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Specificity of FGF signaling in cell migration in *Drosophila*

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SUMMARY

We wanted to investigate the relationship between receptor tyrosine kinase (RTK) activated signaling pathways and the induction of cell migration. Using *Drosophila* tracheal and mesodermal cell migration as model systems, we find that the intracellular domain of the fibroblast growth factor receptors (FGFRs) Breathless (Btl) and Heartless (Htl) can be functionally replaced by the intracellular domains of Torso (Tor) and epidermal growth factor receptor (EGFR). These hybrid receptors can also rescue cell migration in the absence of Downstream of FGFR (Dof), a cytoplasmic protein essential for FGF signaling. These results demonstrate that tracheal and mesodermal cells respond during a specific time window to a receptor tyrosine kinase (RTK) signal with directed migration, independent of the presence or absence of Dof. We discuss our findings in the light of the recent findings that RTKs generate a generic signal that is interpreted in responding cells according to their developmental history.

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

INTRODUCTION

Cell to cell signaling is involved in numerous developmental decisions, ranging from cell growth to cell determination, survival and migration. Recent genetic and molecular analysis has shown that only a limited number of signaling pathways are involved in cell communication and that they are reiteratively used throughout development. Ligands at the outside of the cells ensure the correct activation of receptors at the cell surface by their spatially and temporally restricted availability. However, as the same signaling pathways are reiteratively used, a question regarding their intracellular specificity has been raised. Why do cells react so differently to, for example, various receptor tyrosine kinases (RTKs)? And why is the same signaling pathway interpreted differently in different tissues? Several models have been proposed that can account for the unique developmental decisions taken in response to the activation of different RTKs (Rommel and Hafen, 1998; Simon, 2000; Tan and Kim, 1999). The first model proposes that there are intrinsic differences in the intracellular signaling pathways activated by various RTKs. A second model proposes that specificity arises from differences in the magnitude and/or duration of MAPK activation (Halfar et al., 2001; Marshall, 1995). A third model postulates that RTKs generally act via the same signaling cassette, producing a 'generic' signal, but cells interpret these signals according to their distinct developmental histories (Simon, 2000). In addition, the molecular basis for specificity of RTKs might be increased by the unique abilities of distinct pathways to crosstalk with other signaling pathways.

Recent studies have addressed the specificity of the RTK signal with regard to gene regulation in particular cell fate decisions. It turns out that in many cases, specific transcriptional responses to the activation of different RTKs do not arise from an intracellular signal that is specific to a particular RTK; instead, specific expression in many cases arises through the integration, at the level of response enhancers, of a generic RTK signal with inputs from non-RTK signaling pathways and other cell-type specific transcription factors (Flores et al., 2000; Halfon et al., 2000; Simon, 2000; Xu et al., 2000). Similar roles in generating cell-type-specific responses to signaling have been reported for selector gene products (Guss et al., 2001), including homeotic proteins (Grieder et al., 1997). In line with these experiments is the finding that in many cases one RTK can be functionally replaced in a given developmental context by a different RTK (Fambrough et al., 1999; Ghiglione et al., 1999; Golembo et al., 1996; Perrimon et al., 1995; Reichman-Fried et al., 1994; Schweitzer et al., 1995). Furthermore, the expression of a constitutive active Ras protein can largely compensate for the absence of RTK function in many developmental contexts. Using the recently developed gene chip technology, transcriptome analysis further indicated that different RTKs activate largely overlapping sets of immediate-early genes in a given cell line, again consistent with the idea that RTKs produce a generic signal that is interpreted in responding cells according to their developmental history (Fambrough et al., 1999).

Most of the studies investigating the specificity of RTK signaling have analyzed their capability to induce changes in the transcription profile in responding cells. We thought to investigate the specificity of RTK signaling in a developmental

context in which the signal generated by the RTK induces directed cell migration, a cellular phenomenon that presumably relies on changes in cytoskeletal architecture. During tracheal development in *Drosophila*, a RTK of the fibroblast growth factor (FGF) receptor superfamily encoded by the *breathless* (*btl*) locus is required for the formation of a stereotyped tubular network via directed cell migration and stereotyped cell shape changes (Klambt et al., 1992; Reichman-Fried and Shilo, 1995). The activity of the Btl receptor is triggered by the developmentally controlled local accumulation of its ligand Branchless (Bnl). Bnl functions as a chemoattractant and guides tracheal cells towards their cellular targets (Sutherland et al., 1996). The second *Drosophila* FGF receptor is encoded by the *heartless* (*htl*) locus and is required for directed mesodermal cell migration by a yet unidentified activation mechanism (Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998b; Shishido et al., 1993; Shishido et al., 1997; Wilson and Leptin, 2000). Although RTKs signal generally through an invariant signaling cassette, it turned out that FGF signaling relies on particular cytoplasmic proteins for linking the activated kinase to the downstream signal mediators (Schlessinger, 1993; Schlessinger, 2000). In *Drosophila*, the cytoplasmic protein encoded by the gene *downstream of FGFR* (*dof*; also called *heartbroken* or *stumps*) is required for Btl and Htl signal transduction and acts downstream of the receptors and upstream of Ras (Imam et al., 1999; Michelson et al., 1998a; Vincent et al., 1998). Dof is present exclusively in cells that express FGFRs and is needed in these cells for activation of the MAPK cascade via FGF signaling but not for MAPK cascade activation by other RTK ligands. Therefore, Dof appears to be committed exclusively to FGFR-mediated signal transduction. The difference in the signaling cassette between FGF receptors and other RTKs could be an indication that signaling specificity is in certain cases due to molecules differing between signaling cascades, as outlined in the first model (Luschnig et al., 2000).

To address the question of RTK signaling specificity in the induction of directed cell movement, we have asked whether FGFR signaling provides specificity regarding to its interpretation in tracheal cells or whether the intracellular region of the receptor can be replaced by other RTKs. We find that both Torso (Tor) and, albeit less efficiently, the *Drosophila* EGF receptor (EGFR) can functionally replace Btl and Htl and induce cell migration and activate appropriate cell fate determinants. All three intracellular tyrosine kinase domains are thus capable of triggering cell migration. In addition, the capability of Tor and EGFR to replace the intracellular domain of Btl and Htl allowed us to investigate the role of Dof in FGFR-mediated cell migration. We find that Dof is strictly required for FGFR-mediated cell migration but it is not essential for Tor- or EGFR-mediated migration. These results strongly suggest that Dof is only required for FGFR signaling and does not provide an essential link, allowing tracheal and mesodermal cells to migrate directionally under the control of RTK signaling. Our data are in line with the recent findings that RTKs generate a generic signal that is interpreted in responding cells according to their developmental history (Flores et al., 2000; Halfon et al., 2000; Simon, 2000; Xu et al., 2000) and we extend these findings to two RTK-induced cell migration events in *Drosophila*.

MATERIALS AND METHODS

Fly strains

The following transgenes carrying the chimeric receptors consisting of the extracellular and the transmembrane domain of either Btl or Htl fused to the intracellular domain of Htl, Btl, Tor and EGFR were generated: *UAS btl/ Cyo, UAS btl-htl/ Cyo, UAS btl-tor/ Cyo, UAS btl-egfr/ Cyo, UAS htl/ Cyo, UAS htl-btl/ FM6, UAS htl-tor/ Cyo, UAS htl-egfr/ Cyo*. For each construct, several independent lines carrying insertions on the X or the second chromosome were generated. For the crosses, we used the driver lines *btl*-Gal4 (Shiga et al., 1996) and p(*twist*-Gal4, w^+) (obtained from M. Akam, Wellcome Institute, Cambridge, UK); the *dof* mutant line P1740/ TM3 Sb Ubx lacZ (Karpen and Spradling, 1992; Vincent et al., 1998), which is a lethal P element enhancer trap insertion in 88C; the *btl* mutant line H82Δ3/ TM3 Sb (Klambt et al., 1992), which is an imprecise excision of a P element upstream of *btl*; and the *htl* mutant line *htl*AB42/ TM3; ftz lacZ (Michelson et al., 1998b), which is a null allele. Using the above mentioned *btl* and *dof* alleles, a *btl,dof* mutant chromosome was generated (H82Δ3, P1740/ TM3 Sb). We used as well the marker lines +/ +; 73B/ TM2 $Blue^{69}$ (provided by M. Bienz) and L/ Cyo; rf10/ TM3 (Ubx lacZ) (a gift from L. Keegan).

Constructs

The *btl-htl*, *btl-tor*, *btl-egfr*, resp. *htl-btl*, *htl-tor*, *htl-egfr* fusion constructs were generated by PCR. A primer containing the *Eco*RI site at position 1 of the *btl* sequence (5′-GGGAATTGGGA-ATTCGATTGAAG-3′) or the Asp718 site (5′-GCGGGTACCATCAT-GGCAAAAGTG-3′) and primers containing the end of the transmembrane domain of *btl* and an overlapping region with either the corresponding sequence of *htl*, *tor* or *egfr* were generated: *btl-htl* (5′-TTCATGCTGCGAAAGATGAAACATG-3′), *btl-tor* (5′-GATTT-CGTCTCAGCATGAACGTTATA-3′), *btl-egfr* (5′-CCGACACAGC-ATGAACGTTATG-3′). These primers were used to generate the part of the hybrid receptor cDNAs encoding the Btl extracellular domain and the transmembrane domain.

The second part of the hybrid receptor cDNAs were generated with a primer containing the sequence just after the transmembrane domain of *htl*, *tor* and *egfr*, and an overlapping region with the transmembrane domain of *btl*: *btl-htl* (5′-TTCATGCTGCGAAAGATGAAACATG-3′), *btl-tor* (5′-GTTCATGCTGAGACGAAATCGTTCG-3′) and *btl-egfr* (5′-TTCATGCTGTGTCGGCAAAAGC-3′). The second primers used were: *btl-htl* (5′-CTTTCGCAGCATGAACGTTATG-3′), containing an *Xba*I site and the flanking sequences of pUAST; *btl-tor* (5′-CGGGGTACCGTATTGCACTCGTTC-3′), containing the Asp718 site of pUAST; and *btl-egfr* (5′-CGTCTAGAACAAATCTATGG-GTC-3′), containing also the *Xba*I site of pUAST. The 5′ and 3′ fragments were amplified and fused by PCR using the Expand™ High Fidelity PCR system from Boehringer Mannheim.

The *htl-btl*, *htl-tor* and *htl-egfr* fusion constructs were generated using the same strategy. The following oligos were used: a primer containing the *Not*I site of *htl* (5′-GAGCTGCGGCCGCAAAATGG-CTGCCGC-3′), and a primer containing the *Eco*RI site of *htl* (5′-CGGAATTCAAAATGGCTGCCGC-3′). Primers containing the end of the transmembrane domain of *htl* and an overlapping region with the corresponding sequence of *btl*, *tor* or *egfr*: *htl-btl* (5′-CCGAC-GGATGGCATAGAAGAC-3′), *htl-tor* (5′-TCGTCTGATGGCATA-GAAGAC-3′), *htl-egfr* (5′-CCGACAGATGGCATAGAAGAC-3′). Primers containing the sequence after the transmembrane domain of either *btl*, *tor* or *egfr* and an overlapping region with the transmembrane domain of *htl*: *htl-btl* (5′-CTTTCGCAGCATGAAC-GTTATG-3′), *htl-tor* (5′-TATGCCATCAGACGAAATCGTTCG-3′), *htl-egfr* (5′-TATGCCATCTGTCGGCAAAAGC-3′). The most 3′ primers containing either the sequence of *btl*, *tor* or *egfr*: *htl-btl* (5′-CCGGTACCTTCTTTTTGGTCTCC-3′) and *htl-tor* (5′-CGGG-GTACCGTATTGCACTCGTTC-3′), both containing additionally the

Asp718 site and flanking sequences of pUAST; and *htl-egfr* (5′-CGTCTAGAACAAATCTATGGGTC-3′), containing the *Xba*I site of pUAST.

The chimeric cDNA constructs generated by PCR were subcloned into the pUAST vector in which they were expressed under the control of the hsp70 promoter and the Gal4-binding sites. Transgenic flies were generated by P-element-mediated germline transformation and the insertion sites were mapped genetically according to standard protocols.

Rescue experiments

To test whether the generated chimeric UAS constructs were capable of rescuing the tracheal or mesodermal defects of *btl*, *btl,dof*, *htl*, and *dof* mutant embryos, respectively, the following crosses were set up: *btl*-Gal4 transgenic flies (second chromosome insertion) and the chimeric constructs UAS c (also on the second chromosome) were crossed to L/ Cyo; rf10/ TM3 (Ubx *lacZ*) flies. The progeny *btl*-Gal4/ Cyo; +/ TM3 (Ubx *lacZ*) and UAS c/ Cyo; +/ TM3 (Ubx *lacZ*) were crossed to +/ +; H82Δ3/ TM2 (Blue[69]) flies. Subsequently, *btl*-Gal4/ +; H82Δ3/ TM3 (Ubx *lacZ*) and UAS c/ +; H82Δ3/ TM3 (Ubx *lacZ*) flies were crossed and embryos were collected at 25°C for 15 hours and at 18°C for 30 hours. The Ubx *lacZ* marker on the TM3 balancer chromosome was used to distinguish between homozygous and heterozygous *btl* mutant embryos. The same crosses were repeated using a *btl,dof* chromosome +/ +; H82Δ3, P1740/ TM2 (Blue[69]) in order to test whether the chimeric constructs were able to rescue tracheal defects in *btl,dof* double mutant embryos under the control of the *btl*-Gal4 driver on the second chromosome. We tested the mesodermal rescue capacity of the UAS constructs using the above strategy. To test whether mesodermal defects could also be rescued in *dof* mutant embryos, we crossed *dof* mutant flies to either UAS gene c transgenic flies and to *twist*-Gal4 transgenic flies: UAS c/ +; P1740/ + flies were further crossed to *twist*-Gal4/ +; P1740/ + flies. Embryos were collected at 25°C for 15 hours. Homozygous *dof* mutant embryos were identified by the lack of tracheal cell migration, heterozygous *dof* mutant embryos by a wild-type tracheal system.

Antibody stainings

After collection and dechorionation, the embryos were fixed in 9.6% formaldehyde/heptan solution for 15 minutes. Antibodies used: the mouse mAB 2A12 against the lumen of the trachea (provided by N. Patel), rabbit anti-β-galactosidase (Cappel), mouse anti-DSRF (Guillemin et al., 1996) and mouse anti-Evenskipped (provided by M. Frasch). For confocal analysis the signal was amplified. We used as secondary antibodies peroxidase-conjugated antibodies followed by biotinylated-tyramide (NEN Life Science Product), which is recognized by streptavidin-fluorescein or streptavidin-Texas Red. In addition, rabbit anti-Cy3 affinity pure F(ab′)2 fragments (Jackson) were used as secondary antibodies. Fluorescent images were captured using confocal microscopy (Leica TCS SP2) and processed using the Leica TSC NT 1.6 program and Photoshop 5.5 from Adobe. All images represent projections of sections on one focal plane.

For the other stainings we used as secondary antibodies biotinylated anti-mouse IgG and biotinylated anti-mouse IgM (Vector laboratories).

RESULTS

Hybrid receptors rescue tracheal development in *btl* mutant embryos

In order to investigate whether the FGF receptor Btl can be substituted by other *Drosophila* RTKs, we replaced the endogenous Btl receptor activity by other RTKs. As the local activation of Btl by the spatially restricted ligand Bnl is essential for proper cell migration and tracheal morphogenesis

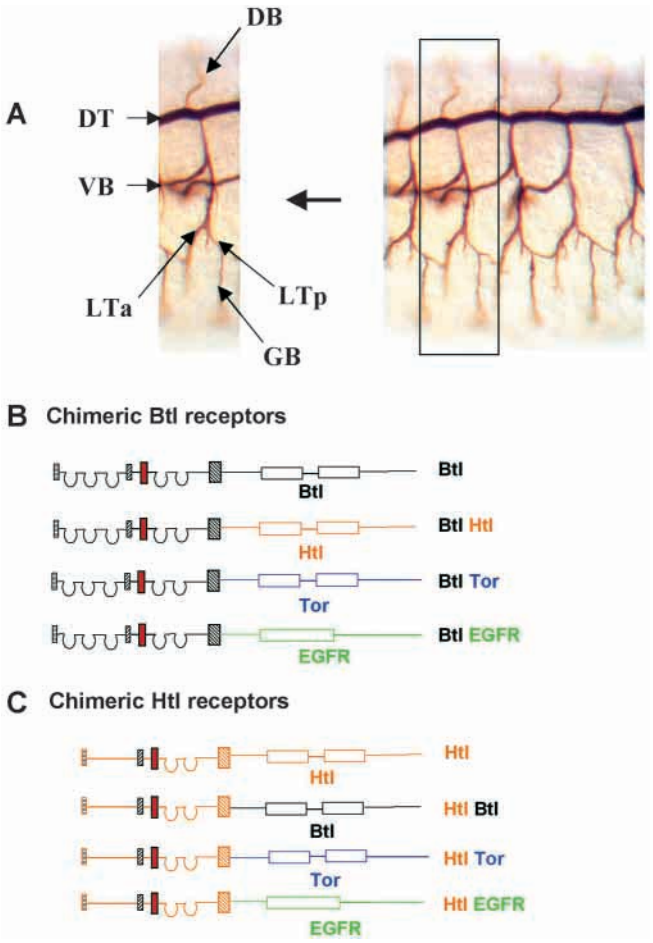

Fig. 1. Receptor constructs tested in vivo. (A) Representation of a tracheal segment indicating the six different primary tracheal branches: DB, dorsal branch; DT, dorsal trunk; VB, visceral branch; LTa and LTp, lateral trunk anterior and posterior; GB, ganglionic branch. (B,C) Wild-type Btl and Htl constructs, and the chimeric receptor constructs. The extracellular domain of Btl is composed from left to right of a N-terminal signal peptide, three Ig domains, an acidic region, a CAM binding domain, two Ig domains and a transmembrane domain. The intracellular domain consists of the juxtamembrane domain followed by the split tyrosine kinase catalytic domain and a short C-terminal domain. Htl contains only two Ig extracellular domains; all the other domains are homologous to Btl. (B) The extracellular and transmenbrane domain of Btl were fused to the intracellular domain of the following RTKs: Htl, Tor and EGFR. (C) Additionally, the extracellular domain and the transmembrane domain of Htl were fused to the intracellular domain of Btl, Tor and EGFR.

(Lee et al., 1996; Sutherland et al., 1996), we fused the extracellular and transmembrane domain-containing portion of the Btl receptor to the intracellular kinase-containing domains of either the other *Drosophila* FGF receptor Htl, the *Drosophila* Tor receptor or the *Drosophila* EGF receptor, respectively (see Fig. 1B). In principle, this should result in the activation of the heterologous kinase domain under the temporal and spatial control of Bnl.

To express these hybrid receptor molecules in the tracheal system, we made use of the UAS Gal4 system (Brand and Perrimon, 1993). Hybrid cDNAs were cloned downstream of

Table 1. Rescue of *btl*⁻ and *btl*⁻*dof*⁻ mutant embryos by expressing either UAS *btl*, UAS *btl-htl*, UAS *btl-tor* or UAS *btl-egfr* in the trachea

Line	*btl*⁻ embryos	*btl*⁻ embryos with trachea rescue
Btl (29b)	35	9 (26%)
Btl (47)	35	10 (29%)
Btl-Htl (19)	70	16 (23%)
Btl-Htl (20)	64	24 (38%)
Btl-Tor (9b)	66	16 (24%)
Btl-Tor (30c)	55	16 (29%)
Btl-Egfr (17)	68	16 (24%)
Btl-Egfr (18)	39	12 (31%)

Line	*btl*⁻ *dof*⁻ embryos	*btl*⁻ *dof*⁻ embryos with trachea rescue
Btl (29b)	44	0 (0%)
Btl (47)	84	0 (0%)
Btl-Htl (19)	32	0 (0%)
Btl-Tor (9b)	43	8 (19%)
Btl-Tor (30c)	33	10 (30%)
Btl-Egfr (17)	52	8 (15%)
Btl-Egfr (18)	43	12 (28%)

the yeast UAS sequences and introduced into the *Drosophila* genome by P-element-mediated germline transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Several independent transformant lines were established for each construct and two of them were tested in rescue assays (see Table 1). Receptor genes were expressed under the indirect control of the *btl* enhancer using a *btl*-Gal4 transgenic driver line (Shiga et al., 1996). In order to verify whether this expression system allowed the timely synthesis of receptors at biologically significant levels, we first tested a *btl* rescue construct for its capacity to rescue *btl* mutant phenotypes. The tracheal system was outlined with a lumen-specific monoclonal antibody (Fig. 2; see Materials and Methods). *btl* mutants are characterized by the complete absence of tracheal cell migration resulting in the lack of all tracheal branches (compare Fig. 2B with Fig. 2A); although tracheal cells are specified and properly invaginate, the tracheal system fails to undergo tracheal morphogenesis (Fig. 2B). When a *btl* cDNA was expressed under the control of Gal4 in the tracheal system of *btl* mutant embryos, tracheal morphogenesis was completely restored and all aspects of tracheal development were rescued, both at 18°C and 25°C (Fig. 2D,E; see Table 1). All six tracheal branches developed, fusion of the dorsal trunk and the lateral trunk occurred properly and terminal cells, as visualized by the expression of the *Drosophila Serum Response Factor/blistered* (*DSRF/bs)* (Affolter et al., 1994; Guillemin et al., 1996), were induced in the correct number and positions (Fig. 2C,F). Clearly, the expression system we used allowed a complete rescue of all tracheal defects of *btl* mutant embryos by a *btl* transgene.

Using the same experimental conditions, we tested the rescue capacity of the three hybrid receptors, Btl-Htl, Btl-Tor and Btl-EGFR (Fig. 2, see Table 1.). In all cases, we tested the rescue capacity at 18°C and 25°C and analyzed tracheal morphogenesis as well as terminal cell determination. For each temperature, a representative embryo is shown.

The expression of a Btl-Htl fusion protein in a *btl* mutant, both at 18°C and at 25°C, allowed a complete rescue of tracheal cell migration and correct fusion of the relevant tracheal branches (Fig. 2G,H). In addition, terminal tracheal cells were determined at the appropriate positions and in correct numbers as assayed by DSRF/Bs antibody staining (Fig. 2I). The rescue efficiency was high, both at 18°C and at 25°C. This result was anticipated as the tyrosine kinase domains of Btl and Htl are both derived from FGF receptors and share 79% identity at the protein level (Shishido et al., 1993).

The expression of the Btl-Tor fusion protein also allowed a substantial rescue. Tracheal cell migration was clearly triggered by the Btl-Tor receptor, such that most tracheal branches developed properly and fusion between adjacent metameres occurred in most segments (Fig. 2J,K). Occasionally, dorsal branches were missing and the lateral trunk failed to fuse in between some tracheal metameres. Altogether, tracheal morphogenesis was restored with great accuracy by the Btl-Tor fusion protein. However, the tracheal tree had sort of a ‘felted’ phenotype with fine branches extending from many positions (Fig. 2K and data not shown). Based on previously reported data, such ectopic fine branches could represent ectopic differentiation of terminal cells by a hyperactive receptor (Lee et al., 1996). In order to investigate whether the expression of the Btl-Tor fusion protein indeed led to the formation of ectopic terminal cells, we analyzed the expression of *DSRF/bs* in *btl* mutant embryos expressing the Btl-Tor protein. Clearly, such embryos had a much larger number of *DSRF/bs*-expressing terminal cells in the tracheal system (Fig. 2L); most tracheal cells forming the lateral trunk expressed *DSRF/bs*, and even cells of the transverse connectives in close proximity to the dorsal trunk accumulated *DSRF/bs*. We think that the ectopic induction of terminal cells could be the cause for the somewhat reduced efficiency to rescue dorsal branch and lateral trunk formation; fusion cells might be respecified to become terminal cells, resulting in tracheal defects. Nevertheless, we conclude that the intracellular domain of the Tor receptor can functionally substitute for the intracellular domain of the Btl receptor, both with regard to cell migration and with regard to the induction of terminal cell differentiation.

In addition to Tor and the two FGF receptors, the *Drosophila* EGFR has been extensively studied (Freeman and Bienz, 2001). To assess whether EGFR could substitute for Btl, we replaced the intracellular domain of Btl with the intracellular domain of EGFR. At 18°C and at 25°C, this hybrid receptor was clearly capable to rescue all primary branches, albeit at reduced frequencies (Fig. 2M,N; compare with Fig. 2B). Many of the rescued embryos show dorsal and ganglionic branch formation defects in half or more of the segments. In addition, the lateral trunk fused only sporadically, in contrast to the dorsal trunk, in which fusion was complete in most embryos. We also analyzed the expression of *DSRF/bs* in *btl* mutant embryos expressing the Btl-EGFR fusion protein and found a significant reduction in the number of terminal cells (Fig. 2O). These results suggest that EGFR signaling is weaker than Btl signaling in the case of tracheal guided cell migration and that the differences between the rescues of the different hybrid receptors might be quantitative rather than qualitative (Ghiglione et al., 1999) (see Discussion).

Hybrid receptors rescue tracheal development in *btl,dof* double mutant embryos

We have previously reported that FGF signaling in *Drosophila*

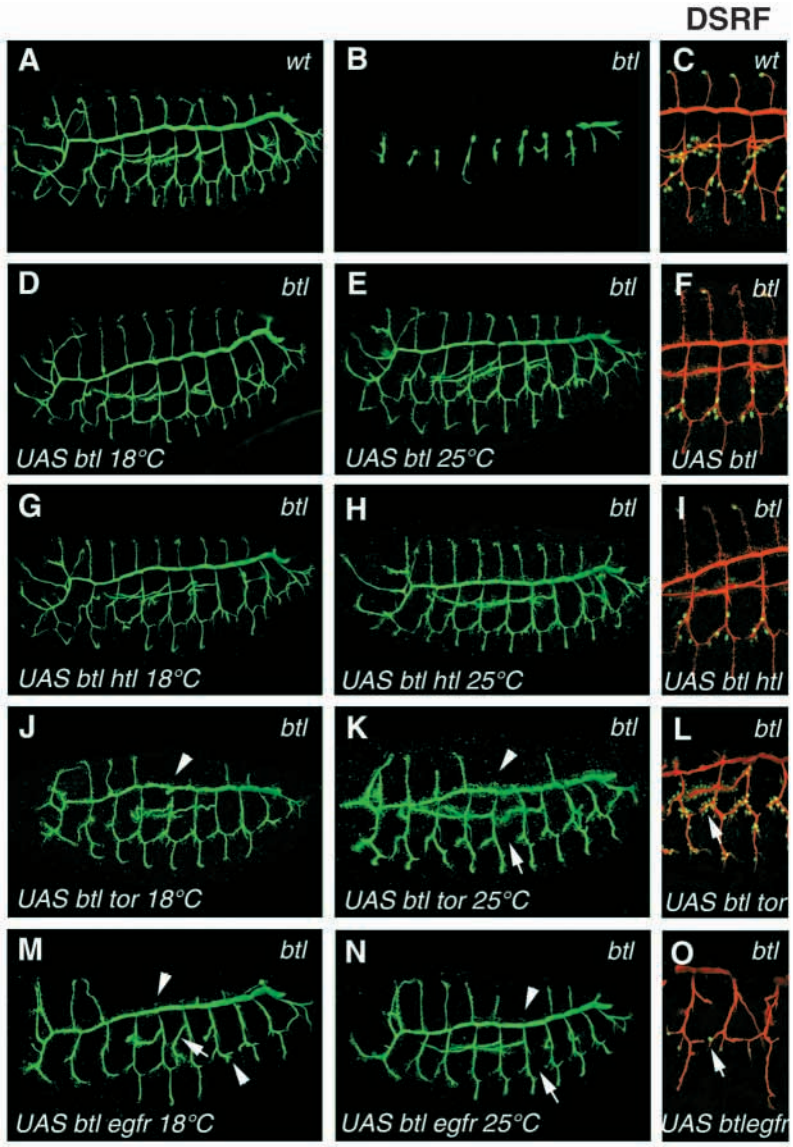

Fig. 2. Rescue of tracheal cell migration and patterning defects by hybrid receptors. The lumen of the tracheal system of stage 15 embryos was visualized with the 2A12 antibody (green, A,B,D,E,G,H,J,K,M,N; red, C,F,I,L,O) and terminal cells were visualized with the anti-DSRF antibody (green, C,F,I,L,O). Embryos obtained from the crosses as described in the Materials and Methods were collected at 18° C and 25°C. (A) Tracheal system of a wild-type embryo. (B) In homozygous *btl* mutant embryos, tracheal cells failed to migrate completely. (C) Tracheal system of a wild-type embryo outlining the *DSRF/bs*-expressing terminal cells. (D,E) All aspects of tracheal cell migration were fully rescued in *btl* mutant embryos expressing a *btl* transgene under the control of *btl*-Gal4. (F) The correct number of terminal cells differentiated at the proper positions as in wild-type embryos. (G,H) The Btl-Htl chimeric receptor construct was able to fully substitute for Btl and rescued tracheal cell migration. (I) The Btl-Htl chimeric receptor construct was able to induce proper terminal cell fates. In F,I, *DSRF/bs* expression in visceral branches is out of the focal plane. (J,K) The Btl-Tor fusion protein was able to rescue tracheal cell migration in *btl* mutant embryos. Some dorsal branches were missing (arrowheads) and the lateral trunk did not fuse in each segment (arrow). Apart from these defects, dorsal trunk fusion, visceral branch and ganglionic branch formation was not affected and the general pattern of the tracheal system was restored. Note the felted phenotype of the tracheal system, owing to the fine ectopic terminal branches (see K). (L) The Btl-Tor construct led to the ectopic formation of terminal cells (arrow). (M,N) The Btl-EGFR fusion protein was able to rescue tracheal cell migration in *btl* mutant embryos but less efficiently than Btl-Tor. Some dorsal branches were missing (dorsal arrowheads), the lateral trunk did not fuse (ventral arrow, N), ganglionic branches often failed to migrate (ventral arrowhead, M) and in some embryos, the visceral branches were misguided (arrow, M). (O) *DSRF/bs* expression and terminal cell formation was strongly reduced (arrow) when compared with wild type (C).

requires the Downstream of FGFR (Dof) protein (Vincent et al., 1998). Mutations allelic to *dof* have also been named *stumps* (Imam et al., 1999) or *heartbroken* (Michelson et al., 1998). In *dof* mutant embryos, tracheal cells fail to migrate, resulting in tracheal phenotypes identical to those seen in *bnl* and *btl*. Dof is an intracellular protein that is essential for signal transmission of both *Drosophila* FGF receptors (Btl and Htl) and acts downstream of the activated receptors and upstream of Ras. Surprisingly, Dof is needed for activation of the mitogen-activated protein kinase (MAPK) cascade via the FGF receptors, but not for MAPK activation via other RTKs. Strikingly, and in contrast to other RTK signaling components, Dof is expressed only in those tissues in which either one of the two *Drosophila* receptors is expressed. Although the molecular function of Dof has not been investigated yet, several models regarding its function have been proposed. On the one hand, Dof could be an adaptor protein, allowing the FGF receptor to connect to the Ras-MAPK pathway. Vertebrate FGF receptors do rely on specific linker proteins, such as SNT/FRS2 (Kouhara et al., 1997) to connect to the MAPK cascade. On the other hand, it has been proposed that Dof might be a key component that confers specificity to FGFR signaling. In both mesodermal and tracheal development, FGF receptors and EGF receptors, both members of the RTKs superfamily, carry out essential functions but elicit unique biological responses. The availability of a dedicated RTK signal transducer like Dof, in addition to shared components such as RAS1, could ensure that the appropriate output is generated by each RTK.

To investigate whether Dof is essential for tracheal cell migration, regardless of which RTK triggers migration, we made use of the observation that, when fused to the extracellular/transmembrane domain, tyrosine kinase domains unrelated to those of FGFRs can interpret the distribution of Bnl and activate the intracellular events resulting in directed migration. Therefore, we expressed the hybrid receptors Btl-Tor and Btl-EGFR in *btl*,*dof* double mutants and assayed the capacity of these receptors to rescue tracheal cell migration and patterning.

The absence of Dof interferes with signaling via both

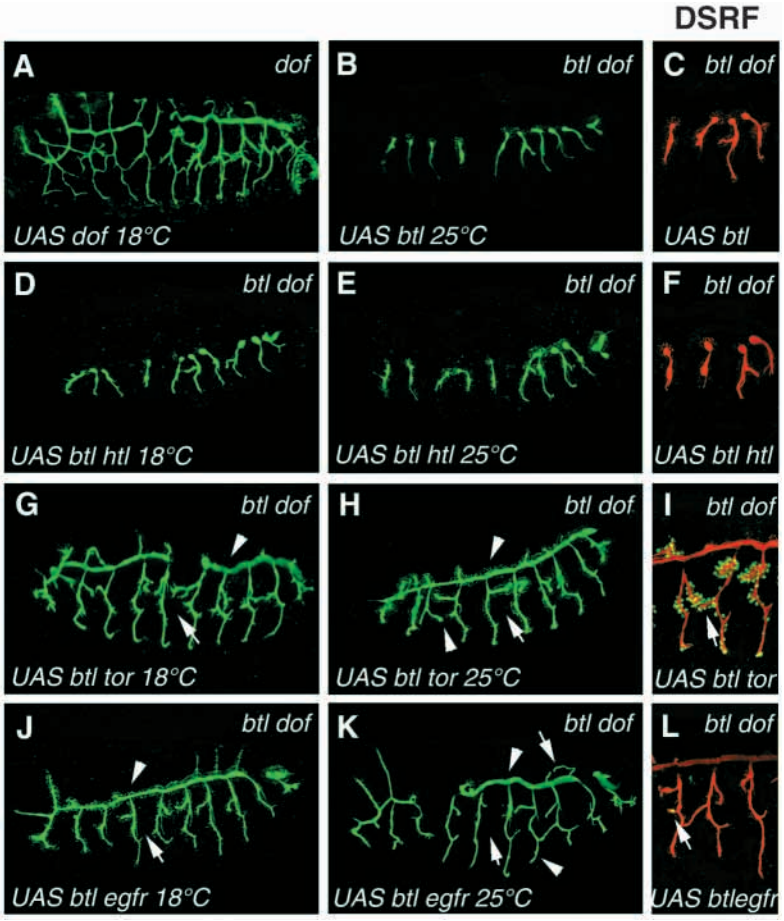

Fig. 3. Rescue of tracheal cell migration in the absence of *dof*. Whole-mount antibody staining for the tracheal lumen of stage 15 embryos using the 2A12 antibody (green, A,B,D,E,G,H,J,K; red, C,F,I,L) and for the terminal cells using the anti-DSRF antibody (green, C,F,I,L). Embryos from crosses described in the Materials and Methods were collected at 18°C and 25°C. (A) Tracheal system of a homozygous *dof* mutant embryo upon the expression of a *dof* transgene driven by *btl*-Gal4. Apart from defects that are due to the malformation of the mesoderm in *dof* mutant embryos (for example, truncation of visceral branches), the tracheal system was fully rescued. (B) The Btl wild-type receptor construct was not able to rescue tracheal cell migration in *btl,dof* double mutant embryos. (C) *DSRF/bs* expression was not induced in any tracheal cell in *btl,dof* mutant embryos expressing the *btl* transgene. (D,E) The Btl-Htl chimeric receptor construct was not able to rescue tracheal cell migration in *btl,dof* mutant embryos. (F) *DSRF/bs* expression was not induced in the tracheal cells of *btl,dof* mutant embryos expressing Btl-Htl. (G,H) In contrast to Btl and Htl, the Btl-Tor fusion protein was able to rescue tracheal cell migration in *btl,dof* mutants. The general pattern of the tracheal system was rescued. Dorsal trunk fusion occurred efficiently but some dorsal branches failed to form (dorsal arrowheads). The lateral trunk did not fuse (arrows) and depending on the embryo, one or two ganglionic branches failed to form (ventral arrowhead). (I) Expression of the Btl-Tor construct led to ectopic activation of *DSRF/bs* and terminal cell formation in *btl,dof* mutant embryos (arrow). (J,K) The Btl-EGFR construct was able to rescue tracheal cell migration in *btl,dof* mutant embryos to a certain extent. General tracheal patterning was rescued, although the tracheal tree was more affected. In some embryos the dorsal trunk was disrupted, some dorsal branches were missing (dorsal arrowheads) or misguided (dorsal arrow) and the lateral trunk failed to fuse (ventral arrows). In contrast to the rescue by Btl-Tor, some of the ganglionic branches failed to form and did not migrate in proper direction (ventral arrowhead). (L) *DSRF/bs* expression was strongly reduced in *btl,dof* mutant embryos expressing Btl-EGFR (arrow).

Drosophila FGF receptors Btl and Htl, and therefore *dof* mutant embryos display defects not only in the tracheal system but also in mesoderm-derived structures (Imam et al., 1999; Michelson et al., 1998a; Vincent et al., 1998). As mesodermal cells are required for certain aspects of tracheal development (Boube et al., 2001; Wolf and Schuh, 2000), we compared the tracheal rescue efficiency of our receptor constructs to the rescue capacity of *dof*, driven under the indirect control of the *btl* enhancer. As previously reported (Vincent et al., 1998), *dof* rescues most aspects of tracheal patterning when expressed exclusively in the tracheal system of *dof* mutant embryos (Fig. 3A). With the exception of the visceral branches, all aspects of the tracheal system were rescued; the visceral branches did not extend properly because the underlying visceral mesoderm is not properly specified and therefore lacks as a support for migrating cells (Boube et al., 2001; Vincent et al., 1998). In addition, the outline of the tracheal network in rescued embryos was generally less well organized, and many branches, although present, did not follow the stereotyped pathways. This is most probably due to the absence or to defects in the mesodermal cell layer, which helps in the correct tracing of tracheal migratory routes (Franch-Marro and Casanova, 2000).

When either the Btl or the Btl-Htl receptors were expressed in the developing trachea of *btl,dof* mutant embryos, no rescue of any aspect of tracheal development was observed (Fig. 3B,D,E; see Table 1); tracheal cells completely failed to migrate and *DSRF/bs* expression was not induced in any tracheal cells (Fig. 3C,F). This result confirms the strict requirement of Dof for FGF signaling, both in cell migration and in transcriptional regulation.

In sharp contrast to the complete failure to rescue tracheal cell migration in *btl,dof* mutant embryos by Btl and Btl-Htl, the expression of Btl-Tor allowed a significant rescue of tracheal patterning (compare Fig. 3B,D,E with Fig. 3G,H; see Table 1). Ganglionic branches were rescued almost completely, dorsal branches developed in more than half of the segments and dorsal trunk fusion occurred efficiently. The only aspect of tracheal development that was rescued with less efficiency (besides the formation of the visceral branches; see above) was the fusion of the lateral trunk. Also in sharp contrast to the rescue with Btl and Btl-Htl, the expression of Btl-Tor in *btl,dof* mutants resulted in the efficient and ectopic induction of *DSRF/bs* expression in a large number of tracheal cells (Fig. 3I, compare also with Fig. 2L). Very similar results were obtained when the Btl-EGFR hybrid receptor was expressed in *btl,dof* mutant embryos; tracheal patterning was restored to a significant extent (Fig. 3J,K; compare with Fig. 3B,D,E; see Table 1). However, and as already observed in the rescue experiments in *btl* mutants, the rescue was less efficient both

with regard to migration and with regard to induction of gene transcription.

From comparing the rescue capacity of Btl-Tor and Btl-EGFR in either *btl* single or *btl,dof* double mutant embryos, we conclude that induction of cell migration by RTKs carrying intracellular kinase domains unrelated to FGF receptors is not or at least not strongly dependent on the presence of Dof; in sharp contrast, the absence of Dof is detrimental to the induction of cell migration by the FGF receptors Btl and Btl-Htl.

Hybrid receptors rescue mesodermal spreading and migration in *htl* mutant embryos

In order to confirm the conclusion we draw from the tracheal rescue experiments in a different developmental context, we generated a new series of hybrid receptors using the extracellular and transmembrane domain of the *Drosophila* FGF receptor Htl (Fig. 1C). As already mentioned, Htl function is essential for mesodermal cell spreading and migration and later during embryonic development for the proper induction of different mesodermal cell fates (Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998b; Shishido et al., 1997; Wilson and Leptin, 2000). In order to assay the rescue capacity of the different receptors or receptor hybrids, we monitored the development of pericardial cells using Evenskipped (Eve) as a marker protein (Azpiazu et al., 1996; Frasch, 1995). As mesodermal spreading and migration is a prerequisite for normal induction of pericardial cells in the dorsal region of the embryo, both *htl* and *dof* mutant embryos lack all 20 Eve-expressing pericardial cells along the dorsal midline on either side of the embryo (compare Fig. 4B,C with the wild-type embryo shown in Fig. 4A). Mesoderm migration and pericardial development was fully rescued in *htl* and *dof* mutant embryos by expressing Htl or Dof, respectively, under the indirect control of the *twist*-enhancer (Fig. 4D,E; see also Materials and Methods and Table 2).

In line with the experimental results described above using the tracheal system, replacing the intracellular domain of the Htl receptor by either Btl (Fig. 4G), Tor (Fig. 4I) or EGFR (Fig. 4K) rescued pericardial development in *htl* mutant embryos. The rescue with the Htl-Btl and the Htl-EGFR hybrid receptors was somewhat suboptimal. In most cases, only 12 to 18 (instead of 20) pericardial cells were properly determined, and they often formed small clusters instead of being aligned along the anterior-posterior axis (see Table 2). In contrast, the Htl-Tor construct led to the development of an excess of pericardial cells, resulting in up to 30 antigen-positive cells along the dorsal midline on each side of the embryo (see Table 2). Although the final pattern of pericardial cells was somewhat irregular, these experiments demonstrate that all three tyrosine kinase domains can signal to the cell migration machinery and to the nucleus in a similar or identical manner in mesodermal

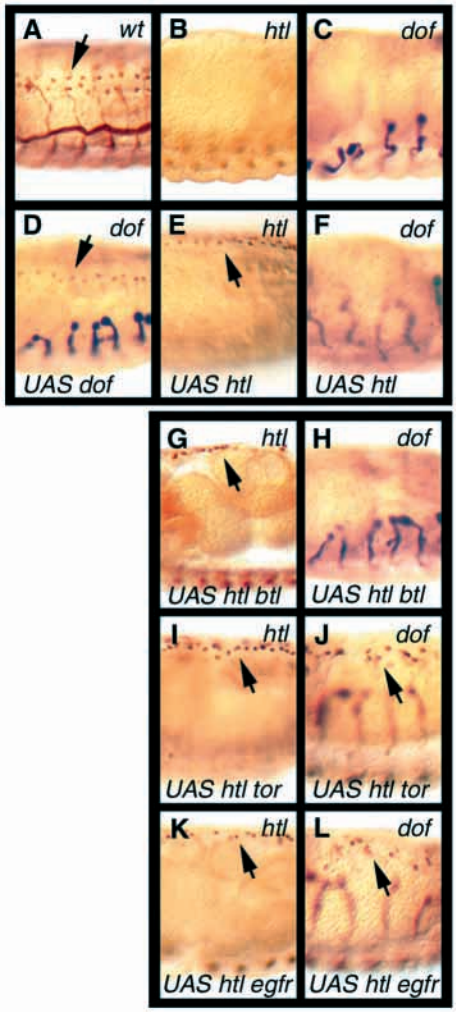

Fig. 4. Rescues of mesodermal cell migration defects by hybrid receptors. The embryos were generated in the crosses described in the Materials and Methods and collected at 25°C. The tracheal system was visualized with the 2A12 antibody. Mesoderm migration and pericardial cell formation was analyzed with an anti-Eve antibody (see also Materials and Methods). Evenskipped is specifically expressed in the pericardial cells and was used as a marker for these mesodermal cells. Arrows point to the pericardial cells. (A) Pericardial cells arranged in two rows at the dorsal side of a wild-type embryo. (B) Mesodermal cells were not able to migrate properly in *htl* mutant embryos. Consequently, pericardial cell fate determination did not occur. (C) Mesodermal cells did not migrate properly in *dof* mutant embryos and pericardial cells failed to form. (D) Mesodermal cell migration and pericardial cell differentiation were rescued in *dof* mutant embryos expressing the *dof* transgene. The arrangement of the cells and cell number were identical to wild-type embryos. (E) Pericardial cell formation was fully rescued by the Htl wild-type construct expressed in *htl* mutant embryos. (F) By contrast, the Htl wild-type construct was not able to rescue mesodermal cell migration and pericardial cell formation in the absence of Dof. (G) The chimeric receptor construct Htl-Btl was not able to fully replace the endogenous Htl receptor when expressed in *htl* mutant embryos. Only 12-16 pericardial cells were formed on each side of the embryo (instead of 20). (H) In *dof* mutant embryos, Htl-Btl failed to rescue pericardial cell formation. (I) Expression of the Htl-Tor fusion protein in *htl* mutant embryos led to excess cardiac cell formation. Nineteen to 30 scattered cells were observed along the midline in the dorsal part of the embryo. (J) The rescue of pericardial cells in *dof* mutant embryos expressing Htl-Tor was almost complete. 18-20 pericardial cells were arranged in a slightly disordered manner at the dorsal side of the embryo. (K) 17-20 pericardial cells were formed in *htl* mutant embryos expressing the Htl-EGFR receptor construct. (L) In *dof* mutant embryos, the Htl-EGFR construct was able to rescue 12-18 pericardial cells on each side of the embryo but the cells failed to arrange in an anterior-posterior row.

Table 2. Rescue of *htl*⁻ and *dof*⁻ mutant embryos by expressing either UAS *htl*, UAS *htl-btl*, UAS *htl-tor* or UAS *htl-egfr* in the mesoderm

Line	*htl*⁻ embryos	*htl*⁻ embryos with pericardial cell rescue
Htl (33)	84	20 (24%) 20 pericardial cells
Htl-Btl (27)	86	15 (17%) 12-16 pericardial cells
Htl-Btl (28)	97	27 (28%) 12-16 pericardial cells
Htl-Tor (29b)	97	20 (21%) 19-30 pericardial cells
Htl-Tor (30a)	98	20 (21%) 19-30 pericardial cells
Htl-Egfr (24)	113	18 (16%) 17-20 pericardial cells
Htl-Egfr (25)	86	25 (29%) 17-20 pericardial cells

Line	*dof*⁻ embryos	*dof*⁻ embryos with pericardial cell rescue
Htl (33)	20	0 (0%)
Htl-Btl (27)	34	0 (0%)
Htl-Tor (29b)	20	3 (15%) 18-20 pericardial cells
Htl-Tor (30a)	38	11 (29%) 18-20 pericardial cells
Htl-Egfr (24)	60	14 (23%) 12-18 pericardial cells
Htl-Egfr (25)	50	13 (26%) 12-18 pericardial cells

cells. The observed differences appear to be more quantitative than qualitative.

Hybrid receptors rescue mesodermal spreading and migration in *dof* mutant embryos

Using tracheal development as an assay system, we found that the intracellular domain of the Tor and the EGF receptors were able to replace the intracellular domain of the FGF receptor Btl and replace its function in cell migration and gene transcription. In addition, this replacement resulted in the induction of cell migration and gene induction in a *dof*-independent manner. To confirm the uncoupling of cell migration and *dof* function, we compared the rescue capacity of Htl and Htl-Btl to Htl-Tor and Htl-EGFR in a *dof* mutant embryo; because *dof* and *htl* map close to each other, we did not generate a double mutant chromosome and assayed the rescue potential in *dof* single mutants.

The expression of either Htl or Htl-Btl in a *dof* mutant embryo under the indirect control of the *twist*-enhancer did not result in the rescue of Eve-expressing pericardial cells (Fig. 4F,H, respectively; see Table 2). However, both Htl-Tor (Fig. 4J) and Htl-EGFR (Fig. 4L) efficiently rescued migration of mesodermal cells. Both hybrid receptors appeared to function with somewhat reduced activity in the *dof* mutant background in comparison with the *htl* mutant background. While Htl-EGFR was somewhat less efficient in *dof*, the formation of supernumerary Eve-expressing cells by Htl-Tor in *htl* mutants was not observed in the *dof* mutant; the rescue resulted in the formation of approximately 20 pericardial cells aligned along the anterior-posterior axis on either side of the embryo (see Table 2). These results obtained in the mesodermal rescue assays are in line with those obtained for the tracheal system and demonstrate that cell migration can be triggered by non-FGF RTKs in the absence of Dof.

DISCUSSION

In the present study, we have addressed the role of FGF signaling specificity in trachea and mesoderm development. We have swapped the intracellular domain of the two *Drosophila* FGF receptors, or replaced them by the intracellular domain of Tor or EGFR. We found that all four hybrid receptors can functionally substitute for the endogenous receptors, albeit with somewhat varying efficiencies. Using these chimeric receptors we have been able to address the functional requirement of Dof, an intracellular protein committed to FGFR-mediated signal transduction, with regard to cell migration. We will discuss our results in the context of the recent findings that RTKs produce generic signals that are interpreted in responding cells according to their developmental histories (Flores et al., 2000; Halfon et al., 2000; Simon, 2000; Xu et al., 2000).

Specificity of FGF signaling in cell migration and transcription regulation

The function of FGFR signaling specificity in tracheal cell migration has been addressed previously (Reichman-Fried et al., 1994). In the referenced study, the authors expressed hybrid proteins consisting of the intracellular domains of the RTKs Tor, EGFR and Sevenless fused to the extracellular and transmembrane domain of a Tor dominant allele, and assayed the rescue potential of these receptors in a *btl* mutant background. These hybrid receptors were ubiquitously expressed and partially rescued the cell migration defects; similar or identical rescues were seen upon the expression of activated Ras or Raf proteins (Reichman-Fried et al., 1994). However, as obvious from the results shown, these partial rescues consisted mainly in the formation of the dorsal trunk (Reichman-Fried et al., 1994); dorsal branches, ganglionic branches and the lateral trunk formed only rarely under these assay conditions (Reichman-Fried et al., 1994) (C. D., unpublished data). However, more recent studies have shown that the development of the dorsal trunk is very particular with regard to its requirement for Bnl (Wolf and Schuh, 2000). Although the outgrowth of the dorsal trunk requires Bnl-induced Btl activity, dorsal trunk guidance does not require localized Bnl/Btl signaling but relies on a so-called ‘bridge cell’. As the studies of Reichman-Fried and collaborators demonstrated that dorsal trunk outgrowth and fusion can be brought about by unrelated RTKs and by Ras, little can be infered from these studies with regard to guidance.

Because we wanted to investigate the signaling specificity of Btl and Dof in Bnl-guided cell migration, we generated proteins containing the extracellular domain of the Btl receptor and the intracellular domain of different RTKs. These hybrid proteins were expressed in the target tissues exclusively, mimicking the wild-type situation as closely as possible. We found that the intracellular domain of Tor can functionally substitute for the corresponding region of the Btl receptor. All six major branches including the dorsal branches and the ganglionic branches form with high efficiency. In addition, the nuclear target gene *DSRF/bs* is activated by the Btl-Tor hybrid, albeit with increased efficiency. As the Bnl-induced transcriptional activation of *pointed* (*pnt*) is a prerequisite for the activation of *DSRF/bs* (Sutherland et al., 1996), we presume that *pnt* is also activated by Btl-Tor. Therefore, both the cellular events linked to guided migration as well as the nuclear events required for further cell fate specifications can be brought about by the Btl-Tor hybrid protein. We conclude that the specific interpretation of the RTK signal in tracheal cells, namely its translation into

a migratory response and the activation of the appropriate differentiation program, is not due to the specificity of the Bnl/Btl signaling system but rather is a property of tracheal cells. It is possible that tracheal cells (and mesodermal cells) express a particular protein(s) that allows them to trigger migration upon RTK signaling. It has been demonstrated previously that tracheal cells do express distinct proteins that allow them to react to the activated Btl receptors with migration; Dof is activated specifically in tracheal and mesodermal cells to allow FGF signal propagation. However, and as we argue below, Dof itself is not an essential tracheal factor that allows to link RTK activation to the cell migration machinery.

Role of Dof in FGF-mediated cell migration and transcription regulation

Besides the essential role of Dof in FGF receptor signaling in *Drosophila*, which we confirm in this work, little is known about the actual cellular or biochemical function of Dof. As Btl, Htl and Dof activity are required for guided cell migration, we were wondering whether cell motility and guidance induced by the hybrid proteins in the absence of endogenous Btl activity required Dof. None of the FGFRs could induce guided migration in *dof* mutants, while both the Tor and the EGFR intracellular domain were capable of rescuing migration in the absence of *dof*; the rescue efficiency of these hybrid receptors was similar to their rescue efficiency in *btl* mutants, which do have Dof protein. This result clearly demonstrates that tracheal and mesodermal cells respond to an RTK signal with directed migration, independent of the presence or absence of Dof. Dof activity appears to be required for FGF signaling per se, possibly relaying the signal from the activated receptor to downstream components, including Ras. It occurs that within a specific developmental window, tracheal cells respond to RTK activity with directed migration. Later in development, specific genes (*pnt*, *DSRF/bs*) are transcriptionally induced as a response to RTK activation. It will be most interesting to identify the cellular factors that impose this specificity and see whether their expression in other cell types results in motility.

Recently, it has been shown that border cells rely for their guided migration during oogenesis on EGFR activity (Duchek and Rorth, 2001). To mediate this guidance function, EGFR signals via a pathway that is independent of Raf-MAP kinase. In addition, the EGFR guidance appears to be receptor-specific, as Htl signaling can not substitute for EGFR signaling. It will be interesting to see whether similar or divergent signaling pathways are required for RTK-mediated migration of border cells, mesodermal and tracheal cells.

Quantitative versus qualitative differences in the cellular response to RTK signaling

As mentioned throughout the Results section, we did observe differences in the rescue efficiencies when we compared the function of the three receptors in the tracheal system and in the mesoderm. The differences we observe might be due (to some extent) to quantitative effects. However, it is also possible that qualitative differences exist and that some of the receptors (for example, EGFR) are less efficient in part because specific, non-essential signal adaptor proteins are absent or reduced in some of the tissues at the time we examined them. Without knowing the precise composition of the entire signaling pathways of all three receptors in the tracheal system and in the mesoderm, it is impossible to determine whether the differences in efficiencies we observe are due to quantitative and/or qualitative aspects of signaling.

That quantitative differences in signaling strength can indeed lead to qualitatively different outcome has been shown previously. In *Drosophila*, this has been demonstrated most clearly in those cases in which RTK ligands act as morphogens (Ghiglione et al., 1999; Greenwood and Struhl, 1997). However, the qualitative aspects of the tissue and stage specificity of the response to RTK signaling (for example the range of possible target genes to be affected) is controlled by the developmental history of the responding cells and not by the intracellular sequence of the RTK involved.

Although it has now been shown in many developmental contexts that the intracellular domains of different RTKs are largely interchangeable, it is worth emphasizing that in many cases FGF receptors can not functionally substitute for other *Drosophila* receptor tyrosine kinases. This is most probably due to the fact that the essential Dof protein is only present in a subset of cells in *Drosophila* (Vincent et al., 1998). Thus, the spectrum of cells in which FGF receptors can function is limited to those cells that express Dof and maybe other FGFR-specific signaling components.

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