that eliminate development of the single somatic muscle and subset of pericardial cells that express EVEN-SKIPPED (EVE) in the dorsal region of the embryonic mesoderm (Gisselbrecht et al., 1996; Fig. 1A,B). An independent complementation group with a mesodermal EVE phenotype identical to that of htl was obtained in the same genetic screen (Fig. 1C). As occurs for htl, the development of other dorsal mesodermal derivatives is severely reduced in this new mutant, including the cardial cells of the heart (Fig. 1D-I), most dorsal somatic muscles (Fig. 1D-I), pericardial cells in addition to those expressing EVE (Fig. 1J-L) and the midgut visceral mesoderm (Fig. 1M-O). On the basis of these and other phenotypic similarities to htl (see below), we have named this new gene heartbroken (hbr).

Recombination and deficiency mapping indicate that hbr is located on the right arm of the third chromosome within the 88B-E cytological interval (data not shown). One allele, hbrYY202, was obtained in the original screen and two additional alleles, hbrems6 and hbrems7, were obtained in a subsequent mutagenesis. Of the three, hbrYY202 has the strongest mesodermal phenotype (see below), although comparison to a deficiency that covers hbr indicates that this allele is not null but rather is a strong hypomorph.

**hbr exhibits dosage-sensitive genetic interactions with htl**

HTL signalling is essential for normal mesoderm development (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997; Michelson et al., 1998). Since hbr and htl have very similar phenotypes, we were interested in whether hbr interacts genetically with htl, implying a possible role for HBR in HTL signalling. We investigated this possibility in two ways.

First, we examined whether hbr is capable of dominantly enhancing a partial loss-of-function htl allele. In contrast to a null allele of htl, the hypomorphic htlYY262 allele (Gisselbrecht et al., 1996) does not completely eliminate mesodermal EVE expression (Fig. 2A). On average, each htlYY262 mutant embryo has 1.5 hemisegments with at least one EVE-positive dorsal mesodermal cell. This is reduced to 0.8 EVE-positive hemisegments in the presence of one mutant copy of hbr, a highly significant difference (P<10^-5). Thus, hbr not only has a htl-like mesodermal phenotype, but it also exhibits a dosage-sensitive genetic interaction with htl.

Second, we determined if hbr can dominantly suppress a hyperactivated form of HTL. The latter was generated by replacing the extracellular domain of the HTL receptor with

![Fig. 1. Abnormal development of dorsal mesodermal derivatives in hbr mutant embryos. Wild-type (A,D,G,J,M), htlAB42 (B,E,H,K,N) and hbrYY202 (C,F,I,L,O) embryos were immunostained for expression of EVE (A-C), myosin heavy chain (MHC; D-F), DMEF2 (G-I) and FAS III (M-O). EVE is normally expressed in a subset of pericardial cells (PC) and a single somatic muscle (SM; Frasch et al., 1987), MHC and DMEF2 in all myofibres and the cardial cells (CC) of the heart (Bate, 1993; Bour et al., 1995; Lilly et al., 1995), and FAS III in the visceral mesoderm (Bate, 1993). All EVE-positive cells are missing from both htl and hbr mutants (see quantitation in Fig. 3O). In addition, most of the dorsal somatic muscles, cardial and pericardial cells fail to form when either htl or hbr function is reduced. Large gaps are also found in the visceral mesoderm of mutant embryos. Dorsal views of all embryos are shown with anterior to the left.](image-url)
Fig. 2. Genetic interactions between hbr and htl in mesoderm development. (A) A strain with a recombinant third chromosome containing both hbrYY202 and htlYY262 alleles was generated. This line was crossed to the hypomorphic htlYY262 mutant alone and embryos of the genotype hbrYY202 htlYY262 were examined for mesodermal expression of EVE. The numbers of EVE-positive hemisegments (T2-3 and A1-7 on both sides of each embryo) were counted for at least 80 embryos of each genotype. The data are expressed as the percentage of all analyzed embryos having the indicated numbers of EVE-positive hemisegments. Note that one mutant copy of hbr combined with homozygosity for the hypomorphic htl allele caused a marked shift in the distribution toward embryos having fewer hemisegments with EVE cells. A statistical comparison of the average number of EVE-positive hemisegments per embryo of each genotype revealed a highly significant difference (see text). (B) Wild-type EVE expression in dorsal mesodermal founder cells at stage 11. (C) Activated HTL generates an increased number of EVE founders when expressed in the mesoderm under twi-GAL4 control. (D) Heterozygous hbrYY202 has no effect on either the number of EVE-positive hemisegments per embryo nor the number of EVE-positive cells per hemisegment. (E) hbrYY202 dominantly suppresses the effect of activated HTL on the specification of extra EVE founder cells.

Mesodermal migration is disrupted in hbr mutant embryos

The earliest phenotype observed in htl mutant embryos is a lack of dorsolateral migration of the invaginated mesoderm (Beiman et al., 1996; Shishido et al., 1997; Gisselbrecht et al., 1996; Michelson et al., 1998; Fig. 3B,D). This abnormality accounts for the later absence of dorsal mesodermal derivatives since these structures require induction by DPP which is supplied by the dorsal ectoderm (Staehling-Hampton et al., 1994; Frasch, 1995). Since the phenotype of later stage hbr embryos is very similar to that of htl (Figs 1, 3A,C,E), we examined the effect of a hbr mutation on earlier stages of mesoderm development. As revealed by transverse sections of stage 10 Twist (TWI)-stained embryos, there is a severe defect in the dorsolateral spreading of hbr mutant mesodermal cells (Fig. 3F). Thus, as is the case for htl, the heart, dorsal somatic muscles and visceral mesoderm fail to develop properly when hbr function is reduced because mispositioned mesodermal progenitors are not exposed to DPP.

Constitutively activated RAS1 but not HTL rescues the hbr mesodermal phenotype

RAS1 is a key signal transducer acting downstream of all RTKs, including HTL (Fanti et al., 1993; van der Geer et al., 1994; Gisselbrecht et al., 1996). Since htl and hbr mutants have similar mesodermal phenotypes and our genetic interaction studies suggested a functional relationship between the products of these genes, we were interested in whether hbr could also be related to RAS1 function. We have shown previously that targeted mesodermal expression of a constitutively activated form of RAS1 can partially rescue the htl mutant phenotype (Gisselbrecht et al., 1996; Michelson et al., 1998). This conclusion was reached by examining both the activated RAS1-induced migration of TWI-expressing cells and the recovery of dorsally restricted EVE-positive muscle and cardiac progenitors in htl embryos. Using these same assays, we found that activated RAS1 is capable of partially rescuing the strong hbrYY202 mutant (Fig. 3G,H). Quantitation of the number of hemisegments in which EVE expression was recovered indicated that hbrYY202 is even more efficiently rescued by activated RAS1 than is htlAB42 (Fig. 30), likely because hbrYY202 is a hypomorph while htlAB42 is a null allele. We also used this quantitative assay to compare the relative strengths of the three hbr alleles and found that their mesodermal phenotypes can be arranged in the following order of increasing severity – hbr<sub>ems</sub>2/hbr<sub>ems</sub>1/hbrYY202 (Fig. 30).

The above results suggest that hbr acts upstream of RAS1 or on a parallel pathway involved in either initiating or transducing the HTL signal. We next asked where hbr functions in relation to the receptor by determining if a constitutively activated form of HTL (see above) can rescue the hbr phenotype. When expressed in the mesoderm of wild-
type embryos, activated HTL induced the formation of additional EVE founder cells, as previously noted, but had no effect on mesoderm migration (Fig. 3I,J). In a htl mutant background, activated HTL partially corrected the mesoderm migration defect and contributed to the specification of significant numbers of EVE progenitors (Fig. 3K,L). Quantitation of the latter effect revealed that activated HTL was significantly more efficient at rescuing loss of htl function than was activated RAS1 (Fig. 3O). In contrast, the influence of activated HTL was completely blocked by a homozygous hbr mutation (Fig. 3M-O). These results, as well as the dominant suppression of activated HTL by hbr (Fig. 2E), argue that hbr acts downstream of or parallel to this mesodermal FGF receptor.

**HTL-dependent activation of MAPK in the mesoderm is dependent on hbr**

MAPK is another important component of the RTK signalling cascade (Seger and Krebs, 1995). A monoclonal antibody specific for the dual phosphorylated, activated form of MAPK (diphospho-MAPK) has recently been shown to be highly effective for monitoring the activity of RTK pathways during *Drosophila* development (Gabay et al., 1997a,b). Using this reagent, high levels of activated MAPK were localized to the leading edge of the migrating mesoderm, with much lower levels present at more ventral positions (Gabay et al., 1997b; Fig. 4A). Activation of MAPK is very weakly enhanced in the ventral mesoderm by *twi-GAL4*-induced expression of a constitutive form of HTL, although the normal gradient of diphospho-MAPK expression does not appear to be significantly altered by this manipulation (Fig. 4B).

Activated MAPK is completely absent from the early mesoderm of *hlt* mutants, confirming that this mesodermal expression of diphospho-MAPK is entirely HTL-dependent (Gabay et al., 1997b; Fig. 4C). Moreover, no activated MAPK is detectable at comparable stages in the mesoderm of *hbr* mutant embryos (Fig. 4D). Activated HTL expressed in a null *hlt* mutant generates a low, uniform level of diphospho-MAPK throughout the mesoderm (Fig. 4E). In addition, reduction of *hbr* function is capable of completely blocking MAPK activation by constitutive HTL (Fig. 4F). These results suggest that *hbr* acts upstream of MAPK in the HTL signal

**Fig. 3.** Dependence of mesoderm migration on *hbr* function and epistasis between *hbr, hlt* and Ras1. Lateral views of stage 11 EVE-stained embryos of the indicated genotypes (A,C,E,G,I,K,M). Transverse sections of TWI-stained stage 10 embryos (B,D,F,H,J,L,N) of the same genotypes as the adjacent EVE-stained embryos. As previously described, no EVE cells are specified in the complete absence of *hlt* function (C) and mesodermal cells remain close to the ventral midline rather than migrating toward the dorsal ectoderm (compare B and D). Similar defects in the formation of EVE founders and migration of TWI cells are observed in the strong *hbrY202* mutant (E,F). As is the case for *hlt* (Gisselbrecht et al., 1996; Michelson et al., 1998), activated RAS1 partially rescues both the migration and EVE cell specification abnormalities seen in *hbrY202* (G,H). *twi-GAL4*-induced expression of activated HTL generates additional EVE founders but does not perturb mesoderm migration (I,J). Activated HTL can partially rescue the mesodermal phenotype of *hlt* (K,L) but not *hbr* (M,N) mutants. Arrowheads indicate the positions of the dorsal ectoderm in the sectioned embryos. (O) Quantitation of *hlt* and *hbr* rescue by activated RAS1 (RAS1Act) and activated HTL (HTLAct) expressed in mutant embryos under *twi-GAL4* control. EVE expression in the dorsal mesoderm was used as a measure of both the mesodermal cell migration and progenitor specification functions of HTL. For each genotype, the percentage of hemisegments containing at least one EVE-positive cell is indicated. The data for *hltAB42* and activated RAS1 rescue of this mutant have been reported previously (Michelson et al., 1998) and are included here for comparative purposes.
transduction pathway, a hypothesis that is consistent with the findings of the above genetic epistasis experiments.

**Competence to respond to DPP induction is independent of hbr**

The inability of hbr mutant cells to migrate into the DPP expression domain in the dorsal region of the embryo can account for the associated absence of dorsal mesodermal structures. However, hbr might also be required in order for mesodermal cells to respond to this growth factor. We investigated this possibility by assessing the effects of ectopic DPP on the expression of the DPP target gene, bagpipe (bap; Azpiazu and Frasch, 1993; Staehling-Hampton et al., 1994; Frasch, 1995).

bap normally is expressed in a set of dorsally restricted, segmentally repeated patches of cells that give rise to the visceral mesoderm (Azpiazu and Frasch, 1993; Fig. 5A,C). This expression is markedly reduced in a hbr mutant (Fig. 5B,D), consistent with the previously documented defect in visceral mesodermal development (Fig. 1O). In an otherwise wild-type genetic background, ectopic DPP induces bap transcription in ventral and lateral mesodermal cells (Staehling-Hampton et al., 1994; Frasch, 1995; Fig. 5E). The same response to ectopic DPP is seen in hbr mutant embryos (Fig. 5F). Thus, competence to be induced by DPP does not require hbr. This mesodermal response to DPP also was found to be independent of htl (Gisselbrecht et al., 1996).

**hbr participates with btl in tracheal development**

A second *Drosophila* FGF receptor is encoded by the btl gene (Glazer and Shilo, 1991; Klämbt et al., 1992). BTL activity is required for the migration of tracheal cells to form primary branches, and for the subsequent induction of secondary and terminal tracheal cell fates (Klämbt et al., 1992; Reichman-Fried et al., 1994; Reichman-Fried and Shilo, 1995; Lee et al., 1996b; Samakovlis et al., 1996; Sutherland et al., 1996). Mutations in btl are associated with a marked inhibition of tracheal branching (Fig. 6A-C). Given the involvement of hbr in the HTL FGF receptor signalling pathway, we examined whether HBR might also function with BTL in the tracheal system. Reduction of hbr function is indeed associated with significant defects in tracheal development. In *hbr*YY202 mutant embryos, numerous primary and secondary tracheal branches are missing and the extension of those that do form is frequently stalled (Figs 6D, 7B). These results imply that hbr is necessary both for tracheal cell migration and for the acquisition of secondary tracheal cell fates. *hbr*ems7 exhibits a very similar tracheal phenotype to *hbr*YY202 (Fig. 6E), whereas *hbr*ems6 has a more severe reduction in tracheal branching (Fig. 6F). Consistent with our earlier findings for the mesoderm, both *hbr*YY202 and *hbr*ems6 are hypomorphic with respect to their effects on tracheal development since more severe phenotypes occur when either allele is in *trans* to a deficiency (Fig. 6G,H). Homozygosity for the only available deficiency that covers the *hbr* locus results in severely dysmorphic embryos (Bilder and Scott, 1995; data not shown), making it impossible to use this stock to assess the tracheal phenotype associated with complete absence of hbr function. Interestingly, although *hbr*ems6 has the strongest tracheal
phenotype, its mesodermal defects are the least severe of the three hbr alleles (Fig. 3O; see Discussion).

As was the case with htl and hbr, btl and hbr exhibit strong genetic interactions. Thus, hbr is capable of dominantly enhancing a hypomorphic btl allele (Fig. 6I) and btl can dominantly enhance the hbr tracheal phenotype (Fig. 6J). Together, these genetic interaction experiments suggest that hbr participates in both the HTL and BTL signalling pathways.

**hbr suppresses hyperactivated BTL**

hbr completely blocks the effects of activated HTL in the mesoderm (Fig. 3M,N). We also investigated the potential requirement for hbr in mediating the effects of BTL hyperactivation. Ectopic ectodermal expression of BNL, the BTL ligand, leads to widespread BTL activation, which causes a strong inhibition of primary tracheal branching, accompanied by the induction of disorganized networks of secondary and terminal tracheal branches (Sutherland et al., 1996; Fig. 7C). A homozygous hbr mutation strongly suppresses this effect of ectopic BNL – the formation of long primary branches is recovered and the additional fine, higher order branches are markedly reduced in number (Fig. 7D). Thus, as with activated HTL in the mesoderm, a hypomorphic hbr mutation is capable of at least partially suppressing the effect of BTL hyperactivation.

**hbr is required for the BTL- but not DER-dependent activation of MAPK in tracheal cells**

Expression of activated MAPK can be used to follow RTK involvement in tracheal development. Specific tracheal cell fates are established initially under the influence of DER, whose activity is reflected in the expression of activated MAPK in the tracheal placodes at stage 10 (Gabay et al., 1997b; Wappner et al., 1997; Fig. 8A). By stage 11, BTL-dependent expression of diphospho-MAPK occurs in the tracheal pits prior to the onset of tracheal branch migration (Gabay et al., 1997b; Fig. 8B). In either btl (Gabay et al., 1997b; data not shown) or bnl (Fig. 8C,D) mutant embryos, the DER-dependent expression of activated MAPK in the tracheal placodes is not affected, while the later expression of activated MAPK in the tracheal pits is largely but not completely eliminated (Fig. 8C,D). With reduced hbr function, the DER-dependent diphospho-MAPK pattern at stage 10 is normal, while BTL-dependent expression in stage 11 is very weakly but significantly reduced (Fig. 8E,F). The

![Fig. 6. btl-dependent tracheal cell migration is disrupted in hbr mutant embryos.](image)

**Fig. 6. btl-dependent tracheal cell migration is disrupted in hbr mutant embryos.** Lateral views of stage 15 embryos immunostained with 2A12, a monoclonal antibody specific for a tracheal lumenal antigen. Anterior is to the left in all panels. (A) Wild-type embryo showing the normal pattern of tracheal branches. (B) Homozygous null btl<sup>LG19</sup> embryo with tracheal cells confined to elongated sacs having occasional short extensions. (C) A homozygous hypomorphic mutant, btl<sup>H82Δ</sup>, has a less severe inhibition of tracheal branching than the null allele. (D) A homozygous hbr<sup>YY202</sup> embryo exhibits tracheal migration defects, including missing dorsal and lateral trunk primary branches, stalled primary ganglionic branches and absent secondary branches. These defects are seen more clearly in the higher magnification view of the same embryo shown in Fig. 7B. (E) hbr<sup>ems7</sup> has a similar tracheal phenotype to that of hbr<sup>YY202</sup> (compare with D). (F) hbr<sup>ems6</sup> has a more severe reduction in tracheal branching than the other two hbr alleles. (G,H) The tracheal phenotypes of both hbr<sup>YY202</sup> and hbr<sup>ems6</sup> are enhanced when these alleles are in trans to a deficiency that covers this locus, Df(3R)<sup>500–85C</sup>. (I) One copy of hbr<sup>YY202</sup> in a homozygous btl<sup>H82Δ</sup> embryo enhances the tracheal phenotype of the latter (compare with C). (J) Reducing btl function in a homozygous hbr<sup>YY202</sup> embryo causes less tracheal branching than seen in the hbr mutant alone (compare with D).

![Fig. 7. hbr acts downstream of btl in tracheal development.](image)

**Fig. 7. hbr acts downstream of btl in tracheal development.** Lateral views of stage 15 embryos of the indicated genotypes stained with the 2A12 antibody. (A,B) High magnification views of the same wild-type and hbr<sup>YY202</sup> embryos as shown in Fig. 6A and B, respectively. Note the missing dorsal trunk (DT), lateral trunk (LT) and secondary (2°) branches, as well as the stalled ganglionic branches (GB) in the hbr embryo. (C) 69B-GAL4-targeted ectodermal expression of BNL causes a reduction in primary branching and an increased formation of fine secondary and terminal branches, as previously described (Sutherland et al., 1996). (D) Ectopic BNL expressed in a homozygous hbr<sup>YY202</sup> embryo causes a less severe disruption of primary branching and a marked reduction in the number of secondary and terminal branches than seen in an otherwise wild-type background (compare with panel C).
extent to which tracheal pit MAPK expression is affected in the bnl and hbr mutants appears to be commensurate with the relative severities of their tracheal migration defects.

**hbr is not required for DER signaling during embryogenesis**

Having established that hbr is not involved in DER-dependent MAPK activation in the tracheal placodes, we next determined if hbr is required for any other DER-mediated process in embryogenesis. DER has many critical embryonic functions (Schweitzer and Shilo, 1997), as reflected in the multiple sites of DER-dependent expression of diphospho-MAPK (Gabay et al., 1997b). However, none of these sites of diphospho-MAPK expression is affected in hbr mutant embryos, including the head folds, cephalic furrow, dorsal folds and ventral ectoderm at stage 8 (Fig. 9A-C), the ventral midline at stage 11 (Fig. 9D-F) and the segmental epidermal pattern at stages 12/13 (Fig. 9G-I). Moreover, DER-dependent patterning of the ventral ectoderm occurs normally in hbr mutant embryos, as determined from the wild-type cuticle pattern (data not shown) and normal expression of FASICLIN III (FAS III) in the ventral epidermis of the three thoracic segments (Fig. 9J-L). Finally, we found that a constitutively activated form of DER is able to partially rescue mesodermal EVE expression equally well in both htl and btl mutants (Fig. 9M-O), an effect that is due to the capacity of DER to activate the RAS/MAPK cascade (which also functions downstream of HTL; Gisselbrecht et al., 1996), in a HBR-independent manner. This is in contrast to the ability of the same hbr mutation to completely block the mesodermal effects of constitutively activated HTL (Fig. 3M). That is, a mutation in hbr interferes with mesodermal HTL but not DER signaling. Thus, by multiple criteria, hbr functions in the HTL and BTL but not in the DER signaling pathways.

**DISCUSSION**

The diversity of biological responses elicited by RTKs with common signal transduction pathways presents a challenging problem for the generation of receptor specificity. In only a few cases have unique signalling mechanisms been identified that can contribute to the generation of specific outputs (Mason, 1994; White, 1994; Marshall, 1995; Songyang et al., 1995; Kouhara et al., 1997; Weiss et al., 1997; Claidinin et al., 1998). We have isolated and characterized a *Drosophila* mutant, hbr, whose embryonic phenotype includes features of both htl and btl mutations. Genetic interaction and epistasis experiments, together with analysis of activated MAPK expression patterns, suggest that hbr participates in the HTL and BTL signalling pathways. In contrast, hbr does not appear to be involved in embryonic EGFR signalling. Thus, hbr may represent a novel signalling component that is specific to FGFR responses in the *Drosophila* embryo.

**hbr functions in the HTL and BTL signalling pathways**

hbr was initially identified as a lethal mutation in which the heart and dorsal somatic muscles fail to form due to a defect in mesoderm migration. Since this phenotype is identical to that associated with loss of HTL FGFR activity (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997; Michelson et al., 1998), we sought and found the following functional relationships between hbr and htl. (1) hbr is capable of dominantly enhancing a hypomorphic htl allele. (2) hbr can dominantly suppress a constitutively active form of HTL. (3) Whereas constitutive HTL can partially rescue loss of htl function, it is completely unable to bypass the mesodermal requirement for hbr. (4) Activated RAS1 can partially rescue the mesoderm migration defects of both htl and hbr mutants. (5) hbr is required for the HTL-dependent activation of MAPK during mesoderm migration. (6) Like htl, hbr is not required for the competence of mesodermal cells to respond to DPP, an inducer of dorsal mesodermal identity. Collectively, these results suggest that hbr acts in the HTL signalling pathway to facilitate the migration of the embryonic mesoderm. Moreover, since heterozygous hbr modifies the capacity of activated HTL to induce the formation of additional heart and muscle progenitors under conditions where mesoderm migration is not affected, hbr must also participate in the cell fate specification function of HTL (Michelson et al., 1998).

hbr also affects branching morphogenesis of the tracheal system, another FGFR-mediated process in *Drosophila*. The migration of tracheal cells in the formation of primary branches is markedly reduced in hbr mutant embryos. In addition, secondary branches are frequently missing, suggesting that hbr also is required for determination of the specialized cells that give rise to these structures. Since both primary and higher order tracheal branching are BTL-dependent processes (Klämbt et al., 1992; Reichman-Fried et al., 1994; Reichman-Fried and Shilo,
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1995; Lee et al., 1996b; Samakovlis et al., 1996; Sutherland et al., 1996), hbr might be involved in signalling by this FGFR. Consistent with this possibility, hbr exhibits dominant genetic interactions with btl, is capable of suppressing the effects of BTL hyperactivation and is associated with a weak but significant reduction of BTL-dependent MAPK activation in tracheal precursor cells. Thus, by multiple criteria, hbr is implicated in the functions of two FGFRs in Drosophila embryogenesis.

Our findings are most readily reconciled by a model in which hbr participates in the signalling cascade acting downstream of both HTL and BTL. Two alternative possibilities are consistent with the available data. In the first case, hbr could function downstream of the receptors but upstream of RAS in a simple linear pathway (Fig. 10A). This would explain why hbr is epistatic to constitutive receptor activity, activated RAS is epistatic to hbr, and hbr is required for HTL- and BTL-dependent MAPK activation. However, these results are equally compatible with the possibility that hbr functions downstream of the receptors in a pathway that is initially parallel to but ultimately convergent with the RAS cascade at (or above) the level of MAPK (Fig. 10B). In either case, there could be additional branching pathways emanating from hbr that contribute to the responses elicited by these FGFRs.

FGFR signalling is involved in mesodermal patterning during vertebrate embryonic development (Mason, 1994; Yamaguchi and Rossant, 1995; Green et al., 1996), and in branching morphogenesis in formation of the mammalian lung and vasculature (Hanahan and Folkman, 1996). Given the involvement of hbr in similar HTL- and BTL-dependent processes in Drosophila, a hbr-related gene(s) likely exists in these other species. In fact, candidates for both models of hbr function already have been characterized for several RTKs in mammals. For example, mammalian FGF and insulin receptors each have substrates, FRS2/SNT and IRS1, respectively, that serve as dedicated adapters to couple receptor stimulation to
activation of RAS (White, 1994; Wang et al., 1996; Kouhara et al., 1997). Such an adapter could fulfill the function of hbr illustrated in Fig. 10A. Another mammalian protein that may be a specific FGFR substrate and function upstream of RAS, p90/80K-H, might also correspond to the hbr product (Goh et al., 1996).

On the other hand, a branching pathway leading to a novel mode of MAPK activation has been characterized downstream of the mammalian nerve growth factor receptor (York et al., 1998). This pathway, which involves the GTP-binding protein RAP1, its guanine nucleotide exchange factor C3G and the adapter protein CRK-L, acts in parallel to and independently of RAS and its effectors. However, the activities of both RAS and RAP1 converge on MAPK, an arrangement that is similar to the alternative scheme of hbr function that we have considered (Fig. 10B). hbr could also be involved in a RAS1-independent mechanism of RAF activation (Hou et al., 1995). Other novel functions of hbr are, of course, not excluded. The cloning of hbr and functional analysis of its protein product will be required to resolve this issue.

All three of our hbr mutants have stronger mesodermal than tracheal phenotypes when compared to null alleles of htl and btl. In addition, a hbr mutant completely blocks the effects of hyperactivated HTL in the mesoderm, whereas the same mutant only partially suppresses hyperactivated BTL in the trachea. These phenotypic differences may reflect allele-specific effects of hbr on each of the FGFR pathways, or may indicate a true differential involvement of hbr in signaling by these two RTKs. In this regard, it may be relevant that the hbrmut6 allele has a more severe tracheal but less severe mesodermal phenotype than the other two hbr alleles, raising the possibility that HBR has independently mutable domains that function differentially in HTL and BTL signaling. Additional genetic and molecular analyses will be required to assess these various hypotheses.

Role of HTL in mesoderm migration

HTL provides an instructive signal for the migration of primary tracheal branches through the localized expression of its activating ligand (Sutherland et al., 1996). Does HTL have a similar function in mesoderm migration? Although the HTL ligand has not yet been identified, the HTL-dependent, graded activation of MAPK in the migrating mesoderm suggests that HTL is indeed instructive for this process (Gabay et al., 1997b). Consistent with this hypothesis, we found that a constitutively active form of HTL, which generates uniform, albeit low, MAPK activation throughout the mesoderm, induces only partial rescue of a null htl mutant. This is in marked contrast to the complete rescue obtained by wild-type HTL expressed in an identical temporal and spatial manner (Michelson et al., 1998). Thus, graded activation of HTL is likely to be essential for the proper directional migration of mesodermal cells. In contrast, constitutive receptor activity may facilitate random cell movement, increasing the probability that some cells reach the dorsal ectoderm, but not reconstituting a normal pattern of cell migration. This could explain why activated HTL did not perturb migration in an otherwise wild-type background since, under these conditions, we still observed graded diphospho-MAPK expression. To further test the importance of graded signalling in mesoderm migration, it will be necessary to achieve uniform MAPK activation at a level comparable to that normally found in cells at the leading edge.

Does hbr mediate FGFR specificity?

Although hbr is important for signalling by two FGFRs, it is not required for FGFR responses in the Drosophila embryo. This conclusion is based on the normal ventral ectodermal patterning and the completely wild-type DER-dependent expression of diphospho-MAPK seen in hbr mutant embryos. In addition, a hbr mutant fails to block the mesodermal effects of constitutive DER, which is able to partially substitute for HTL due to its ability to activate the common RAS/MAPK cascade in a HBR-independent manner. This is in contrast to the markedly reduced effects of constitutive HTL and BTL in the same hbr mutant background. Thus, hbr may be a key component that confers specificity to FGFR signalling. This is of particular interest since EGF and FGF receptors both function in the mesoderm, but with different biological outcomes (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997; Buff et al., 1998; Michelson et al., 1998). Both types of receptor also function in tracheal cells, but again each elicits a unique response (Klämbt et al., 1992; Reichmann-Fried and Shilo, 1995; Lee et al., 1996b; Wappner et al., 1997). The availability of a dedicated RTK signal transducer like HBR, in addition to shared components such as RAS1, could insure that the appropriate output is generated by each receptor.

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