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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 ouput 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 tracheal 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 tracheal and glial cell migration, and also participates in the movement of border cells. Ectopic expression of a constitutively active BTL receptor suggested that proper tracheal 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 tracheal system where new branches form (Sutherland et al., 1996). The dynamic pattern of BNL expression guides primary tracheal branching and ectopic BNL is capable of redirecting normal tracheal migration. In addition, BNL activates BTL in the determination of the tracheal 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 tracheal 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 (Wappner 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^{YY262} (Gisselbrecht et al., 1996), btl^{LG19}, $btl^{H82\Delta3}$ (Klämbt et al., 1992), bnl^{P1} (Sutherland et al., 1996) and flb^{1K35} (Clifford and Schüpbach, 1994). The hbr^{YY202} 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^{ems6} and hbr^{ems7}, were obtained in a separate EMS mutagenesis designed to isolate mutations that fail to complement hbr^{YY202}. *hbr* also fails to complement Df(3R)$ry^{506\text{-}85C}$ 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 *twi*-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. Balancer 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 cI repressor was obtained as a 0.6 kb *Not*I-*Bgl*II restriction fragment from the plasmid, pHSλbtl (Lee et al., 1996b). Using the polymerase chain reaction (PCR), a *Bgl*II 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 *Not*I 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* *Not*I-*Bgl*II fragments were cloned into the *Not*I site of pUAST (Brand and Perrimon, 1993) to generate pUAS-λhtl. DNA sequencing confirmed that an in-frame fusion had been generated at the *Bgl*II site between the λ repressor and *htl* coding regions.

Germline transformation

pUAS-λhtl 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, hbr^{YY202}, was obtained in the original screen and two additional alleles, hbr^{ems6} and hbr^{ems7}, were obtained in a subsequent mutagenesis. Of the three, hbr^{YY202} 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 htl^{YY262} allele (Gisselbrecht et al., 1996) does not completely eliminate mesodermal EVE expression (Fig. 2A). On average, each htl^{YY262} 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

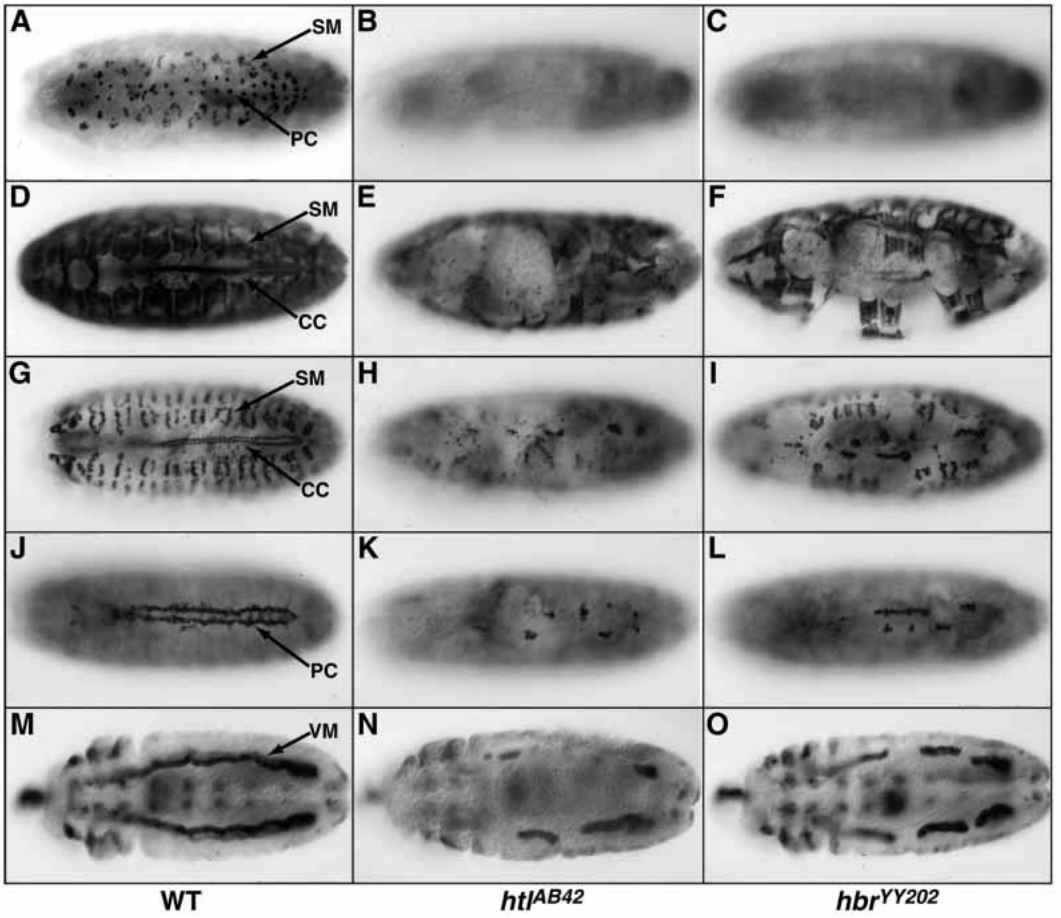


Fig. 1. Abnormal development of dorsal mesodermal derivatives in *hbr* mutant embryos. Wild-type (A,D,G,J,M), htl^{AB42} (B,E,H,K,N) and hbr^{YY202} (C,F,I,L,O) embryos were immunostained for expression of EVE (A-C), myosin heavy chain (MHC; D-F), DMEF2 (G-I), a pericardial cell antigen (J-L) and FAS 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.

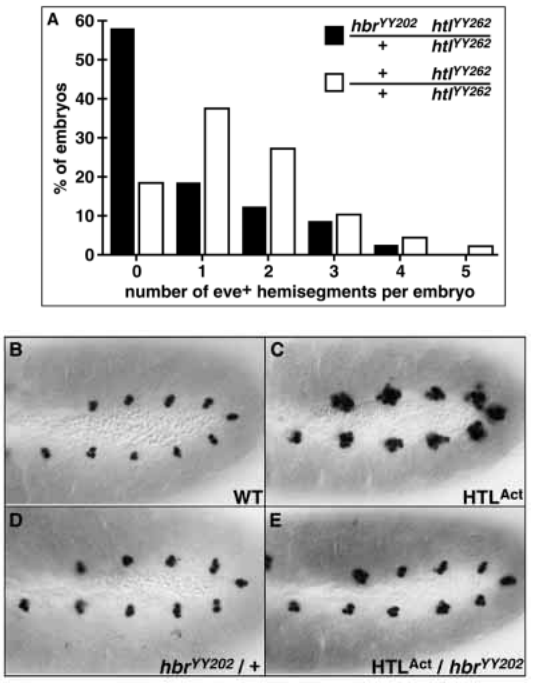


Fig. 2. Genetic interactions between *hbr* and *htl* in mesoderm development. (A) A strain with a recombinant third chromosome containing both hbr^{YY202} and htl^{YY262} alleles was generated. This line was crossed to the hypomorphic htl^{YY262} mutant alone and embryos of the genotype hbr^{YY202} htl^{YY262}/+ htl^{YY262} 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 hbr^{YY202} has no effect on either the number of EVE-positive hemisegments per embryo nor the number of EVE-positive cells per hemisegment. (E) hbr^{YY202} dominantly suppresses the effect of activated HTL on the specification of extra EVE founder cells.

the dimerization domain of the bacteriophage λ cI repressor. This strategy constitutively activates RTKs by causing ligand-independent receptor dimerization (Lee et al., 1996b). Such a construct was placed under GAL4 UAS control and ectopically expressed in transgenic embryos using a *twi*-GAL4 driver (Greig and Akam, 1993).

Under the influence of activated HTL, an increased number of EVE-expressing muscle and cardiac progenitors is formed in an otherwise wild-type genetic background (Fig. 2B,C). This response is equivalent to that obtained with activated forms of RAS1 or DER and reflects the involvement of both RTKs in mesodermal cell fate specification (Gisselbrecht et al., 1996; Buff et al., 1998; Michelson et al., 1998). Heterozygous *hbr* alone does not affect mesodermal EVE expression or mesoderm migration (Fig. 2D and data not shown). However, one mutant copy of *hbr* markedly suppresses the influence of activated HTL on the formation of EVE-positive mesodermal cells (Fig. 2E). This suggests that *hbr* is required for the cell fate specification function of HTL, in addition to its involvement in HTL-dependent cell migration (see below). These findings establish a strong genetic interaction between *hbr* and *htl*, suggesting that *hbr* is involved in mesodermal FGFR signalling.

Mesodermal migration is disrupted in *hbr* mutant embryos

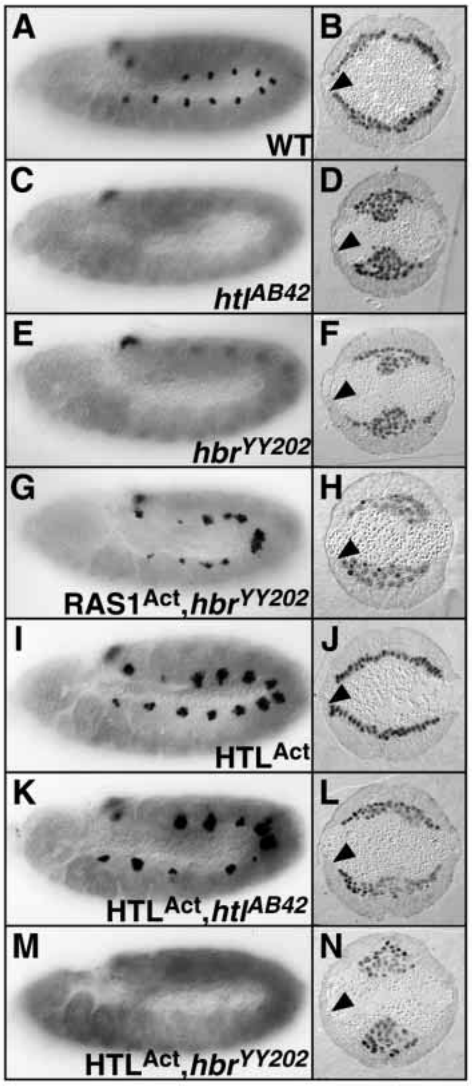
The earliest phenotype observed in *htl* mutant embryos is a lack of dorsolateral migration of the invaginated mesoderm (Beiman et al., 1996; Shishido et al., 1997; Gisselbrecht et al., 1996; Michelson et al., 1998; Fig. 3B,D). This abnormality accounts for the later absence of dorsal mesodermal derivatives 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.

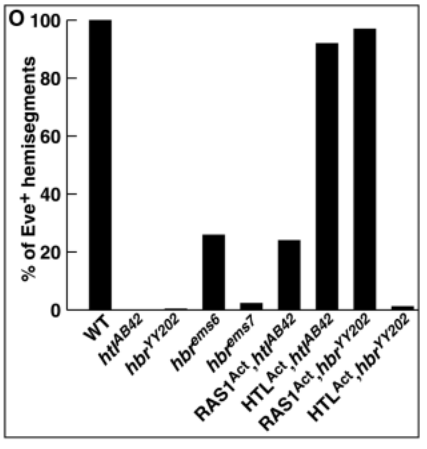
Constitutively activated RAS1 but not HTL rescues the *hbr* mesodermal phenotype

RAS1 is a key signal transducer acting downstream of all RTKs, including HTL (Fantl 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 hbr^{YY202} mutant (Fig. 3G,H). Quantitation of the number of hemisegments in which EVE expression was recovered indicated that hbr^{YY202} is even more efficiently rescued by activated RAS1 than is htl^{AB42} (Fig. 3O), likely because hbr^{YY202} is a hypomorph while htl^{AB42} 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 – $hbr^{ems6} < hbr^{ems7} < hbr^{YY202}$ (Fig. 3O).

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 *htl* 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 *htl* 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*, *htl* 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 *htl* 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 hbr^{YY202} mutant (E,F). As is the case for *htl* (Gisselbrecht et al., 1996; Michelson et al., 1998), activated RAS1 partially rescues both the migration and EVE cell specification abnormalities seen in hbr^{YY202} (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 *htl* (K,L) but not *hbr* (M,N) mutants. Arrowheads indicate the positions of the dorsal ectoderm in the sectioned embryos. (O) Quantitation of *htl* and *hbr* rescue by activated RAS1 ($RAS1^{Act}$) and activated HTL (HTL^{Act}) 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 htl^{AB42} 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 mesoderm 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).

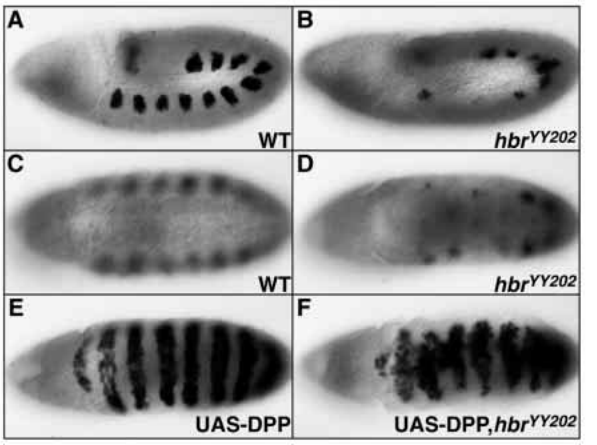


Fig. 5. Mesodermal cells in *hbr* mutant embryos are competent to respond to DPP. Lateral (A,B) and ventral (C,D) views of early stage 10 wild-type and hbr^{YY202} embryos showing expression of *bap* mRNA. The normal dorsal mesodermal expression of *bap* in segmentally repeated patches of cells is markedly reduced in *hbr* embryos. (E) *twi*-GAL4-directed ectopic expression of DPP induces *bap* transcription in stripes that span the entire dorsoventral axis (Gisselbrecht et al., 1996; Staehling-Hampton et al., 1994). (F) Ectopic DPP also activates *bap* expression in the ventral mesoderm of a *hbr* mutant.

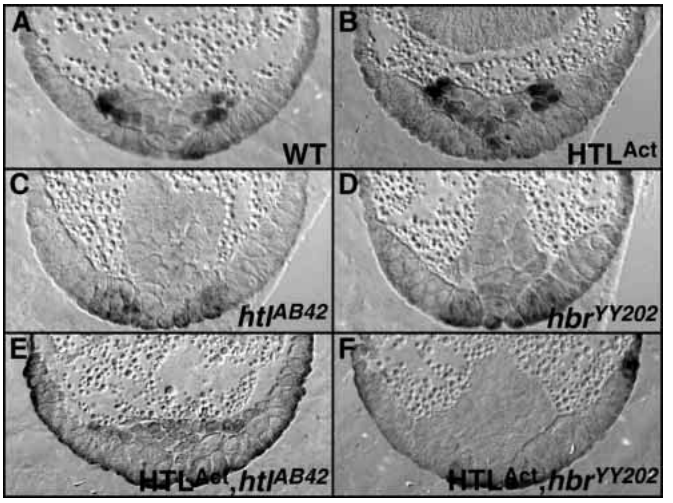


Fig. 4. Activation of MAPK at the leading edge of the migrating mesoderm depends on *hbr*. Transverse sections of embryos immunostained with an antibody against the diphosphorylated, activated form of MAPK are shown during the process of mesoderm migration. (A) In wild type, the highest levels of diphospho-MAPK in the migrating mesoderm are localized at the leading edge, as previously reported (Gabay et al., 1997b). More ventral cells have barely detectable levels. Expression is also seen in the ventral ectoderm. (B) *twi*-GAL4-mediated expression of activated HTL (HTL^{Act}) leads to slightly higher levels of diphospho-MAPK in ventral mesodermal cells, but the overall graded expression is not significantly perturbed. (C,D) Activated MAPK is completely lost from the mesoderm but not from the ventral ectoderm in both htl^{AB42} and hbr^{YY202} mutants. Note that the mutant mesodermal cells remain aggregated at this stage, as opposed to the wild-type mesodermal cells that have already dissociated and begun to spread dorsolaterally. (E) HTL^{Act} in a *htl* mutant background leads to a low, uniform activation of MAPK and partial rescue of cell migration. (F) In a *hbr* mutant, HTL^{Act} does not induce MAPK phosphorylation and does not rescue mesoderm migration.

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.

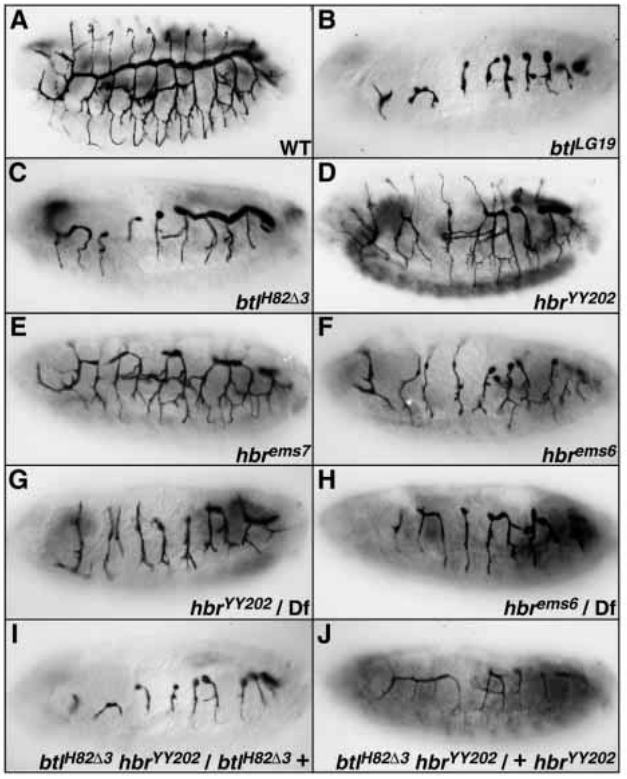


Fig. 6. *btl*-dependent tracheal cell migration is disrupted in *hbr* mutant embryos. Lateral views of stage 15 embryos immunostained with 2A12, a monoclonal antibody specific for a tracheal lumenal antigen. Anterior is to the left in all panels. (A) Wild-type embryo showing the normal pattern of tracheal branches. (B) Homozygous null btl^{LG19} embryo with tracheal cells confined to elongated sacs having occasional short extensions. (C) A homozygous hypomorphic mutant, $btl^{H82\Delta3}$, has a less severe inhibition of tracheal branching than the null allele. (D) A homozygous hbr^{YY202} 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^{ems7} has a similar tracheal phenotype to that of hbr^{YY202} (compare with D). (F) hbr^{ems6} has a more severe reduction in tracheal branching than the other two *hbr* alleles. (G,H) The tracheal phenotypes of both hbr^{YY202} and hbr^{ems6} are enhanced when these alleles are in *trans* to a deficiency that covers this locus, Df(3R)$ry^{506\text{-}85C}$. (I) One copy of hbr^{YY202} in a homozygous $btl^{H82\Delta3}$ embryo enhances the tracheal phenotype of the latter (compare with C). (J) Reducing *btl* function in a homozygous hbr^{YY202} embryo causes less tracheal branching than seen in the *hbr* mutant alone (compare with D).

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

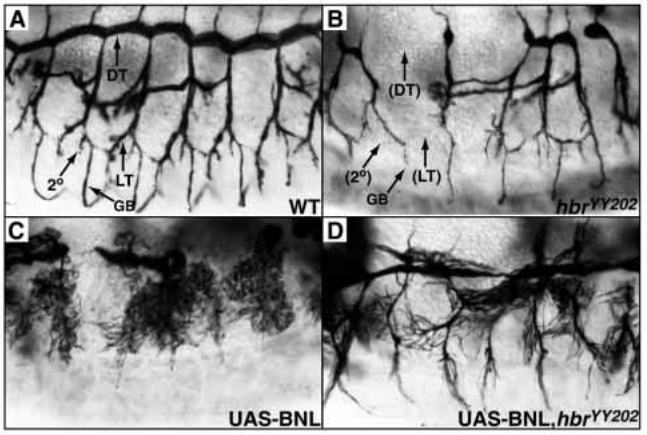


Fig. 7. *hbr* acts downstream of *btl* in tracheal development. Lateral views of stage 15 embryos of the indicated genotypes stained with the 2A12 antibody. (A,B) High magnification views of the same wild-type and hbr^{YY202} embryos as shown in Fig. 6A and B, respectively. Note the missing dorsal trunk (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^{YY202} 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).

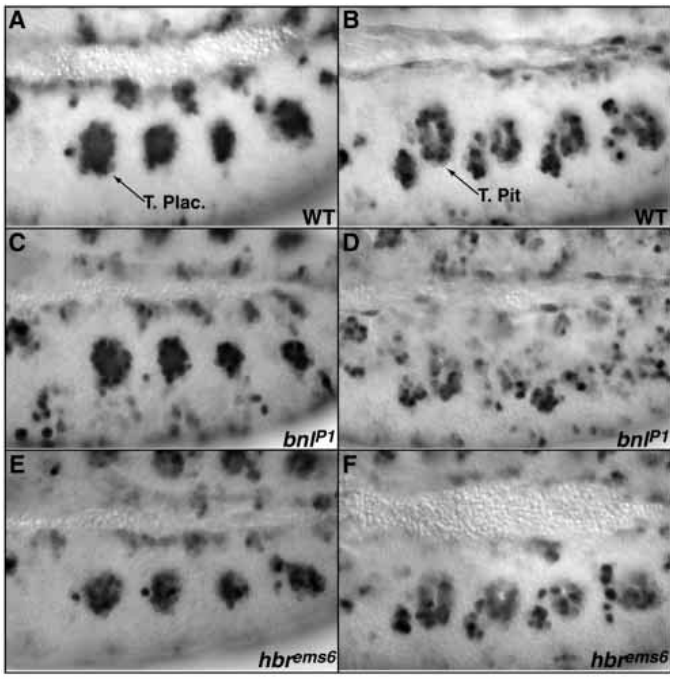


Fig. 8. *hbr* is involved in the BTL but not the DER-dependent activation of MAPK in tracheal cells. Lateral views of stage 10 (A,C,E) and stage 11 (B,D,F) embryos of the indicated genotypes immunostained for expression of diphospho-MAPK. In wild type (A,B), MAPK is activated by DER in cells of the tracheal placodes at stage 10 and by BTL in cells of the tracheal pits at stage 11 (Gabay et al., 1997b). Neither *bnl* (C) nor *hbr* (E) mutants alter DER-dependent MAPK activation. In contrast, MAPK activation by BTL is strongly reduced in *bnl* (D) and weakly reduced in *hbr* (F) mutants.

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., 1997a,b). 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 FASCICLIN 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 *hbr* mutant embryos (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; Clandinin 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,

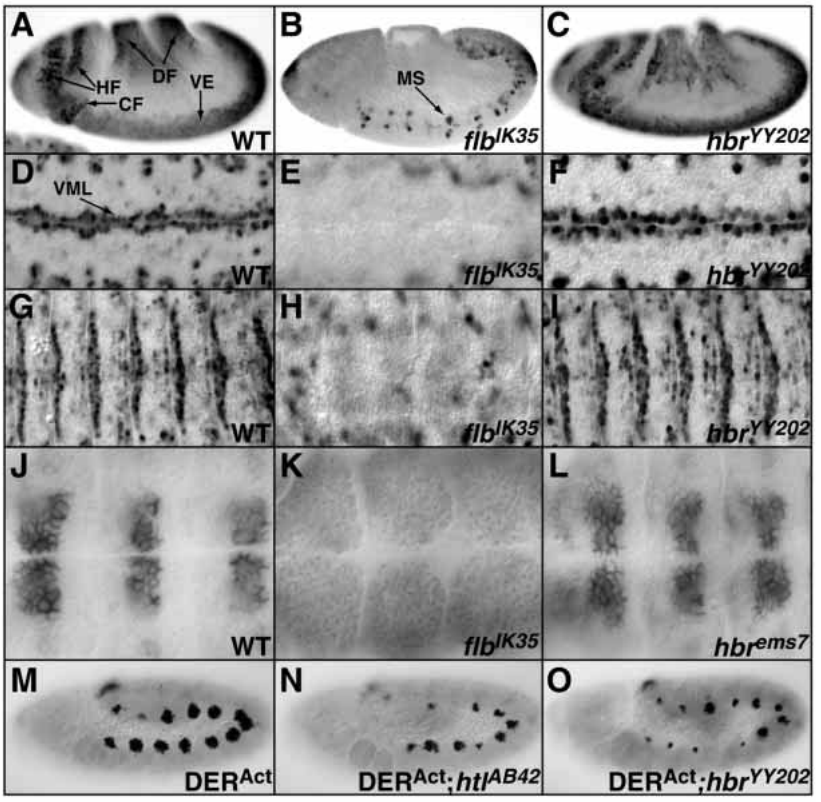


Fig. 9. *hbr* is not involved in DER-dependent embryonic signaling. Embryos of the indicated genotypes were stained for expression of diphospho-MAPK (A-I), FAS III (J-L) or EVE (M-O). (A-C) MAPK activation in the head folds (HF), cephalic furrow (CF), dorsal folds (DF) and ventral ectoderm (VE) of stage 8 embryos is absent from *flb* but unaffected in *hbr* mutant embryos. The residual diphospho-MAPK seen in *flb* embryos is due to normal HTL activity in the migrating mesoderm (MS, panel B). (D-F) Diphospho-MAPK expression in the ventral midline (VML) is eliminated in *flb* but not in *hbr* embryos. (G-I) Activation of MAPK in a segmentally repeated pattern in the ventral epidermis of stage 12/13 embryos is dependent on *flb* but not on *hbr* function. (J-L) FAS III expression in the ventral ectoderm of the three thoracic segments in stage 11 embryos is entirely normal in the *hbr* mutant but is absent from the *flb* mutant. (M-O) *twi*-GAL4-mediated ectopic expression of a constitutive form of DER in an otherwise wild-type genetic background causes an overproduction of EVE-expressing mesodermal progenitors (Buff et al., 1998). In both *htl* and *hbr* mutant embryos, constitutive DER induces a variable recovery of dorsal mesodermal EVE cells due to its ability to activate the RAS/MAPK cascade that also acts downstream of HTL (Gisselbrecht et al., 1996). This is in contrast to the inability of an activated form of HTL to rescue the mesodermal phenotype of a *hbr* mutant (compare with Fig. 3M). That is, the *hbr* mutation blocks HTL but not DER signaling in the mesoderm.

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

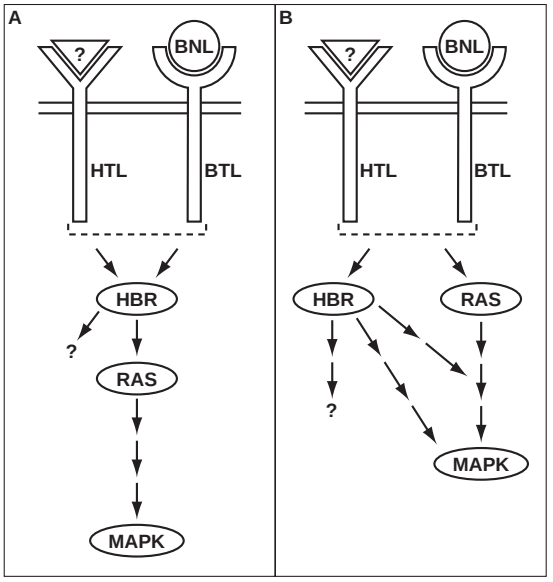


Fig. 10. Alternative models for *hbr* function in the HTL and BTL signalling pathways. The genetic data indicate that HBR acts downstream of both HTL and BTL FGFRs, and either in a linear pathway leading to RAS and MAPK activation (A), or in a pathway parallel to RAS (B). In the latter case, the HBR and RAS branches must converge on MAPK, with HBR or its downstream effectors acting either directly or indirectly on MAPK. In either model, unique signalling pathways also may be mediated by HBR.

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 signalling by these two RTKs. In this regard, it may be relevant that the hbr^{ems6} 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

BTL 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 EGFR 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; Reichman-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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