Functional dissection of the *Drosophila melanogaster* Fibroblast Growth Factor signalling pathway in branching morphogenesis of the developing tracheal system

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Caroline Dossenbach

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Ausgeführt unter der Leitung von

Prof. Markus Affolter

Abteilung Zellbiologie

Biozentrum der Universität Basel

Klingelbergstrasse 50-70

CH-4056 Basel

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auf Antrag von:

Prof. Dr. Markus Affolter	Prof. Dr. Walter J. Gehring	Prof. Dr. Silvia Arber
(Dissertationsleiter)	(Korreferent)	(Vorsitzende)

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Prof. Dr. Marcel Tanner
(Dekan)

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Summary

Fibroblast Growth Factor (FGF) signalling is involved in numerous developmental processes ranging from cell determination to mitogenesis, and cell survival to cell migration. Interestingly, the same signalling pathway is used reiteratively throughout development and the question regarding the intracellular specificity is raised.

Little is known about the intracellular signalling events of the FGF signalling pathway leading to specific cellular responses. Since the FGF signal is essential throughout embryonic and adult development and plays a role in many pathogenical processes, it is important to identify the factors, which determine the differential responses.

We were interested to investigate the specificity of FGF signalling in a developmental context in which the signal induces directed cell migration, a cellular phenomenon that relies on changes of the cytoskeletal architecture.

During gastrulation in early embryonic development, but also during the formation of organs in mammals and in *Drosophila*, FGFs have been shown to act as chemoattractants and guide cells toward their targets. In these contexts, FGF signalling has been shown to induce filopodia, which are long cellular extensions containing parallel actin bundles.

Using *Drosophila* tracheal and mesodermal cell migration as model systems, we found that the intracellular domain of the two *Drosophila* FGF receptors (FGFRs) Breathless (Btl) and Heartless (Htl) can be replaced by the equivalent domains of Torso and EGFR, and yet these hybrid receptors will rescue cell migration in *btl* or *htl* mutant embryos, respectively. These chimeric receptors rescued cell migration even in the absence of Downstream-of-FGFR (Dof), a scaffolding protein that has been shown to be essential for FGF signalling in *Drosophila*. Thus, Dof acts specifically in the FGF signalling pathway. The functional characterization of Dof has demonstrated that Dof is indeed a FGFR specific phosphotarget and forms a complex with both FGFRs, but it is not a substrate of Torso.

We performed a functional deletion analysis of the Btl receptor to define the interaction domains of Dof and other putative adapter proteins essential in the process of cell migration. Deletion of all putative interaction domains outside of the kinase domain did not affect the rescue capacity of the truncated Btl receptors in vivo suggesting that the kinase domain is sufficient for transmitting the signal. In line with this interpretation, results from S2 cell culture experiments revealed that Dof interacts with the kinase domain, and it does so independently of the activation state of the receptor. Surprisingly, in S2 cells, Btl receptors lacking the C-terminus did not auto-phosphorylate, as consequence we could not observe phosphorylation of Dof. We assume, that the short C-terminus is required for conformational changes of the kinase activation loop upon dimerization of the receptors to enable trans-phosphorylation.

Dof belongs to a distinct family of adapter proteins than its functional homologue, the vertebrate FGFR adapter protein FRS2 that has been shown to constitutively interact with the juxtamembrane domain of the FGFRs. We could show that the human FGFR2, when expressed in the tracheal system, is only able to rescue cell migration defects effectively in the presence of Dof. These results suggest that Dof is able to interact with human FGFRs. At present, there is no evidence for a FRS2 homologue in *Drosophila* that might act as substitute for Dof.

Upon receptor activation, Dof recruits the phosphatase Corkscrew (Csw), the *Drosophila* Shp2 homologue. Csw recruitment represents an essential step in FGF induced cell migration and transcriptional activation via the Ras/MAPK cascade. However, our results indicate that the activation of Ras is not sufficient to activate the migration machinery in tracheal and mesodermal cells. Ectopic activation of the Ras/MAPK cascade partially rescued tracheal cell migration in *btl* or *dof* mutant embryos. But high levels of sustained activation of Ras or the MAPK in wild-type tracheal cells did not disturb the migratory behaviour of the cells in contrast to ectopic activation of Branchless (Bnl), the *Drosophila* FGF homologue, which completely impaired primary branch outgrowth. In a wild-type tracheal system, MAPK activity is restricted to the tracheal tip cells. Single cell rescue experiments indicate that Bnl induces the migratory response

exclusively in the tip cells of the outgrowing tracheal branches; the stalk cells are pulled forward by cell-cell adhesion contacts.

The small GTPases of the Rho family have been shown to regulate cytoskeletal rearrangements. For tracheal development, Dcdc42 could function in collaboration with Drac in the regulation of actin dynamics according to our experiments. Additional proteins linking either Dof or Csw to the small GTPases have to be identified.

Introduction FGF signalling

The Fibroblast Growth Factors (FGFs) constitute a large family of proteins that act in a wide range of developmental and pathological processes. The biological functions of FGFs include the regulation of cell proliferation, differentiation, survival, motility and tissue patterning.
In early and late embryogenesis, FGF signalling is essential for mesoderm induction, midbrain development and patterning of the neural plate, proper development and branching of the lung, the development of the skin and the growth and patterning of the limbs and skeletogenesis.
In the adult, FGFs are involved in tissue repair, wound healing and neurite outgrowth, as well as in the maintenance of the vasculature system and the migration in angiogenesis.

By their spatially and temporally restricted availability, FGFs act as ligands at the outside of the cells ensuring correct activation of the FGF receptors (FGFRs) that belong to the receptor tyrosine kinase (RTK) family. However, FGF signalling is reiteratively used and intracellular signalling components are highly conserved within the RTK signalling. The question arises, how intracellular specificity is achieved resulting in all the distinct cellular responses. Why is the same signalling pathway interpreted differently in different tissues? One model proposes intrinsic differences in the intracellular signalling pathway. A second model suggests that specificity arises from differences in the magnitude or duration of MAPK activation, one of the conserved components of RTK signalling (Halfar et al., 2001; Marshall, 1995). And finally, a third model postulates that RTKs generally act via the same signalling cassette, producing a generic signal, but cells interpret these signals according to their distinct developmental histories (Simon, 2000).

This introduction focuses on one specific cellular response to FGF signalling, namely the induction of cell motility during distinct processes of embryonic development.

Diverse migratory processes of vertebrate embryonic development are compared to the fruit fly *Drosophila melanogaster,* a model organism to study the basic strategies underlying development. Most developmental processes have been conserved among multicellular organisms.

Furthermore, the introduction gives an overview about the molecular players and regulators in the FGF signalling pathway acting upstream and downstream of the FGFR in Vertebrates and in *Drosophila*.

I. Characterisation of the FGFRs and downstream signalling cascades

In human and mice, 23 FGFs exist which are ca. 30-70% identical in their primary amino acid sequences and are proteolytically processed. So far 4 FGFs have been identified in zebrafish, 7 in Xenopus and 7 in chicken. In invertebrates only one FGF homologue Branchless (Bnl) has been identified in *Drosophila* and two in *Caenorhabditis elegans* (Egl17 and Let756) (Ornitz and Itoh, 2001).

Most FGFs have amino-terminal signal peptides and are secreted from the cells. Some FGFs are not secreted, but however, are found on the cell surface within the extracellular matrix (FGF1 or acidic FGF, FGF2 or basic FGF). Some remain intracellular because they lack the classical signal sequence that allows efficient export from the cell. FGF1 stimulates in vitro endothelial cell migration and proliferation. FGF2 exists as a cytoplasmic 18-kDa isoform and four high molecular weight (HMW) isoforms that are nuclear based. A new identified protein called translokin, which is associated with the microtubular network, specifically binds FGF2. Mutation of the nuclear targeting sequence of translokin or RNAi interference abrogates the intracellular translocation of FGF2 (Auguste et al., 2003). HMW isoforms but not the 18-kDa isoform of FGF2 have a N-terminal sequence responsible for the nuclear targeting/retention signal. Dominant negative strategies in cultured cells demonstrated that HMW FGF stimulate DNA synthesis independent of cell surface receptors to induce the mitogenic response, but the exact role of intracellular FGF still has to be elucidated (Auguste et al., 2003; Ornitz and Itoh, 2001; Powers et al., 2000).

In vertebrates, 4 structurally related FGFR exist and alternative splicing results in even more receptor variations.

The expression patterns of the FGFR are distinct but overlapping during embryonic development. In the mouse embryo FGFR1 is expressed in the mesenchyme and predominantly in the brain, FGFR2 in several epithelial

tissues, FGFR3 predominantly in the brain, spinal chord and cartilage rudiments of the developing bone and FGFR4 in tissues of endodermal and mesodermal origin (Klint and Claesson-Welsh, 1999).

1. The extracellular domain of FGFRs

All receptors consist of an extracellular domain with 3 Immunoglobulin-like domains (Ig-like domains), a stretch of acidic amino acids between Ig-like domain 1 and 2 that is unique for FGFRs and a heparin-binding domain. FGFR1-3 have different splice variants, these receptors either have two or three Ig-like domains. Two Ig domain variants may or may not have the acidic box and the receptors may even lack the transmembrane and intracellular parts. A splice variant of FGFR3 for example, in which the transmembrane domain is deleted, was reported to be localised into the nucleus (Klingelberg Diss). The third Ig-like domain can be spliced into IIIb or IIIc isoforms that have different ligand binding specificity. Ligand binding and specificity resides in IgII and IgIII and the linker that connects them (Plotnikov et al., 2000).

Heparan sulfate proteoglycans (HSPGs) such as syndecan and glypican are a class of molecules that act as coreceptors of FGFRs and stabilise ligand-receptor complex formation. Proteoglycans are abundant components of the extracellular matrix and are composed of core proteins with covalently attached modified glycosaminoglycan (GAG) polysaccharide polymer side chains. All heparan GAG chains undergo some N-deacetylation/N-sulfation and O-sulfation and they are referred as heparan sulfate (HS). Sulfation is responsible for the majority of the structural diversity of HS chains and different FGFs have different affinities for unique HS sequences. This suggests that tissue specific pattern of O-sulfation and the local concentration of HS can regulate the activity and specificity of FGFs (Nybakken and Perrimon, 2002; Ornitz, 2000).

Crystallography experiments have shown that a minimal complex of FGF2/FGFR1 in the absence of heparin/HS is formed (Plotnikov et al., 2000; Plotnikov et al., 1999). This minimal complex allows transient receptor dimerization and may signal at high ligand concentrations. Heparin/HS and a second FGF molecule are required to stabilise the active complex to gain a

sustained specific intracellular response. One model suggests that heparin interacts with both FGF and FGFR and promotes the formation of a stable 1:1:1 FGF:FGFR:heparin ternary complex. A second ternary complex is then recruited to the first complex via direct FGFR:FGFR contacts, secondary interactions between FGF in one ternary complex and FGFR in the other complex, and indirect heparin-mediated FGFR-FGFR contacts. There are no direct FGF-FGF interactions. One role of the acidic box laying between IgI and IgII could be that it competes for the binding of heparin to the basic heparin binding domain of FGFR thereby providing an autoinhibitory effect (Plotnikov et al., 1999).

In *lazy mesoderm (lzme)* mutant mice, the UDP-glucose dehydrogenase, an enzyme required for the synthesis of the glycosaminoglycan side chains of the proteoglycans is not produced. In this mutant, GAG production is blocked and FGF signalling is disrupted downstream of FGF ligand expression since expression of FGF signalling target genes is reduced as for example *mspry2* comparable to $FGF8^{-/-}$ embryos (Garcia-Garcia and Anderson, 2003).

2. The intracellular domain of FGFRs

FGFRs have a transmembrane domain followed by the intracellular part that consists of a long juxtamembrane domain, a split tyrosine kinase domain with an N-terminal lobe and a C-terminal lobe and a short C-Terminus.

Binding of two FGFs lead to receptor dimerization, conformational changes and autophosphorylation of the receptors on 7 conserved tyrosines.

The kinase domain contains two functionally important binding sites, the ATP binding site and the substrate binding site. In the unphosphorylated FGFR1 the ATP binding site is accessible while the substrate binding site is blocked in the so-called activation loop. When another FGFR molecule comes close, the activation loop changes its conformation, the two conserved tyrosine residues in the activation loop (Y653 and Y654) get trans-phosphorylated thereby upregulating the kinase activity and the phosphorylation of the other 5 phosphotyrosine residues (Klingenberg Dissertation). Two tyrosine residues Tyr

677 and 701 in the C-terminal lobe are not sites of autophosphorylation but they play a role in the stabilisation of the activation loop (Foehr et al., 2001).

The remaining 3 phosphotyrosines are potential binding sites for Src homology 2 (SH2) or phosphotyrosine binding domain (PTB) containing adapter proteins.

SH2 domains are 100 aa residue long conserved motifs that are found in different enzymes or in proteins that lack enzymatic activities denoted as adapters. SH2 containing proteins are affected by binding to the receptor, either by tyrosine phosphorylation, and conformational changes and by increased availability of their substrates giving rise to a cellular response. The same is true for PTB domain proteins that contain the 100-150aa conserved domain.

In the juxtamembrane domain of FGFR1 and FGFR2, there is one phosphorylated tyrosine motif (Y463) that serves as binding site of the adapter Crk (FGFR3 and FGFR4 lack the tyrosine in the juxtamembrane domain). Crk binds the guanine nucleotide exchange factor C3G that has been shown to activate the monomeric GTPase Rap1 to mediate sustained activation of the Mitogen-activated protein kinase (MAPK) ERK1 and 2.

Two phosphorylated tyrosines are in the kinase insert domain of FGFR1 and 2 (Y583, Y585), one in FGFR3 and none in FGFR4. The kinase insert tyrosine residues appear to be dispensable for FGFR1. Finally, the C-terminus (Y766) serves as binding site for PLCγ and the adapter Shb (Klint and Claesson-Welsh, 1999). Binding of PLCγ to the activated FGFR1 leads to its translocation to the plasmamembrane and subsequent phosphorylation upon FGFR1. At the plasmamembrane, PLCγ catalyses the hydrolysis of phosphoinositol lipids to inositol phosphates and diacylglycerol, which in turn stimulate the release of intracellular Ca^{2+} and activation of the protein kinase C (PKC).

Experiments have shown that PLCγ-activation is neither required for FGFR1 mediated mitogenesis, nor for chemotaxis nor for neurite outgrowth (Klint and Claesson-Welsh, 1999).

Shb is an ubiquitously expressed adapter protein containing SH2 and PTB domains. Interestingly, Shb binds to the same tyrosine motif at the C-terminus as PLCγ and according to experiments they don't compete for each other (Cross et al., 2002).

3. Different cellular responses elicited by FGFR1

The FGFR1 is the best-characterised FGFR and intensive studies about the interaction with downstream signalling components have been done.

3.1 The MAPK cascade

In contrast to other RTKs, all FGFRs lack a Grb2 binding site. The adapter Grb2 is constitutively bound to the guanine nucleotide exchange factor (GEF) Son of Sevenless (SOS). SOS catalyses the exchange of GDP for GTP on Ras, thereby activating Ras which is tethered to the membrane by farnesylation. GTP-bound Ras binds the N-terminal domain of the serine/threonine kinase Raf targeting Raf to the membrane which is sufficient for its activation. Raf then phosphorylates and activates the dual specificity kinase MEK that finally activates and phosphorylates the MAPK, ERK1 and ERK2. Oligomers of the activated and phosphorylated MAPK ERK1 and 2 enter the nucleus and phosphorylate transcription factors thereby regulating the expression of certain genes for a given cellular response.

3.2 The adapter FGFR substrate 2 (FRS2)

In vertebrate FGF signalling, Grb2 is recruited to the FGFR by FRS2 (FGF receptor substrate 2, also called suc1-associated neurotrophic factor target, SNT-1) (Kouhara et al., 1997). FRS2 is a membrane associated (myristoylated) multiadapter protein that binds constitutively to a stretch of the juxtamembrane domain of the FGFR1 (407-433aa) with its N-terminal PTB domain in a phosphotyrosine independent interaction. PTB domains usually interact with the canonical NPXY motif or asparagine residues, but the juxtamembrane domain lacks this motif or asparagine residues. That is unique and it was suggested that FRS2 transiently contacts the FGFR prior to receptor activation (Ong et al., 2000a; Xu et al., 1998).

Nuclear magnetic resonance (NMR) studies have shown that the PTB domain of FRS2 possesses unique features. The most important difference is that the PTB domain contains an additional β-strand (β8) that is essential for the

interaction with the conserved domain of the juxtamembrane domain of FGFR1 (Dhalluin et al., 2000). At the C-terminus, FRS2 contains multiple tyrosine phosphorylation domains, among those 4 Grb2 binding sites and two binding sites for the protein tyrosine phosphatase Shp2 that carries an additional Grb2 binding site. Thus, Grb2/SOS complexes are recruited either directly or indirectly via Shp2 upon tyrosine phosphorylation of FRS2 in response to FGF stimulation. The C-terminus of FRS2 is required for the recruitment of SOS either to contact SOS directly or through intermediate proteins (Hadari et al., 2001; Xu and Goldfarb, 2001; Xu et al., 1998).

Two isoforms of FRS2 exist, they are highly homologous, but they have a different expression pattern. FRS2α is expressed ubiquitously and can be detected at every developmental stage of the mouse, whereas the expression of FRS2β begins at day 9 and is primarily confined to tissues of neuronal origin. In contrast to FRS2α, FRS2β does not bind constitutively to the juxtamembrane domain of FGFRs but only to activated TrkA receptors to a canonical tyrosine phosphorylated NPXY motif (Lax et al., 2002).

3.3 The mitogenic response

FGFs are potent inducers of DNA synthesis in multiple cell types. For the mitogenic response, complex formation of the two adapters FRS2 at the juxtamembrane domain, Shb at the C-terminus and the phosphatase Shp2 is required for maximal and sustained activation of the MAPK. Shb probably directly regulates phosphorylation and association of Shp2 with FRS2. The mechanism whereby Shp2 regulates FRS2 phosphorylation state and MAPK activation remains unclear, although it has been demonstrated that the catalytic domain of Shp2 is required for sustained MAPK activation (Cross et al., 2002; Hadari et al., 1998). In addition to the plasma membrane Ras activation pathway, Ras is activated on intracellular membranes of the Golgi apparatus and the endoplasmic reticulum upon RTK stimulation. Endomembrane Ras may be particularly important for sustained Ras activation. It has been described that endomembrane Ras activation requires the Src family kinases (SFKs) and recent studies have shown that Shp2 activates the SFKs.

In addition, Shp2 probably dephosphorylates the negative RTK regulator Sprouty (Spry) (Zhang et al., 2004).

There is a first hint that sustained activation of the MAPK by Shp2 results in mitogenic activation of the cell. Also the adapter Crk seems to be involved in mitogenic signalling (Hadari et al., 2001). And the protein kinases C λ and ζ have been shown to interact with FRS2 following FGF stimulation activating mitogenic signalling via the MAPK cascade (Lim et al., 1999).

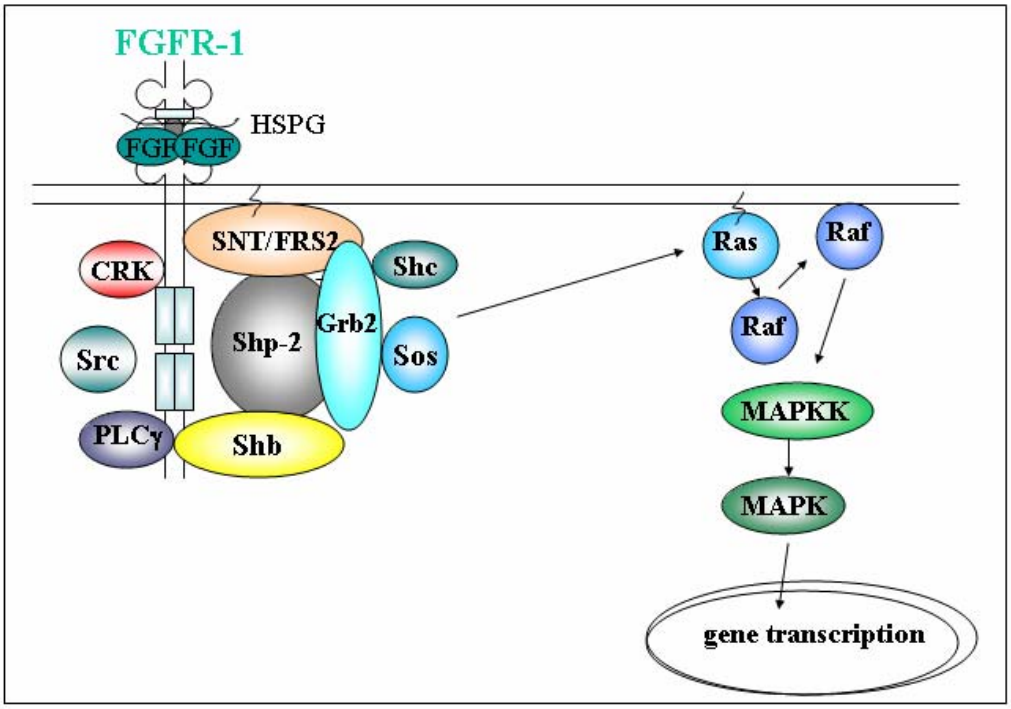


Figure 1: Schematic representation of the mitogenic FGF signalling response.

3.4 The cell survival response

Recruitment and phosphorylation of the docking protein Gab1 to FRS2 associated Grb2 activates the cell survival pathway via the PI3 kinase. Gab1 binds constitutively to the SH3 domain of Grb2. Gab1 like FRS2 belongs to the family of scaffolding adapter proteins that are targeted to specific membrane lipids with their N-terminal pleckstrin homology domain (PH). It contains multiple

potential tyrosine, serine or threonine phosphorylation sites. The *Drosophila* homologue is Daughter of Sevenless (Dos) that was identified as potential substrate of Corkscrew (Csw), the *Drosophila* Shp2 ortholog. Soc1, the *C. elegans* homologue was found in a screen for suppressors of hyperactive Egl-15, the FGF receptor ortholog.

Interestingly, mammalian Gabs and Dos contain two Shp2/Csw binding sites (Gu and Neel, 2003).

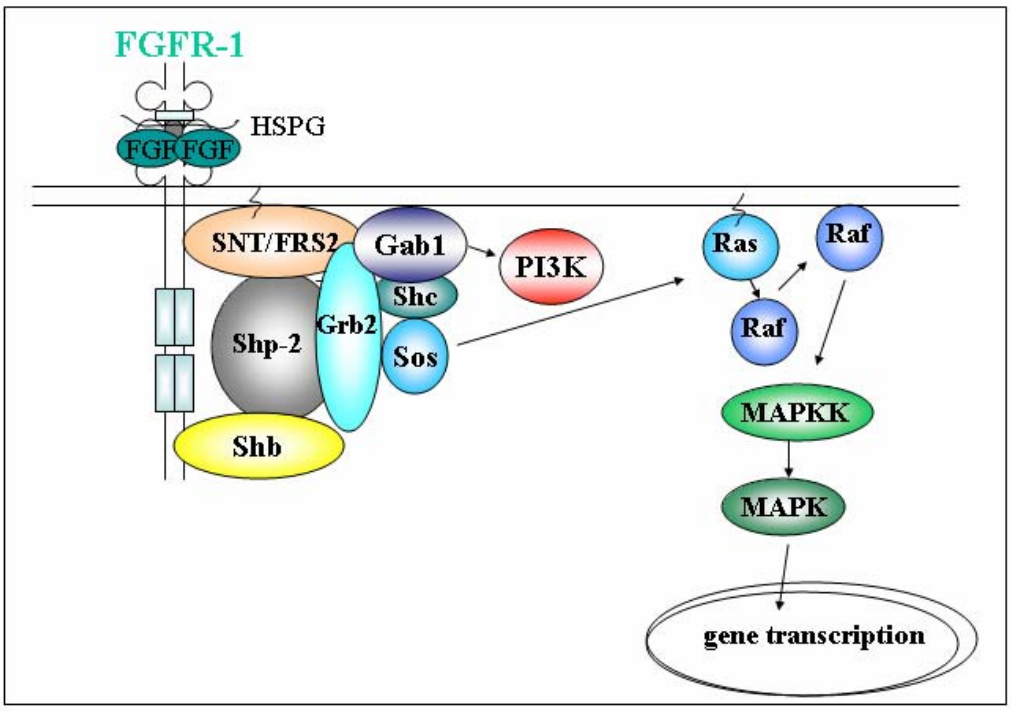


Figure 2: Schematic representation of the cell survival FGF signalling complex.

3.5 The migratory response

There is evidence that activation of Shp2 is required to elicit the migratory response (Hadari et al., 2001; Rosario and Bircmeier, 2003). When the Shp2 binding sites of FRS2 are mutated, Grb2 can partially restore the chemotactic response. Thus, there are some redundancies in the function of the molecules.

It was also shown that the adapter Shb forms additionally to Shp2 a complex with Src and the focal adhesion kinase (FAK), a serine-threonine kinase that interacts with integrins. Integrins play a role in the regulation of focal adhesion assembly and disassembly, a process important during cell movement. Upon activation and phosphorylation of FAK in response to integrin mediated cell adhesion or cellular stimulation by agonists, FAK associates with Src. Shp2 promotes Src activation indirectly. It was shown that Shp2 dephosphorylates PAG , a transmembrane glycoprotein, thereby inhibiting the recruitment of Csk to PAG. Csk is responsible for the C-terminal inhibitory phosphorylation of Src (Zhang et al., 2004).

Dependent on the activity of RhoA, Src transits from the perinuclear region to the cell periphery requiring actin stress fibres to incorporate into focal adhesions or into smaller focal complexes at lamellipodia and filopodia. There, Src phosphorylates a number of focal adhesion components including paxillin, tensin and p130 Crk associated substrate CAS and also the p190RhoGAP (Holmqvist et al., 2003; Timpson et al., 2001). The role of FAK and Src is thought to be in the turnover of focal adhesions (Webb et al., 2004).

It has also been shown that FAK overexpression stimulates cellular migration, whereas removal of FAK decreases migration, which is associated with an increase in the number of focal adhesions and stress fibres (Ilic et al., 1995). Interestingly, cells lacking Shp2 show hyperphosphorylation of FAK, increased numbers of focal adhesions at the cell periphery and a decreased migratory capacity similar to $FAK^{-/-}$ cells (Larsen et al., 2003).

In cell culture experiments, it was also shown that the urokinase type plasminogen activator (uPA), a proteolytic enzyme is upregulated upon FGF stimulation. uPA is involved in the extracellular matrix breakdown. Experiments in L6 myoblasts proposed that the Y463 and Y730 of FGFR1 could be essential for FGF2-mediated uPA induction (Boilly et al., 2000).

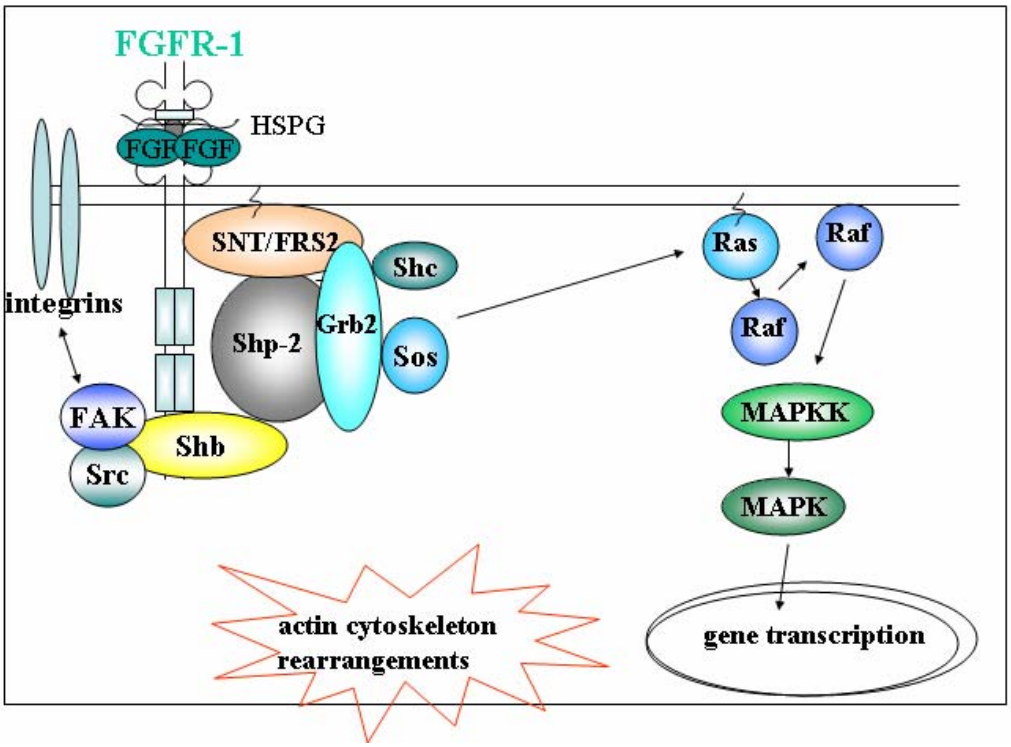


Figure 3: Schematic representation of the migratory FGF signalling response.

4. Negative regulation of FGF signaling

4.1 The ubiquitin ligase Cbl

FGF induced ternary complex formation of FRS2α, Grb2 and the RING type E3 ubiquitin ligase Cbl results in ubiquitination and degradation of FRS2α and FGFR. In vivo, Grb2/SOS and Grb2/Cbl complexes compete for binding to tyrosine-phosphorylated FRS2α.

In Cbl-deficient fibroblasts, the FGFR receptors are internalised in a normal manner, indicating that other mechanisms exist for downregulation of FGFR. (Wong et al., 2002).

4.2 MAP kinase-mediated negative feedback mechanism

MAPK dependent phosphorylation of FRS2α on threonine residues results in reduced tyrosine phosphorylation of FRS2α upon FGF signalling and subsequent reduced recruitment of the Grb2/SOS complex and the phosphatase Shp2. As consequence the MAPK response and other downstream signalling cascades attenuate. Experiments using the Boyden chamber assay have shown that the mobility is enhanced of FRS2$\alpha^{-/-}$ mouse embryonic fibroblasts (MEF) expressing a mutant form of FRS2α in which the threonine sites are mutated to valine. The mutant MEF cells expressing the threonine mutated FRS2α even grew faster than MEFs expressing the wild type FRS2α resulting in a transformed phenotype (Lax et al., 2002).

4.3 PLCγ as negative regulator of FGFR

Mice homozygous mutant for FGFR1 Y766F are viable but show a gain of function phenotype. Phosphorylation at Y766 (the binding site for PLCγ) has been implicated in the internalisation and degradation of the receptor. PKC a target of PLCγ has also been suggested to be involved in phosphorylation and downregulation of Src-family kinases, which are downstream targets of FGFR1 (Partanen et al., 1998).

4.4 Sprouty (Spry)

Another conserved inhibitor of RTK signalling is Sprouty (Spry). Spry was first identified in *Drosophila* as an inhibitor of FGF signalling during tracheal development (Hacohen et al., 1998) and subsequent studies have shown that it also inhibits signalling mediated by the EGFR during eye development and oogenesis in *Drosophila* (Casci et al., 1999; Kramer et al., 1999). Bnl signalling in the tip cells of the outgrowing tracheal branches regulates *spry* expression. In *spry* mutants, Aop/Yan, an ETS domain containing repressor of Bnl induced transcription, is degraded in an expanded domain. Subsequently, expanded domains of the transcription factor Pointed and the *Drosophila* Serum response factor homologue Blistered/DSRF can be observed, two transcriptional targets

that get expressed upon Bnl signalling. Genetic mosaic analysis has shown that Spry function is required in the tip cell and acts non autonomously to inhibit secondary and terminal branching by nearby stalk cells (for more details, see chapter about the development of the tracheal system in *Drosophila*) (Hacohen et al., 1998)
In contrast, during eye development, Spry acts cell autonomously as negative regulator of EGF signalling in R7 cells. Thus it is possible that Spry influences neighbouring cells in the tracheal system maybe indirectly (Casci et al., 1999; Kramer et al., 1999).

In mammals, four Sproutys (Spry1-4) and three Sprouty-related EVH1 domain proteins (Spred1-3) have been identified. In both *Drosophila* and mammals, these proteins all consist of conserved carboxy-terminal cysteine rich domains and highly divergent amino-terminal domains. The four mammalian Sprys are known to be small phosphoproteins that form oligomers through their carboxy-terminal domains. The carboxy-terminal domains are also required for the translocation and for anchoring Spry to the plasma membrane through palmitoylation.
Sprys have been shown to act as inhibitors of the ERK signalling cascade but can also function to positively regulate this pathway. The slightly different activities are probably due to their different interaction partners, including Cbl, Grb2, Raf1, FRS2, caveolin-1, and the *Drosophila* RasGAP, Gap1 and Drk, the Grb2 homologue.
Spry1 and 2 get phosphorylated on Tyr 55 on the N-terminus, which serves as docking site for Cbl. The competitive binding of Cbl prevents the RTK from ubiquination, internalisation and the following degradation. Thus Spry acts as positive regulator resulting in prolonged and sustained signalling activity. Finally, Cbl ubiquinates Spry and Spry gets degraded by the proteasome.
Upon FGF induced tyrosine phosphorylation, Spry translocates to the ruffling membrane region and binds to Grb2 at the same Tyr 55 as Cbl, thereby preventing recruitment of the Grb2/SOS complex to FRS2 or Shp2 and

inhibiting downstream events in the Ras/MAPK cascade (Hanafusa et al., 2002).

Another mechanism how Spry interferes with ERK activation is at the level of Raf. Spry2 and 4 physically associate with Raf1 through the highly conserved Raf binding motif (RBM) in the C-terminus thereby blocking phosphorylation of Ser338 on Raf1 that is essential for Raf activity.

In summary, Sprys have a positive and negative function residing in the N-terminal domain and a negative function in the C-terminal domain (Christofori, 2003).

4.5 Sef (similar expression of *fgf* genes)

The Sef protein is conserved across zebrafish, mouse and humans but not invertebrates. Sef encodes a putative transmembrane protein, with a signal and transmembrane domain. There is similarity to the intracellular region of mouse and human interleukin 17 (IL17) receptor. A putative tyrosine phosphorylation site juxtaposed to the transmembrane domain was shown to be important for FGFR1 and FGFR2 interaction. For the inhibitory effect of Sef, both the extracellular and the intracellular domain have to be functional. The intracellular domain of Sef interacts with the FGFR1 independent of FGFR1 tyrosine kinase activity.

In contrast to Sprouty, Sef seems to specifically repress FGF signalling (Furthauer et al., 2002; Kovalenko et al., 2003; Tsang et al., 2002). Overexpression of mouse Sef in NIH3T3 cells does not inhibit ERK1/2 activation by PDGF, EGF for example.

Coimmunoprecipitation experiments have shown that Sef binds to the FGFR1 and mediates a reduction in tyrosine phosphorylation of FGFR1 and its immediate downstream target FRS2, thereby modulating multiple FGF-mediated signalling pathways as for example regulating the mitogenic response (Kovalenko et al., 2003).

II. Chemotactic cell movement

Cells move by locally extending filopodia (long extensions containing parallel actin bundles) and lamellipodia (dendritic actin network) being protrusions of the leading edge. Lamellipodia formation is driven by localised actin polymerisation under the control of Rho family of small GTPases in conjunction with loosening of the myosin thick filament network in the cortex to allow expansion to occur (Dormann and Weijer, 2003).

To gain traction, the cell has to attach to the substrate and make new contacts at the leading edge. The attachment sites mature to become focal adhesions that allow the cell to exert force upon its surroundings by actomyosin-dependent contraction to pull the cell body forward. Transmembrane adhesion receptors of the integrin family provide a link between the actin cytoskeleton and extracellular matrix (ECM) components and at focal adhesions, stress fibres, which are long bundles of filamentous F-actin, are linked to the integrins.

Sliding of myosin along actin filaments to mediate contraction requires specific phosphorylation of the myosin light-chain (MLC), which is carried out by the myosin light chain kinase (MLCK). The myosin light chain phosphatase (MLCP) opposes the action of MLCK and dephosphorylates MLC. MLCP is phosphorylated and inactivated by Rho kinase (Dossenbach et al.) downstream of Rho signalling to promote membrane ruffling and cell migration.

Finally, the cell contacts at the rear of the cell are released from the extracellular matrix and the membrane receptors are recycled from the rear to the front. Recent evidence indicates that Rho is required for tail retraction. Rho regulates also the phosphorylation of moesin, a plasma-membrane-actin-filament crosslinker (Larsen et al., 2003; Rogers et al., 2003).

Maximal cell migration occurs when cytoskeletal forces are in balance with adhesion. Under conditions in which the adhesive strength is too low, cells are unable to generate enough traction to move, whereas under conditions of high adhesiveness, cells are unable to break cell-substratum attachments.

Chemotactic cell movement involves the detection of gradients of signalling molecules. Interpretation of the signal results in polarisation of the actin and myosin cytoskeleton of the cell along the gradient resulting in directed cell movement (Dormann and Weijer, 2003).

It was also shown that an intact microtubule cytoskeleton is required to maintain the polarised distribution of actin-dependent distributions at the leading edge of migrating fibroblasts. Microtubules are arranged with their minus ends near the cell centre or anchored at the centrosome. The plus ends radiate towards the leading edge, where they display dynamic instability. Microtubules can grow along actin bundles. Behind the lamellum, microtubule breakage and depolymerization occurs as a result of the compressive forces of the converging actin to which they are bound.

One hypothesis is, that microtubule growth could promote local activity of Rac in the cell front to drive lamellipodia protrusion, focal complex formation and further microtubule growth. Microtubule shortening could activate RhoA behind the lamellum to drive actomyosin contraction and promote the stabilisation of the microtubules, to protect them from breakage.

A second hypothesis is, that microtubule-actin interactions orientate towards the leading edge, which could direct the delivery of signalling molecules or membrane components required for lamellipodia protrusion.

It has also been shown that during dynamic instability, microtubules specifically target focal contacts, and that the targeting frequency is inversely proportional to focal contact lifetime. Further evidence indicates that a kinesin microtubule motor may deliver a regulatory factor that promotes focal adhesion disassembly.

The Rho family of small GTPases also regulates the microtubules, for example RhoA mediates microtubules stabilisation. PAK kinases downstream of Rac1 also promote microtubule growth, probably by regulating the microtubule destabilising protein Op18/stathmin (Rodriguez et al., 2003).

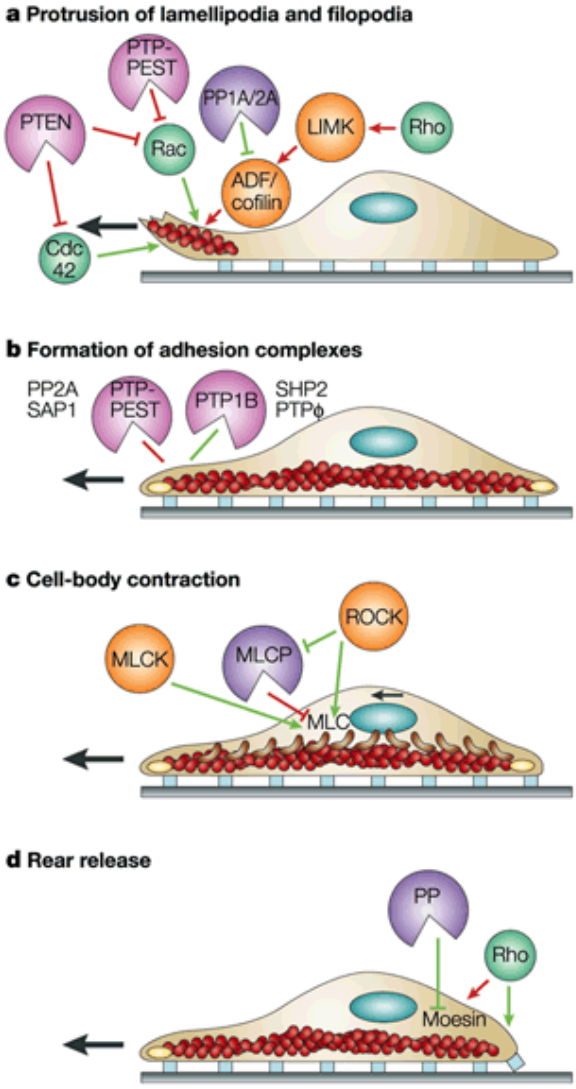


Figure 4: Influence of phosphatases on cell migration. (A) Migration is initiated by protrusion of the leading edge and formation of new actin filaments. Rac induces lamellipodia and Cdc42 stimulates filopodia. Their actions are opposed by phosphatase and tensin homologue (PTEN). Rac activation is also opposed indirectly by protein tyrosine phosphatase (PTP)-PEST (for proline, glutamate, serine and threonine-rich domain). The actin-severing protein, actin depolymerizing factor (ADF)/cofilin, can be dephosphorylated by protein phosphatases (PP)1A and PP2A to stimulate migration. LIM (for LIN11, ISL1 and MEC3) kinase (LIMK), which is activated by Rho, phosphorylates ADF/cofilin. (B) Attachment at the leading edge occurs first with focal complexes, which develop into focal adhesions. Formation/turnover of focal adhesions is regulated by many phosphatases, some of which include: PP2A, SAP1 (for stomach-cancer-associated PTP) and PTP-PEST, all of which are generally inhibitory to migration; and PTP1B, SHP2 (Src-homology-2 domain-containing protein tyrosine phosphatase and PTP$^{\phi}$, all of which are generally stimulatory to migration. (C) Cell-body contraction results from forces generated through actomyosin interactions. Myosin light-chain (MLC) phosphatase (MLCP) dephosphorylates MLC to inhibit migration and oppose the action of MLC kinase (MLCK). Rho kinase (Dossenbach et al.) phosphorylates MLCK and inhibits its activity. (D) Rho stimulates tail retraction. Rho also phosphorylates the actin-binding-protein moesin, which is dephosphorylated at the rear of the cell, before rear release. Moesin is dephosphorylated by a type 1/2A PP-family phosphatase. Coloured lines indicate stimulation (green), or inhibition (red), of fibroblast migration (Larsen et al., 2003).

1. Molecular requirements for actin-based lamellipodia and filopodia formation

New actin filaments are nucleated by the Arp2/3 complex and grow in a polarised fashion with the fast growing barbed ends oriented toward the leading edge. Arp2/3 is activated by the WASP (Wiskott-Aldrich Syndrom protein) and SCAR family of proteins, which are in turn activated through small G proteins. Cortactin also binds and activates Arp2/3 complex and at the same time stabilises branches.

Fed by monomeric actin-profilin from the subunit pool, new branches grow rapidly and push the membrane forward (for the exact process see Fig.1). Profilin allows elongation of barbed ends of filaments, blocks binding to the pointed end and inhibits spontaneous nucleation of actin filaments. Capping by the Capping protein or Gelsolin, another capping protein, limits the length of the growing branches, since short filaments are stiffer and more effective at pushing on the membrane.

The Ena/VASP proteins have been shown to interact with the barbed ends of actin filaments, shielding them from the activity of capping protein while supporting filament elongation which is important for the cell to create more filopodia like structures to sense guidance cues in the environment (Bear et al., 2002). Toward the rear of the lamellipodia actin filaments become debranched, severed and depolymerised by Cofilin-like proteins (ADF/Cofilin) and the released monomeric actin or globular G-actin is recycled into polymer at the leading edge (Petit et al., 2002; Rogers et al., 2003). Furthermore, Cofilin is required at the initiation of protrusion for barbed end mediated actin assembly at the leading edge and thereby increases levels of Arp2/3-mediated polymerisation (Larsen et al., 2003; Pollard and Borisy, 2003).

Arp2/3 has not been detected in filopodia in which actin is arranged in bundles rather than in a branched array as in lamellipodia.

The question remains how external stimuli are converted into signals that regulate nucleation-promoting factors. The strong autoinhibition of WASP is overcome by signalling molecules including Rho family GTPases (see next chapter), PIP2, Profilin, Grb2 and Nck. Requiring several signalling inputs of

different natures is attractive because it makes the mechanism of actin polymerisation sensitive to coincident signals (Carlier et al., 2003).

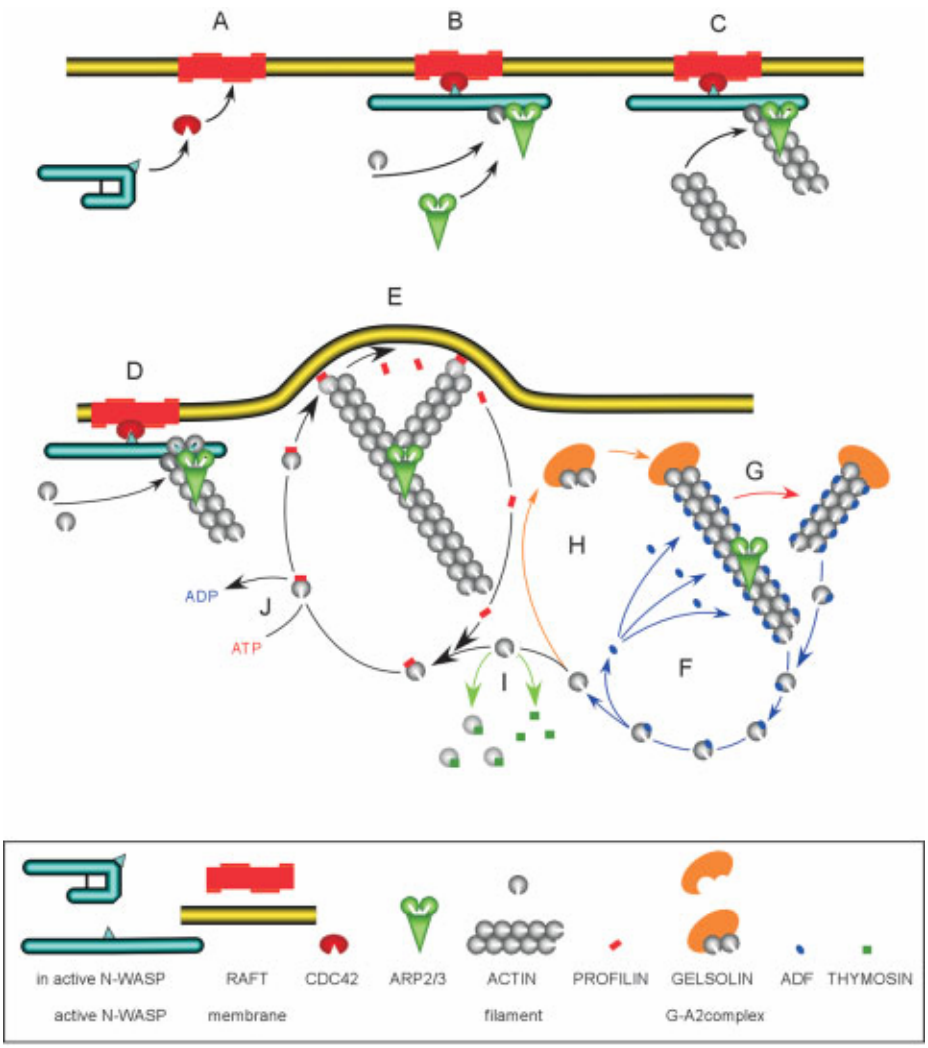


Figure 5: Schematic diagram representing the reactions involved in building a branched filament array at the leading edge. (A) Activation and targeting of N-WASP at the plasma membrane by signalling molecules. (B) Formation of the ternary branching complex G-actin–N-WASP–Arp2/3. (C,D) Association of the branching complex with a nucleus or filament barbed end. (E) Growth of the branched filament from G-actin and profilin-actin. (F,G,H) Regulation of actin dynamics in lamellipodium by Actin Depolymerising Factor and capping proteins (represented by gelsolin, which makes a 1:2 complex with G-actin). (I) Sequestration of actin by thymosinb4. (J) Recycling of G-actin by nucleotide exchange (Carlier et al., 2003).

2. Role of the small Rho GTPases in cytoskeletal reorganization

Members of the Rho (Ras homologous) family of GTPases are major components regulating changes in cell morphology and reorganization of the cytoskeleton in response to external stimuli (see also chapter II). Each Rho-family GTPase contains a membrane-attachment domain, a GTP/GDP binding domain and an effector loop. These molecules cycle between inactive GDP-bound and active GTP-bound forms. The cycling is regulated by guanine nucleotide exchange factors (GEFs), which exchange GDP for GTP, and GTPase-activating proteins (GAPs), which induce the hydrolysis of, bound GTP to GDP. Guanine nucleotide dissociation inhibitors (GDIs) prevent dissociation of GDP and the hydrolysis of bound GTP thereby preventing activation.
In fibroblasts, RhoA is implicated in the formation of actin stress fibres and focal adhesions, Rac1 stimulates membrane ruffling, lamellipodia, focal complex formation and is present at cell junctions, and Cdc42 participate in the formation of filopodia, cell rounding and the loss of actin stress fibres (Van Aelst and D'Souza-Schorey, 1997).

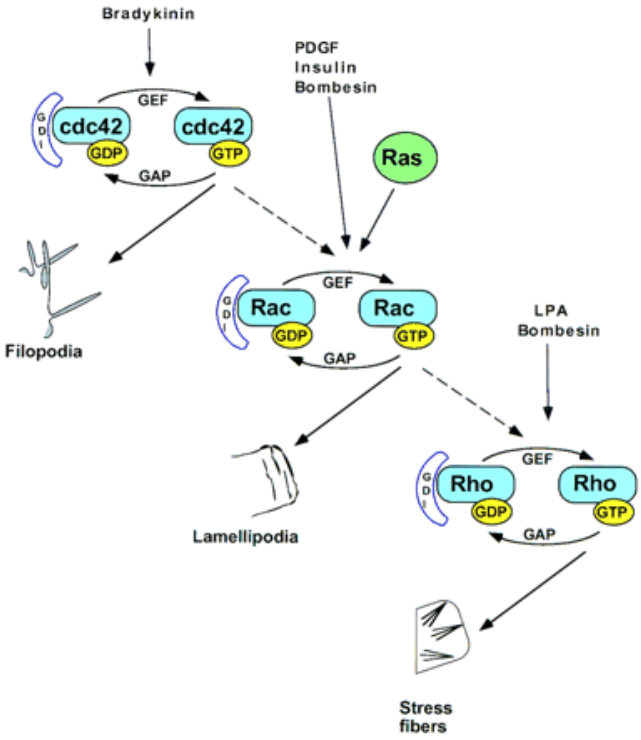


Figure 6: GTPase cascades involved in cytoskeleton organization in fibroblasts. Various extracellular stimuli trigger the activation of Cdc42, Rac, and Rho GTPases and elicit specific short-term responses such as the formation of filopodia, lamellipodia, and stress fibres, respectively. Moreover, Cdc42 appears to activate Rac, which in turn activates Rho. The direct links between these GTPases remain to be clarified. Different agonists can activate the Rho GTPases independently. The mechanism by which these agonists activate Rho GTPases may involve GEFs, GAPs, or GDIs (Van Aelst and D'Souza-Schorey, 1997).

The role of Rho family GTPases is not fully understood, numerous studies using mutants as well as dominant negative and constitutive activated forms of these GTPases support their importance in cell migration in vitro and in vivo (Petit et al., 2002).

One of the targets of Rac1 is the phosphatidylinositol-4-phosphate-5-kinase (PIP 5-kinase). Through the increase of PIP2 levels, PIP5-kinase regulates the function of many actin-associated proteins.

Rac1 mediates initiation of Cadherin-mediated cell-cell adhesion in a PI3-kinase-dependent manner. Rac1 is specifically expressed in initiating areas of contact where lamellipodia are formed, whereas E-cadherin gradually accumulates along the entire contact length. The regulatory subunit of PI3K associates with the E-cadherin/catenin complex and the Rac1 GEF Tiam1, which promotes E-cadherin based cell-cell adhesion, is regulated by PI3K activity (Ehrlich et al., 2002).

Another target of Rac1 is POR1 that specifically interacts with Rac1 in GTP-dependent manner. Deletion mutants of POR1 inhibit the induction of membrane ruffles by RacV12, a constitutive active form of Rac. POR1 interacts with the GTPase Arf6 that has been shown to elicit cytoskeletal rearrangements at the cell surface. But experiments have shown that Arf6 and Rac function on distinct signalling pathways to mediate cytoskeletal reorganisation (Van Aelst and D'Souza-Schorey, 1997).

WASP is regulated by Cdc42. WASP contains multiple domains that interact with different signalling molecules, phosphoinositides and components of the machinery required for actin polymerisation, such as actin monomers and the Arp2/3 complex. Upon activation by GTP-Cdc42, the intramolecular inhibition of

WASP is released and permits the binding of WASP to the Arp2/3 complex. This complex formation activates the actin-nucleation thereby locally increasing actin polymerisation.

Contraction and stabilisation of stress fibres is obtained when phosphorylated myosin light chain (MLC) is associated with actin. Phosphorylation of MLC induces a conformational change in myosin, thereby increasing its binding to actin filaments and subsequently the formation of stress fibres. MLC phosphorylation is regulated by the opposing effect of MLC-kinase (MLCK) and MLC-phosphatase. Some targets of Rho proteins, for example PAK, a serine/threonine kinase, exert their effects by regulating the phosphorylation state of MLC. PAK is required for the disassembly of focal adhesions and promotes lamellipodia formation and membrane ruffling. Moreover PAK activation leads to a decrease in MLC phosphorylation through phosphorylation of MLCK, thus destabilising actin stress fibres.

In contrast, the Rho associated serine/threonine kinases ROKα/β that are targets of the Rho GTPase, phosphorylate MLC and inactivate the MLC-phosphatase thus increasing actomyosin assembly.

III. Developmental role of FGF signalling in Vertebrates

1. Tumorogenesis

Several FGFs were discovered because of their oncogenic potential. For example in human pancreatic cancer and in breast cancer, in lung carcinomas or glioma, FGFs are over expressed.
Some tumours are mainly spread through the lymph, others mainly through the blood stream. A positive correlation between the vascular density of the primary tumour and the number of metastases formed has been reported for several cancers (Klingenberg Dissertation).
Several antagonists of FGF signalling that are of pharmaceutical interest were tested for example antisense molecules, neutralising antibodies, dominant-negative FGFRs or soluble FGF receptors.
Blockade of the FGF pathway in mice by FGF2 or FGFR1 antisense molecules or by FGF2 neutralising antibody inhibits tumour angiogenesis and growth.
In mice, tumour growth can also be inhibited by the adenoviral expression of soluble FGFR1 and VEGFR1. FGFR1 decreases the number of capillaries in tumours suggesting that growth is inhibited through an angiogenesis dependent mechanism, and the coexpression of soluble VEGFR1 has an additive effect in the inhibition of tumour growth (Auguste et al., 2003).

2. FGF-mediated movement of mesoderm cells during gastrulation

Gastrulation is the process that establishes the three-layered body plan, the ectoderm, mesoderm and endoderm of the embryo and is one of the main morphogenetic events that shape the early embryo.
In mammalian and avian embryos first the embryo consists of two layers, the epiblast and the hypoblast. The epiblast gives rise to embryonic and

extraembryonic structures whereas the hypoblast gives rise to extraembryonic structures only. Gastrulation starts with the formation of the primitive streak. The anterior part of the streak is known as Hensen's node. During gastrulation, cells move towards the primitive streak where they undergo an FGF-mediated "epithelial to mesenchymal transition" (EMT) that requires downregulation of E-cadherin, a process probably regulated by FGFR1. Chimeric analysis of $FGFR1^{-/-}$ mice has shown that FGFR1 signalling is required for EMT to occur in mouse embryos and that $FGFR1^{-/-}$ deficient cells have strong migration defects (Ciruna and Rossant, 2001). FGFR1 signalling is required for the expression of *mSnail* that downregulates E-cadherin. Mouse embryos homozygous mutant for mSnail die late during gastrulation with mesodermal cells retaining epithelial characteristics including the expression of E-cadherin. Downregulation of E-cadherin at the primitive streak not only regulates the EMT and migration of mesoderm progenitor cells, but also permits the rapid accumulation of cytosolic β-catenin levels in response to localised Wnt signalling and Wnt target genes are expressed, as for example Brachyury and T box genes that specify mesodermal cell fates (Ciruna and Rossant, 2001).

The protein tyrosine phosphatase Shp2 seems to be required to positively transmit the FGFR1 signal. Shp2 mutant cells in chimeric mice accumulate within the primitive streak because of the incapability of the Shp2-deficient cells to undergo EMT, which involves changes in cell shape, adhesion or migration. These processes are essential for the cells to exit the streak and to contribute to the mesoderm. The behaviour of Shp2 mutant cells looks very similar to the one of $FGFR1^{-/-}$ cells in chimeras suggesting a specific role of Shp2 downstream of FGFR1 to induce chemotaxis (Saxton and Pawson, 1999).

After passing the streak, the cells move out as individual cells into the space between the epiblast and the hypoblast to form the axial and lateral mesodermal, the definitive endoderm and extra-embryonic structures.

The phenotypes of knockout mice already suggested that FGFs are involved in cell movement during gastrulation, since FGF8 and FGFR1 mutants showed severe defects in cell migration at the primitive streak stages (Yang et al., 2002).

On the basis of the expression pattern of FGF8 and FGF4 (both are expressed in the streak and partially overlapping) it has been shown that mesodermal cells are attracted by sources of FGF4 and repelled by FGF8. It was proposed that cells move away for the primitive streak as a result of repulsion by FGF8 and that cells emerging from the anterior streak are attracted back in towards the midline to form somites and lateral plate mesoderm after regression of the node, by a FGF4 signal from the forming notochord. Cells leaving the posterior streak are attracted by a source of unknown chemoattractant originating from the boundary region between the area opaca and area pellucida to form extraembryonic structures. These cells never sense the attractive influence of the forming notochord and somites (Dormann and Weijer, 2003).

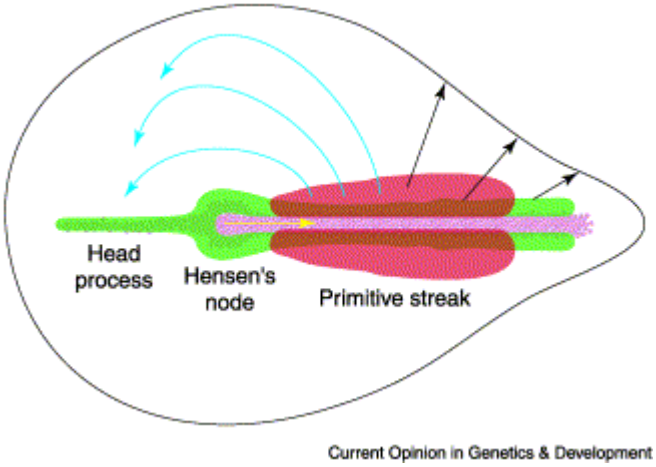


Figure 7: FGF expression directs cell movement in chick embryos. Movement of cells emerging from the primitive streak repelled by FGF8 (orange) produced in the streak. Mesoderm cells are attracted back in towards the midline to form somites and lateral plate mesoderm (blue arrows) by FGF4 (green) produced by the forming head-process. Cells emerging from the posterior streak (black arrows) are attracted by a signal emitted from the boundary of the embryo (Dormann and Weijer, 2003).

3. Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing vessels. Angiogenic factors, as the FGFs and the Vascular Endothelial Growth Factors (VEGFs), stimulate endothelial cells to secrete several proteases and

plasminogen activators resulting in the degradation of the vessel basement membrane, which allows the cells to invade the surrounding matrix. The cells migrate, proliferate and differentiate to form a new lumen-containing vessel. Finally, the endothelial cells deposit a new basement membrane and secrete growth factors such as the platelet-derived growth factor (PDGF), which attract supporting cells, the pericytes, ensuring the stability of the new vessels. This complex process involves other factors, as the angiopoietins and ephrins that act on specific receptors to regulate vessel stability (Cross and Claesson-Welsh, 2001).

FGF2 (also called basic FGF) was the first angiogenic factor identified and the FGFR1 is the main FGFR expressed in endothelial cells in vitro and has also been detected in activated endothelial cells in vivo. In capillary endothelial cell lines, stimulation of FGFR1 induces proliferation, migration, protease production and tubular morphogenesis, whereas activation of FGFR2 increased motility only.

Studies in $FGF2^{-/-}$ mice have shown, that endothelial cells from these mice have a defect in cell migration that can be compensated by the addition of exogenous FGF2 (Auguste et al., 2003).

FGFs probably regulate vascular morphogenesis indirectly by inducing secondary angiogenesis regulators such as VEGF.

Disruption of FGF or FGFR genes was not very informative so far with regard to vessel formation in vivo. $FGF1^{-/-}$ or $FGF2^{-/-}$ mice (single or double knockouts) do not present a defective vascular phenotype during development. This suggests a functional redundancy or a non-essential role of FGFs in vasculogenesis or angiogenesis. Mice with gene knockouts of FGFR1 or FGFR2 yield embryos arrested in their development before the onset of vascularization, because of the lack of mesoderm inducing signals.

But overexpression of FGF1 or FGF2 in mice hearts lead to an increase of vessel density. And transgenic mice that overexpress specifically a dominant-negative FGFR1, truncated at the C-terminal kinase domain, in the retinal pigmented epithelium in the developing eye, show a poorly branched vascular bed in the choroid and an avascular neonatal retina (Auguste et al., 2003).

4. Development of the mammalian lung

4.1 The morphology of the lung

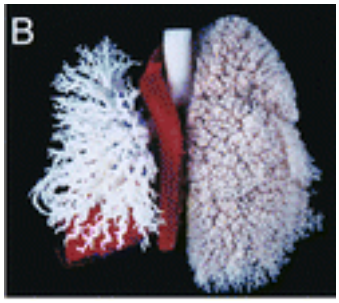


Figure 8: Human lung. (B) In white, preparation of the lung of an adult human using acryl polyester to fill in the airways. View from behind. The left lung has been filled less than the right half. Courtesy of H. Kurz, Anatomical Museum, University of Basel, Switzerland. In red, the descending aorta is visible (Affolter and Shilo, 2000).

The mammalian lung evolved as a system of branched conduits for air and blood coupled to a vast network of alveolar structures designed for gas exchange. In the developing respiratory system, airway branching is a prenatal event and formation of the alveoli spans pre- and postnatal life.

Pulmonary branching is reproducible in its spatial pattern. This suggests that the pattern of respiratory morphogenesis in the first 16 airway generations is genetically predetermined. Between the level of 16 and 23 generations, in which the alveoli are formed, branching appears more randomly.

Primordial lung buds originate as outpouchings of the primitive foregut endoderm and reiterated budding and branching of these tubules generate the airway tree. As the lung develops, vascular and airway components intermingle at the distal end of this tree to form the future alveolar-capillary barrier.

Endodermal cells of the ventral foregut form the epithelial lining of the tube. Lateral plate mesodermal cells migrate and condense around the endoderm to form the mesenchyme.

Left-right differences in pattern are first seen when secondary buds form (see Fig.1).

The genes *lefty-1, lefty-2* and *nodal* are expressed on the left side of mouse embryos and are implicated in the determination of left-right laterality (Warburton et al., 2000). These differences continue to develop as airways

undergo branching and are assembled into lobes, which result in more lobes in the right lung than in the left (mouse and human).

4.2 Cell fate determination

The homeodomain protein Nkx2.1 is essential for the complete induction of the lung branching morphogenesis. In the absence of Nkx2.1, dorso-ventral separation of the trachea from the esophagus, as well as lung branching morphogenensis and epithelial cell lineage determination at an early stage, prior to the specification to peripherial lung cell phenotypes is arrested. The hepatocyte nuclear family (HNF) of transcription factors cooperate with Nkx2.1 to determine pulmonary epithelial cell fate. HNF-3 activates transcription of Nkx2.1 in respiratory epithelial cells. Another important factor is GATA-6, a member of the GATA family of zinc finger transcription factors, induces differentiation of primitive foregut endoderm into respiratory epithelial cell lineages by interacting with HNF3β, Nkx2.1 and GATA family members (Warburton et al., 2000).

4.3 The branching process

A large amount of factors are involved in the branching process. The most relevant will be discussed. In the lung branching process, localised dynamic FGF10 expression in the distal mesenchyme induces chemoattraction and epithelial cell proliferation. Little is known, how specific clusters of mesenchymal cells are determined in a stereotyped fashion to secrete FGF10 and stimulate bud outgrowth.

Experiments have demonstrated directional chemotactic bud outgrowth in mesenchymal free epithelial cell cultures or whole lung cultures toward an implanted FGF10 soaked heparin bead. In FGF10 knockout mice, induction of primary bud outgrowth is disrupted and the mice have a blunt ended tracheal tube (Warburton et al., 2000). Other experiments have shown that in mesenchyme free lung epithelial cultures differential cell proliferation does not seem to be the initial event that triggers lung bud induction (Cardoso, 2001).

FGF10 binds and signals via the FGFR2 that is expressed throughout the respiratory tract epithelium from the earliest stages of lung development and during branching morphogenesis.

There is evidence that also retinoic acid (RA) signalling is required for lung bud initiation. RA results from sequential oxidation of vitamin A from retinol to retinalaldehyde and to the acid form.

Acute vitamin A deprivation in pregnant rats resulted in blunt end trachea and lung agenesis in some embryos, a phenotype similar to $FGF10^{-/-}$ mice. Probably, RA signalling restricts FGF10 expression and has to be inhibited to allow proper distal lung morphogenesis (Cardoso, 2001).

The outgrowing epithelial bud expresses high levels of Sonic hedgehog (Shh) that acts as negative regulator and restricts also FGF10 expression in the immediate vicinity of the outgrowing bud. Shh signalling is initiated upon binding to Ptc1 and results in the activation of Shh target genes by Gli (1,2,3) transcription factors. Glis are expressed in overlapping but distinct domains in the lung mesenchyme. The distinct phenotypes observed when Gli genes are inactivated individually or in combinations suggest that there are complex interactions between Gli members in regulating lung growth and pattern formation.

The Bone Morphogenetic Protein-4 (BMP-4) growth factor that belongs to the TGFβ superfamily is expressed throughout the epithelium but at increased levels at the tip cells of the bud. As the bud approaches the chemotactic source of FGF10, increased BMP-4 levels leading to an inhibition of cell proliferation slowing down bud outgrowth during the branching process. BMP-4 regulates also proximal-distal differentiation.

TGFβ-1 is expressed uniformly in the subepithelial mesenchyme and accumulates later at sites of cleft formation and along proximal airways. TGFβ-1 promotes the synthesis of extracellular matrix that when deposited in the epithelial-mesenchymal interface prevents local branching. TGFβ-1 has also been identified as negative regulator of epithelial cell proliferation and differentiation.

Finally during postnatal life, FGFR3 and FGFR4 play an essential role in regulating alveolization (Affolter et al., 2003; Cardoso, 2001; Warburton et al., 2000)

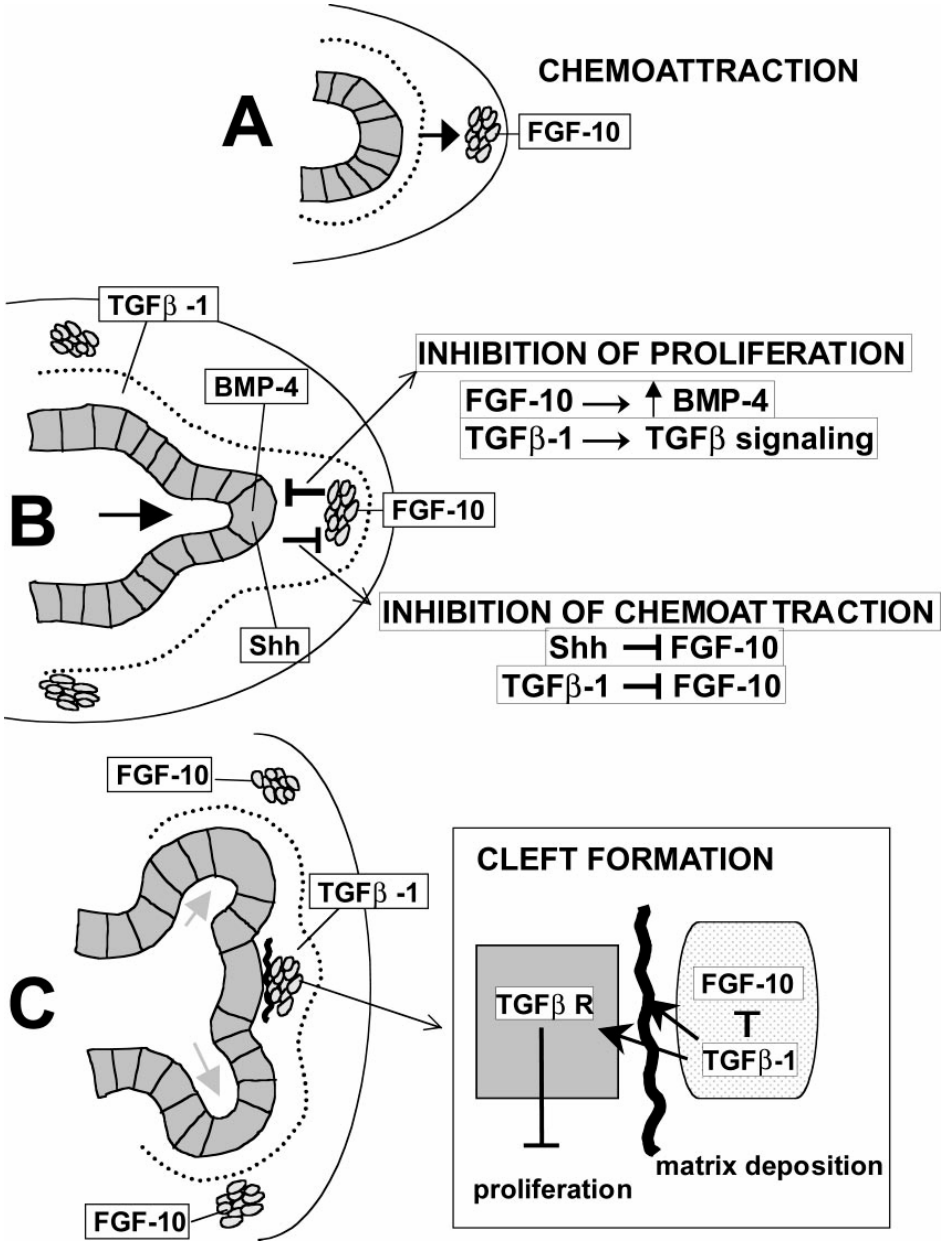


Figure 9: Control of bud formation during branching morphogenesis. Diagram incorporates models proposed by Bellusci and co-workers and Lebeche and co-workers. (A) Local expression of FGF-10 in the mesenchyme induces chemo-attraction and epithelial growth. (B) As the bud is induced, FGF-10 is inhibited by Shh expressed at the tips and by TGF_-1 expressed throughout the subepithelial region. Concomitantly, proliferation is inhibited at the tips by FGF-10-mediated up-regulation of BMP-4. (C) These mechanisms limit bud outgrowth and expansion, resulting in cleft formation. FGF-10-expressing cells appear at other sites to induce a new generation of buds. At the cleft, low levels of FGF-10 are maintained by subepithelial TGF_-1, which also induces synthesis of extracellular matrix components deposited in the epithelial-mesenchymal interface and prevents local budding (Cardoso, 2001).

IV. *Drosophila melanogaster* as model organism to study FGF-mediated chemotaxis and cell motility

Many studies of cell movement are carried out on cultured cells because the migration can be observed in real time and the culture medium can be altered to examine the factors that affect cell motility as the FGFs do.
During embryonic development, cell migration must be regulated temporally and spatially and this behaviour must be co-ordinated with cell fate specification and differentiation of the motile cell.
In vitro studies can only suggest, but not establish the factors that control or guide cell migration in vivo. Furthermore, cellular behaviour can depend upon the precise composition of the cellular environment. Knockout mice have the disadvantage that usually several gene copies of essential factors that for example control cell migration exist and the generation time, until the siblings are born, is long.
The fruit fly *Drosophila melanogaster* is a genetically tractable organism with only four chromosomes and many components of the mammalian signalling pathways are highly conserved in *Drosophila*. Saturating genetic screens for mutants were done and have resulted in the identification of genes required for cell migration. The strength of *Drosophila* is that combined cellular and genetic approaches are possible which have begun to elucidate the cellular mechanisms. Additional advantages of the fruit fly are the easy way to generate transgenic flies and the short generation time (10 days).

1. FGF signalling pathway in *Drosophila*

1.1 Sugarless (Sgl) and Sulfatless (Sfl)

In *Drosophila,* FGF signalling is impaired when HS synthesis is compromised as in vertebrate FGF signalling. Two enzymes encoded by the genes *sugarless* and *sulfatless* are critical for the biosynthesis and modification of HS GAGs.

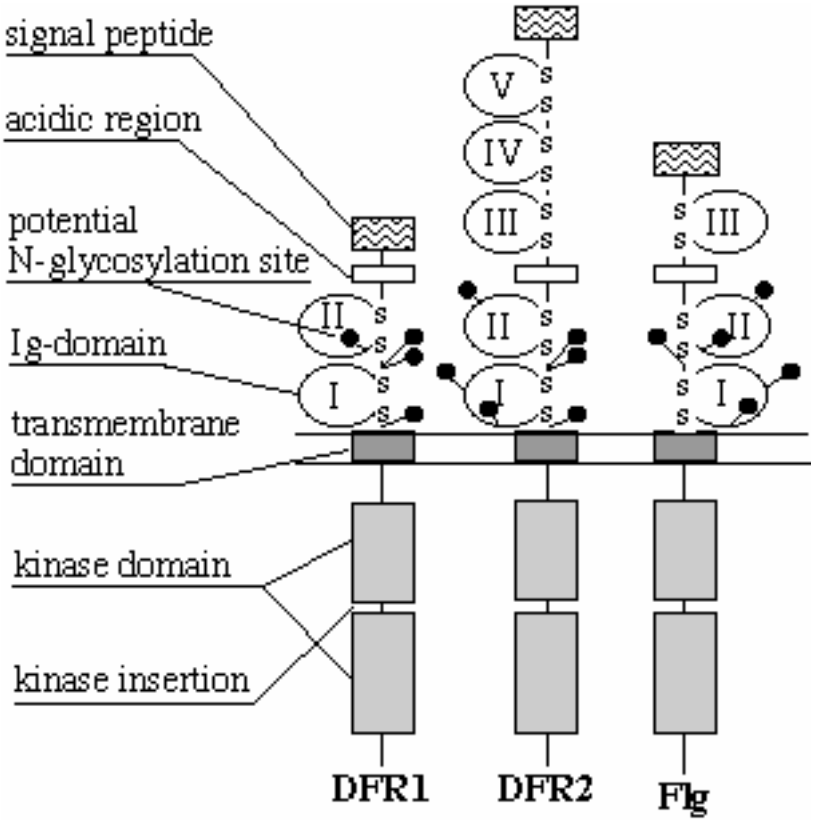
Sugarless encodes an enzyme which catalyses formation of UDP-D-glucoronic acid, an essential building block of heparan and *sulfatless* encodes an enzyme which catalyses the N-deacetylation/N-sulfation of heparan. Loss of function alleles of both *sfl* and *sgl* induce mesodermal migration defects, the heart does not form and tracheal development is disturbed and also Htl and Btl dependent MAPK activation is significantly reduced. Epistasis studies demonstrated that *sfl* and *sgl* function upstream of *btl* and *htl* and downstream of *bnl* expression. Overexpression of *bnl* partially rescues the *sfl* and *sgl* phenotypes. These experiments demonstrate that in vivo high levels of FGF can activate the FGFR in the absence of heparin (Lin et al., 1999).

1.2 Breathless (Btl) and Heartless (Htl)

There are two FGF receptor homologues in *Drosophila* encoded by the genes *breathless* and *heartless* (Klambt et al., 1992; Shishido et al., 1993). *Btl* is expressed in the anterior and posterior midgut and in midline glial cells. During stage 10-12, *btl* starts to be expressed on the surface of all tracheal cells. During stage 12, *btl* expression declines and gets restricted to the outgrowing tracheal branches, the DB, VB and anterior and posterior lateral trunks and is regulated by a positive feedback mechanism (Ohshiro et al., 2002). Btl is a receptor molecule of 1053 aa and Htl of 715 aa. The structure of Btl is homologous to the one of the vertebrate FGFR2. The N-terminus contains a highly hydrophobic signal peptide (2-20aa) 5 Ig-like domains of which two were found in the region flanked by the acidic domain and the transmembrane domain. These Ig-like domains are suggested to serve as binding sites for the only known FGF homologue in *Drosophila* Branchless (Bnl). In contrast to FGFR2, Btl is not modified by alternative splicing in the extracellular domain. Htl contains only 2 Ig domains.

The juxtamembrane domain of Btl and Htl is highly divergent from FGFR1, the region used by FGFR1 to recruit FRS2. A distant relative of FRS2 exists in *Drosophila*, but is not required for the transduction of the FGF signal (Battersby et al., 2003). A split tyrosine kinase domain follows the juxtamembrane domain. Except for the kinase insert domain, the overall sequence homology of the

kinase domain between Btl, Htl and the vertebrate receptors is about 60%. The homology between the kinase domain of Btl and Htl is 79%. The kinase insert domain is also positioned similar to the vertebrate receptors (Btl: 22aa, FGFR2: 14 aa) but the sequence conservation is low. As the vertebrate FGFRs, Btl and Htl lack a Grb2 binding site and the link to the conserved MAPK, encoded by the gene *rolled,* cascade is not known.



1.3 Downstream-of-FGFR (Dof)

Interestingly, the FGF signalling pathway must have existed in a common precursor of vertebrates and insects but as we have seen the adapter protein FRS2 is not conserved in its function in insects and a functional different adapter molecule called Downstream-of-FGFR (Dof), also known as Stumps or

Heartbroken, seem to fulfil a similar role to FRS2 (Imam et al., 1999; Michelson et al., 1998a; Vincent et al., 1998).

Dof is expressed in tracheal cells, mesodermal cells and a subset of glial cells that express either Htl or Btl. In *dof* mutant embryos the tracheal cells invaginate but fail to migrate and the induction of secondary and terminal branch markers is lost. This phenotype is similar to the one of *btl* and *bnl* mutants and *csw* mutants.

Dof gets phosphorylated upon receptor activation and acts downstream of Htl and Btl and upstream of Ras. It is also essential for MAPK activation (Vincent et al., 1998). The gene encodes two transcripts to produce proteins of more than 1000 aa that differ slightly at the N-terminus. The Dof protein from transcript II is a cytoplasmic protein and contains two ankyrin repeats and a coiled coil domain. Strikingly, the protein has no structural domains typical of molecules involved in signal transduction, as for example SH2 and PTB domains.

The most closely related vertebrate protein identified is the B lymphocyte signal transduction molecule (BCAP), which has been found in chicken, human and mouse and acts as an adapter in B-cell receptor signal transmission. Upon phosphorylation it recruits the PI3 kinase. BCAP and another signalling molecule in B cells, BANK share the overall structure of Dof (Battersby et al., 2003).

In a yeast two-hybrid screen it was shown, that Dof probably interacts with its N-terminal domain with the C-terminal lobes of the kinase domain of both FGF receptors, Btl and Htl.

The same N-terminal domain, that is highly conserved between Dof and BCAP, and defined as Dof/BCAP/BANK (DBB) motif, can interact with itself and with the C-terminal part of the protein leading probably to a folded form in vivo that can be specifically regulated thereby modifying the affinity of binding molecules. The DBB motif also promotes intermolecular interactions with another Dof molecule leading to dimerization of Dof (Battersby et al., 2003).

In the Dof protein, the environments of the tyrosines at positions 97, 914, 930 could act as binding sites for the SH2 domain of Drk. In addition, the context of the tyrosine 486 could serve as binding site for the SH2 domain of the

regulatory subunit of Phosphatidyl 3-kinase and the tyrosine at position 844 could represent a binding site for RasGAP. And finally, the tyrosine at position 515 is a potential binding site for Shp2 homologue encoded by the gene *corkscrew (csw)* in *Drosophila*. Recruitment of Csw is essential for MAPK activation and transcriptional activation. Drk can either bind directly to Dof or be recruited indirectly by Csw as it was shown for the Torso receptor (Cleghon et al., 1998). Experiments have shown that the interaction of Dof and Csw is probably also essential to induce cell migration (Petit et al., 2004).

To get a clue about migratory response and how cytoskeletal rearrangements are induced it is essential to understand the role of Dof and its interaction partners.

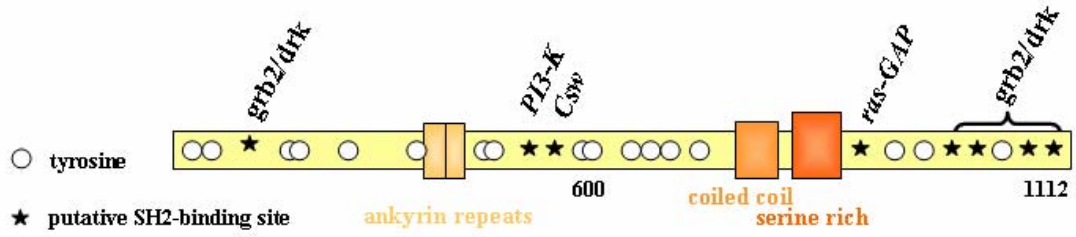


Figure 11: Schematic representation of Dof indicating the phospho-tyrosine motifs and putative SH2-interaction domains. Conserved domains are the ankyrin repeats, the coiled-coil domain and the serine rich domain.

1.4 Corkscrew (Csw)

Corkscrew (Csw) encodes a nonreceptor protein tyrosine phosphatase (PTPase) that is maternally contributed and homologous to the Shp2 protein in vertebrates (Perkins et al., 1992). Csw contains two N-terminal SH2 domains, a

C-terminal catalytic region, and a poorly conserved C-terminal region that contains a proline rich segment and a phospho-tyrosine motif that is capable of binding Drk. In addition, Csw contains a domain of about 150 aa that interrupts its PTP domain and is not present in Shp2. The crystal structure of Shp2 has revealed that the N-terminal SH2 domain binds to the catalytic domain, which keeps Shp2 inactive. The same could be true for Csw. Originally it was identified as a positive transducer of the Torso, EGF and Sevenless signals. Probably it activates Ras by bringing the Drk/SOS complex in close proximity to the receptor. But this model does not account for the still unknown function of the catalytic activity of Csw. Dominant negative phenotypes are observed when catalytic dead Csw proteins are expressed during embryogenesis (Johnson Hamlet and Perkins, 2001b; Perkins et al., 1996b).

In *csw* mutant embryos, tracheal cell migration is impaired similarly to the one of *btl, bnl* and *dof* mutant embryos. This suggests that Csw operates positively in Btl signalling.

In *csw* mutants also the dorsal mesodermal precursors cells are missing similar to *htl* and *dof* mutant embryos. In hemizygous csw mutant embryos, some pericardial precursor cells can be observed. Thus, Csw probably has a more prominent role in Htl dependent migration rather than Htl dependent pericardial cell specification (see next chapter).

Csw physically interacts with the DIM-7 protein, encoded by the gene *moleskin (msk)*, a nuclear import protein. DIM-7 likely transports activated MAPK into the nucleus (Lorenzen et al., 2001).

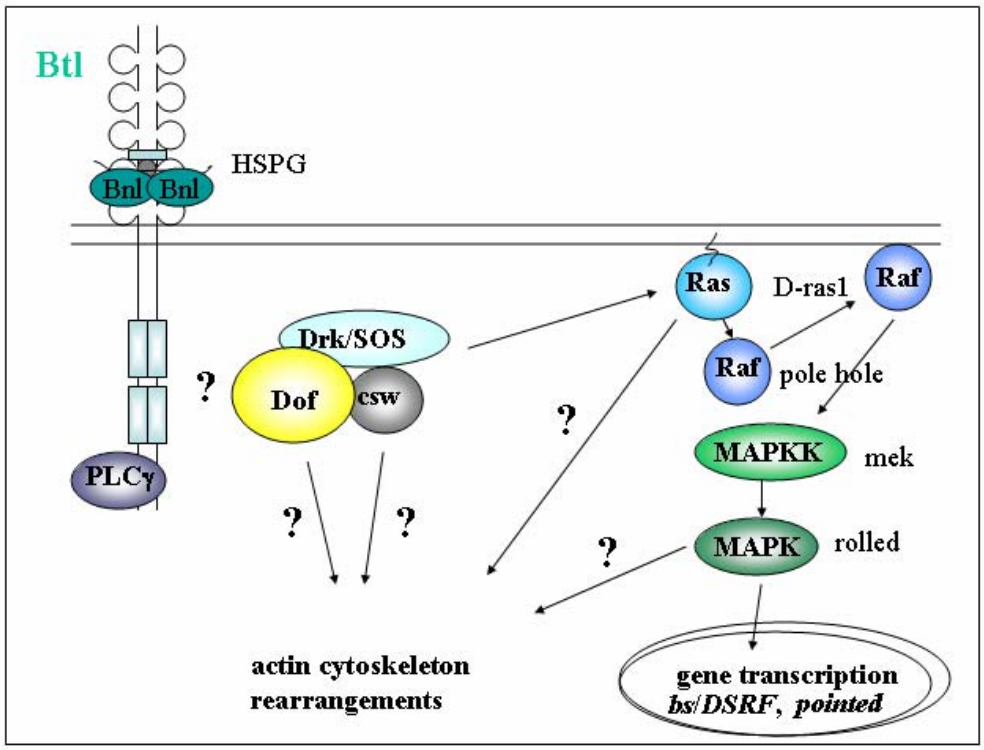


Figure 12: Schematic representation of the Bnl signalling pathway, the *Drosophila* FGF homologue.

2. Developmental role of FGF signalling in *Drosophila*

2.1 Htl and its role during gastrulation

Htl is first expressed in the whole mesoderm primordium and later in a subset of heart and somatic muscle precursors and in certain cells of the ventral nerve cord.

During the gastrulation process, the mesodermal and endodermal primordia are internalised by infoldings of the blastoderm epithelium and later disperse into individual cells that spread out to form the germ layers. The ventral blastoderm cells constrict their apical sides and shorten along their apical-basal axis forming a ventral furrow, which is going to be completely internalised. Once inside the embryo, the mesoderm premordium looses its epithelial structure and

disperses into single cells, which divide, attach to the ectoderm and migrate out on the ectoderm to form a single cell layer.

The maternal protein Dorsal has its highest concentration at the ventral side of the embryo and activates the expression of the transcription factor Twist and zinc finger protein Snail that define the mesoderm primordium. Huckebein defines the anterior and posterior borders of the mesoderm and ventral furrow. (Leptin, 1999).

Htl is already expressed during the blastoderm stage and later after the primary fate of the mesoderm is determined by Twist. Finally, in a second round of expression, Htl determines the mesodermal cell fates of the visceral mesoderm, heart and the somatic muscles. The dorsal most two rows of mesodermal cells split from the visceral mesoderm and give rise to the heart precursors. The dorsal row forms the dorsal vessel that are termed cardioblasts and the cells in the second row give rise to the pericardial cells that flank and support the dorsal vessel (Beiman et al., 1996). Pericardial cells express the pair rule gene *evenskipped*.

In *htl* mutant embryos, mesodermal cells do not spread and migrate properly to reach the positions where they receive the patterning cues from the ectoderm.

Such patterning cues are Dpp and Wg. Dpp induces the transcription of the homeodomain protein Tinman (Tin) that is in concert with Htl signalling responsible for inducing dorsal mesodermal fates including the cardiac and visceral derivatives. Wg is necessary for promoting the segregation of specific somatic muscle and cardiac founder cells.

Due to the failure of *htl* mutant cells to reach the dorsal most regions, the heart precursor cells are lost and the visceral mesoderm is reduced.

Unfortunately, the ligand of Htl has still not been identified yet. The differences in the extracellular domain of the receptor and the different expression pattern compared to Btl, suggests that Htl interacts with an Htl-specific ligand. The Htl-dependent, graded activation of MAPK in the migrating mesoderm suggests that Htl has an instructive role in guiding mesodermal cells (Cleghon et al., 1998; Gisselbrecht et al., 1996; Michelson et al., 1998a; Michelson et al., 1998b; Shishido et al., 1997)

2.2 FGF signalling recruits mesodermal cells into the male genital imaginal disc

The *Drosophila* sex determination hierarchy also controls the expression of *bnl* and *btl* in order to recruit larval mesodermal cells to become part of the male genital imaginal disc.

The sex-specific transcription factor encoded by *doublesex (dsx)* that is spliced either to the DsxMale form or DsxFemale form, specifies, together with the homeotic genes Abdominal-A and Abdominal-B, the sexually dimorphic pigmentation patterns of the abdomen through their regulation and modification of *bric-a-brac* to produce either the female or male pigmentation pattern.

There is only a single genital disc comprised of three separate primordia, derived from embryonic segments A8, A9 and A10. Each of these primordia responds in a unique manner to the sex determination hierarchy. In females, the A8-derived primordium grows to dominate the disc and gives rise to most of the female genitalia. The A9 primordium develops into the parovaria, part of the internal female genitalia and the A10 primordium develops into the female analia.

In males, the converse happens. The A9 primordium grows to dominate the disc and gives rise to all of the male genitalia. A8 primordium gives rise to a miniature male eight tergite and the A10 primordium develops into the male analia.

Bnl and *btl* are expressed in the mature male genital disc, but not in the female one. Bnl is expressed in genital disc epithelium and recruits actively *btl*-expressing cells with mesodermal identity into the male genital disc during late larval development. After being recruited into the disc, they define a novel third compartment that is clonally distinct from the anterior and posterior compartments. Subsequently, the *btl* expressing cells become epithelial and give rise to the paragonia and vas deferens.

The male specific deployment of FGF signalling is a consequence of *bnl* expression being repressed in female discs by DsxF. Feminised clones in the male genital disc repressed cell autonomously *bnl* expression and the *btl* expressing cells could not be recruited anymore. Feminised *btl* expressing cells

still reacted to the Bnl signal and migrated toward the disc (Ahmad and Baker, 2002).

2.3 The tracheal system of *Drosophila melanogaster*

Our lab is particularly interested in the formation of the tracheal system, a branched tubular epithelial organ that delivers oxygen throughout the body of the fly to the target tissues.

Oxygen enters the network of hollow epithelial tubes through the spiracles and diffuses passively along the major branches until reaching the fine terminal branches (tracheoles) that end blindly on the surface of all tissues.

The tracheal epithelium is a monolayer with stiff extracellular cuticle that lines its apical (lumenal) surface and prevents the tubes from collapsing.

The pattern of the tracheal branches is bilaterally symmetric and shows a segmental repeated structure. Except for the terminal branches, the pattern of the tracheal branches in each hemisegment is stereotyped and established by a fixed developmental program.

With its small number of cells, about 80 per hemisegment, and the availability of a large battery of cell markers, the embryonic tracheal system provides an excellent model system to define the mechanisms of guided branching morphogenesis and the role of FGF signalling in this process (for reviews see, (Affolter and Shilo, 2000; Manning and Krasnow, 1993; Metzger and Krasnow, 1999; Shilo et al., 1997).

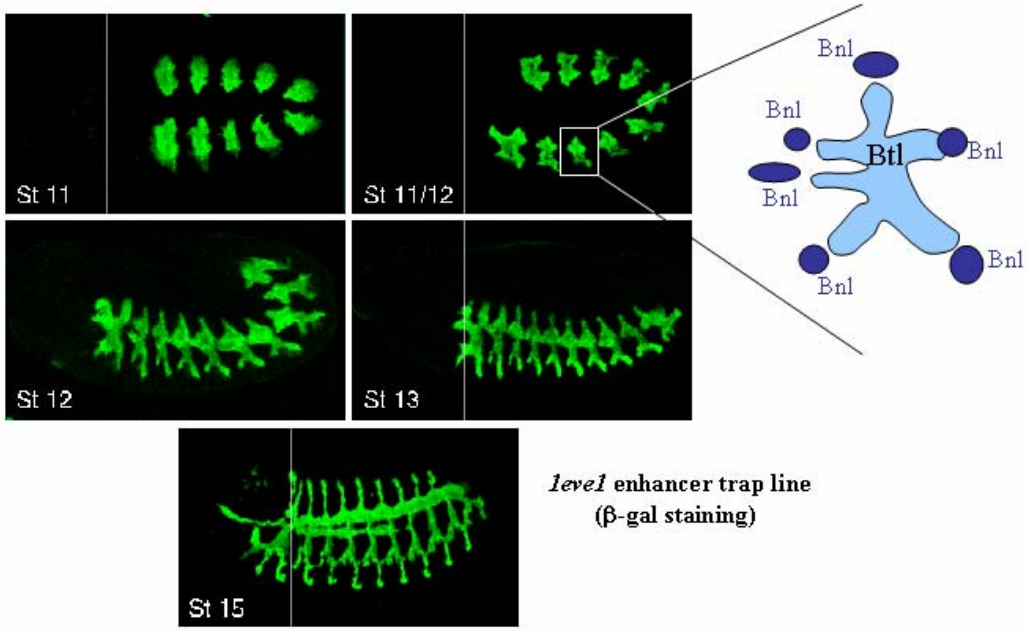


Figure 13: Development of the tracheal system, the respiratory organ of *Drosophila*. Primary branch outgrowth and fusion processes of the embryonic tracheal system are presented from stage 11 until stage 15. One placode from a stage 11 old embryo is outlined in a schematic representation. The FGF receptor tyrosine kinase Breathless (Btl; blue) is expressed in all tracheal cells. The activation of the receptor in the cells at the tip of the outgrowing branches, presumably due to their proximity to the localized source of the FGF ligand Branchless (Bnl), leads to the formation of filopodial cell extensions indicating the Bnl expressing epidermal cells surrounding the placode as six clusters (dark blue) guiding the outgrowing primary tracheal branches in stereotypic directions. The tracheal system of embryos from a 1-eve-1 enhancer trap line was visualized with an anti β-Galactosidase antibody (green) (Figure by Valérie Petit).

a) Tracheal cell fate determination

The tracheal system derives from 10 clusters of ectodermal cells on each side of the embryo, one in each hemisegment. Two regionally transcribed transcription factors, Trachealess (Trh) and Drifter/Ventral veinless (Drf/Vvl) are required to select groups of cells within the large epithelial sheet to define the tracheal placode. Trh is expressed throughout the tracheal development and in complex with its ubiquitously expressed partner Tango, it activates the

transcription of genes encoding proteins essential for the subsequent branching process.

b) Invagination and primary branch outgrowth

After cell fate determination, the tracheal cells invaginate into the underlying mesoderm forming an elongated sac that is still connected to the surface by a thin stalk generating an initial lumen. The invagination process is regulated by Epidermal growth factor (EGF) signalling probably in collaboration with Wingless (Wg) and Hedgehog (Hh) signalling (Glazer and Shilo, 2001; Llimargas and Casanova, 1999). The cells do not undergo an epithelial-mesenchymal transition, retain their epithelial character and undergo few rounds of cell division until they reach the number of about 80 cells within each placode. Without any further cell division, each sac initiate the outgrowth of six primary branches: Dorsal branch (DB), dorsal trunk anterior and posterior (DT), lateral branch anterior and posterior (LT), visceral branch (VB) and ganglionic branch (GB).

The Decapentaplegic (Dpp) signalling pathway specifies the fate of the tracheal branches that will bud from the dorsal and ventral part of the tracheal placode. The activation of expression of the zinc finger protein Knirps (Kni) and Knirps-related (Knrl) by Dpp is essential to determine the correct number of cells in dorsal and ventral branches (Chen et al., 1998). It also allows the tracheal cells to respond to the chemoattractant Branchless (Bnl), the only known FGF homologue in *Drosophila*, which guides the branches in stereotypic directions (Sutherland et al., 1996).

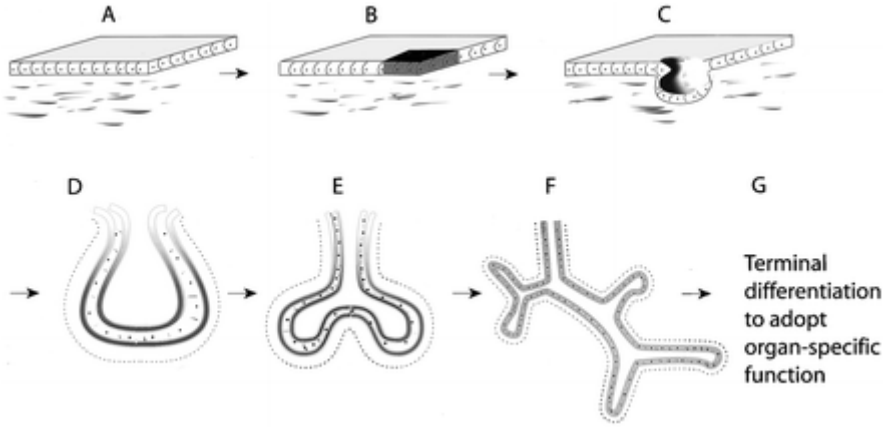


Figure 14: Branching Morphogenesis at the Cellular Level

Schematic representation of a typical branching process. In many cases, a subgroup of cells (schematically illustrated in black in [B]) of a pre-existing epithelium (A) is assigned to undergo branching morphogenesis by the expression of a specific subset of transcription factors and/or signalling mediators. As a consequence of this determination step, these cells invaginate or form a primary bud (C). Branch formation is then initiated in the invaginated (or budded) structure (from [D] to [E]) and the branching process can be reiterated numerous times (F). In addition, lateral branches can be induced. After the branching process, complex processes lead to the development of specialized terminal structures, a process that is different in different branched organs. Because the development of the vascular system does not in general follow the scheme outlined in this figure, we have excluded in this review a description of how the branched aspects of the arterial and venous network arises (Affolter and Shilo, 2000).

c) FGF signalling acts as guidance cue and induces directed cell migration

Bnl is expressed in six dynamic clusters of mesenchymal and epidermal cells around the tracheal placode. Defined numbers of tracheal cells migrate toward these clusters in a stereotyped manner and form the six primary branches. *Bnl* expression is downregulated when the tracheal cells reach the clusters.

The tracheal cells react to Bnl with the formation of dynamic actin containing filopodial extensions. Experiments have shown that FGF signalling induces cytoskeletal dynamics in the absence of transcriptional induction of any known gene. It is suggested that Dpp signalling is required for stabilising the outgrowing branches (Ribeiro et al., 2002).

In *btl* and *bnl* mutants, the specification of tracheal cells is normal and the placode invaginates but the tracheal cells fail to migrate and primary branch outgrowth is blocked. Spatial regulation of Btl activity by the ligand Bnl is essential for the outgrowth of primary branches in the right directions (Lee et al., 1996). In contrast, ectopic Bnl can redirect tracheal cell migration to new sites of expression, demonstrating its role as chemoattractant (Sutherland et al., 1996).

Each of these branches has a defined identity that specifies tube size and the subsequent determination of specialised cell fates at precise positions and in the appropriate number.

Bnl expression near the end of the primary branches not only guides primary branch outgrowth but also activates transcription of genes that activates the program of secondary and terminal branching, such as the ETS domain containing transcription factor Pointed that is required to form secondary branches (Samakovlis et al., 1996) and the *Drosophila* Serum Response Factor homologue Blistered/DSRF required for terminal branch formation (Guillemin et al., 1996).

d) Secondary and terminal branch formation

Secondary branches sprout from the primary branches and are unicellular tubes that subsequently ramify into dozens of terminal branches, which are long cytoplasmic extensions also forming a lumen and transporting oxygen directly to the target tissues. Fusion cells at the extremity of dorsal and lateral branches and the dorsal trunk allow the interconnection of adjacent tracheal metamers, leading to the formation of a continuous luminal network (Samakovlis et al., 1996). The Wg pathway is required for the formation of the dorsal trunk by activating the expression of the transcription factor Spalt (Sal) (Franch-Marro and Casanova, 2002) that specifies DT cell fate and the fusion markers Escargot, Fusion-2 and Fusion-3. Additionally, Wg signalling acts in concert with Bnl to regulate Notch signalling by stimulating Delta expression limiting the number of fusion cells. (Chihara and Hayashi, 2000; Llimargas, 2000). A *hunchback (hb)* expressing bridge cell of mesodermal origin has shown to be essential to complete migration and the fusion process of the dorsal trunk (Wolf and Schuh, 2000).
The dramatic growth of the tracheal network does not involve cell proliferation or apoptosis. The branches form by migration, rearrangement and by cell elongation. These conditions allow a precise investigation of the induction of the cell migratory response in absence of cell division in a developing organ (Samakovlis et al., 1996).

2.4 FGF as chemoattractant of the air sacs

Bnl also plays a key role in the development of the tracheal system of the adult. Metamorphosis transforms the *Drosophila* larvae into an adult fly by consuming larval tissues and creating new organs using imaginal cells. A new tracheal system has to be built that will satisfy the aerobic requirements of the specialised tissues of the adult. But in addition, the organs of the pupa must be kept oxygenated while the adult develops. Transformation of the tracheal system begins during the third larval instar when imaginal tracheoblasts start to divide (reviewed in(Manning and Krasnow, 1993).

The proliferating tracheoblasts spread over the larval tracheal system using it as a scaffold to form an extensive branching network before the larval cells histolyze. Some tracheoblasts elaborate coiled structures those are unique to the pupa. Others grow to form the air sacs of the adult, which are large reservoirs that are juxtaposed to major muscle systems, and to the brain.

The majority of the adult thorax, the epidermis, the wing and flight muscle are produced by the wing imaginal disc (reviewed in (Cohen et al., 1993). This organ arises as a tubular invagination of the epidermis and when it grows and flattens during larval development, it develops four distinct cell types: Squamous peripodial cells on the surface, columnar epithelial cells on the other surface, a distinct group of adepithelial cells that nestle against the most proximal columnar epithelial cells and finally stalk cells that connect the disc to the epidermis. A large tracheal branch attaches to the columnar epithelial surface. It does not ramify to generate contacts with the disc cells. There are some adepithelial cells with tracheal identity (tracheoblasts) that maintain continuity with the cells of the main tracheal branch. These cells are precursors of the adult tracheal air sacs. They extend long cytoneme-like filopodia and migrate and proliferate in response to Bnl that is expressed in small groups of the columnar epithelium. At third larval instar, *btl* expressing adepithelial cells bud from the tracheal branch, increase in cell number and expand posterior toward the region of greatest *bnl* expression. These *btl*-expressing cells do not express *htl* at the same time and in contrast to tracheal cells do not form a lumen. How the air sacs are finally connected to the tracheal lumen is unknown.

Experiments inducing mitotic clones have shown that Bnl is sufficient to induce tracheoblast cell migration by inducing oriented filopodia formation in the tracheoblasts. Thus, spatially regulated Btl activity is required to induce cell migration and proliferation in tracheoblasts.

This process that induces air sac tracheoblasts in the wing disc may be common to other discs as well, since *bnl* and *btl* are also expressed in the leg and eye disc after pupa formation (Sato and Kornberg, 2002).

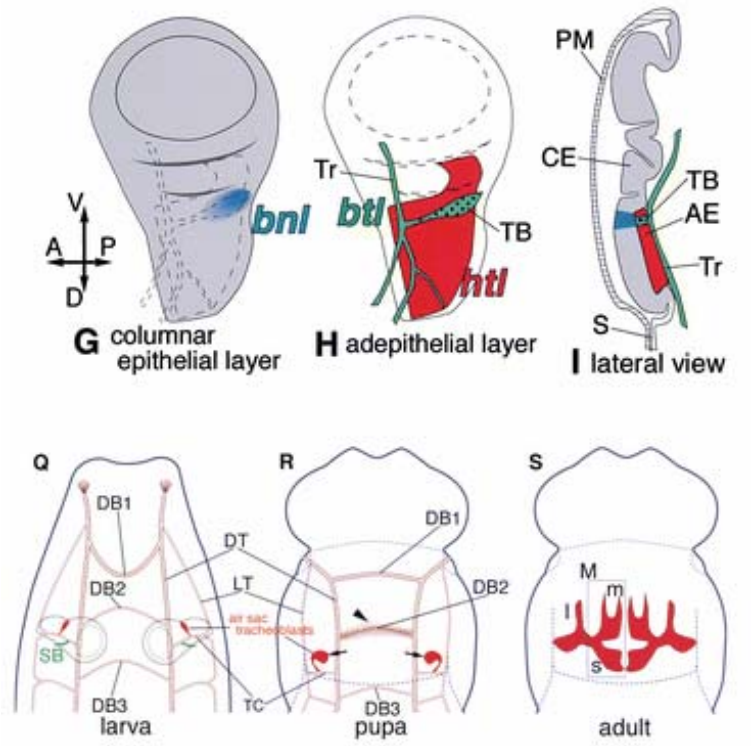


Figure 15: Schematic representation of larval air sac development. (G–I) Schematic drawings showing FGF and FGF receptor expression in late third instar wing discs: TB, tracheoblasts (green spotted area); CE, columnar epithelium; AE, adepithelial cells; PM, peripodial cells; Tr, trachea; S, stalk. Scale bars are 100 µm (A–D) and 50 µm (E and F). (Q–S) Schematic drawings of the formation of thoracic air sacs. Larva (Q), pupa (R), and adult (S) are shown. Tracheoblasts and air sacs, red; spiracular branches (SB), green; both air sac tracheoblasts and spiracular branches connect to transverse connectives (TC), which extend from dorsal trunks (DT) to lateral trunks (LT). Dorsal branches (DB) connect dorsal trunks. Bundles of tracheoles along dorsal branch 2 in (R) are pupal tracheae, which extend to the developing flight muscle prior to air sac formation (Sato and Kornberg, 2002).

Aim of the thesis

FGF signalling is involved in numerous developmental processes ranging from cell determination to mitogenesis, and cell survival to cell migration. Interestingly, the same signalling pathway is used reiteratively throughout development and the question regarding the intracellular specificity is raised.
Little is known about the intracellular signalling events of the FGF signalling pathway leading to specific cellular responses. Since the FGF signal is essential throughout embryonic and adult development and plays a role in many pathogenical processes, it is important to identify the factors, which determine the differential responses.

We were interested to investigate the specificity of FGF signalling in a developmental context, in which the signal induces directed cell migration, a cellular phenomenon that relies on changes of the cytoskeletal architecture.
During gastrulation in early embryonic development, but also during the formation of organs in mammals and in *Drosophila*, FGFs have been shown to act as chemoattractants and guide cells toward their targets (Cardoso, 2001; Dormann and Weijer, 2003; Sutherland et al., 1996; Warburton et al., 2000; Yang et al., 2002).
We were wondering whether the FGF signal not only guides the cells in specific directions, but also whether it directly regulates cytoskeletal rearrangements thereby inducing cell movement.

In *Drosophila*, only the FGF homologue Branchless, the FGF receptor homologue Breathless and the adapter protein Downstream-of-FGFR (Dof) have been identified so far as essential components for tracheal and mesodermal cell migration.

The aim of the thesis was the functional characterization of Breathless to identify domains that specifically interact with adapters eliciting chemotaxis by providing a link to the cytoskeleteton.

To address the question of FGF signalling specificity in the induction of directed cell movement, we asked whether FGF signalling provides specificity due to its specific intrinsic interpretation or whether the intracellular part of the receptor can be replaced by another RTK.

Additionally, we wanted to analyze the role of Dof. We asked whether Dof is a FGF-specific signalling mediator, or whether it is required by other RTKs to transmit the migratory cellular response.
Since we found that Dof acts exclusively in the FGF signalling pathway, we were wondering, whether Dof directly interacts with the FGF receptors and if so, with which domain of the receptors Dof interacts with. We performed a functional deletion analysis of Breathless in vivo and in vitro to address this question.

Another aspect we wanted to investigate was, whether Dof is a functional homologue of the vertebrate scaffolding adapter protein FRS2, which has been shown to recruit the Grb2/SOS complex and the phosphatase Shp2 to the activated FGFRs.

Further, we wanted to investigate other known downstream FGF signalling components for their capacity to induce a migratory response.
Experiments in vertebrates have shown that the Rho family of small GTPases regulate focal adhesion assembly, disassembly and induce filopodia and lamellipodia. It is still not known how RTKs and other downstream targets regulate the small GTPases, neither in vertebrates nor in *Drosophila*. The GTPase homologues in *Drosophila* were tested for their function in migration. Further we tested whether additional components of the conserved MAPK cascade provide a cytoplasmic link to the cytoskeleton.

And finally, we were interested in the question of whether the migratory response is only activated in the tip cells of the outgrowing tracheal branches, the following cells being pulled by cell-cell adhesions, or whether all tracheal cells have the capacity to migrate.

Experiments:

Functional dissection of the FGF signalling pathway

I. The GAL4-UAS system for directed gene misexpression

The GAL4-UAS system is a very effective systematic misexpression system. The yeast transcriptional activator Gal4 can be used to regulate gene expression in *Drosophila* by inserting the upstream activating sequence (UAS), to which Gal4 binds, next to a gene of interest (gene X). The Gal4 gene has been inserted at random positions in the *Drosophila* genome to generate enhancer trap lines that express Gal4 under the control of nearby genomic enhancers. There is now a large collection of lines that express Gal4 in a variety of cell-type and tissue specific pattern. The expression of gene X can be driven in any of these patterns by crossing the appropriate Gal4 enhancer-trap line to flies that carry the UAS-gene X transgene (Brand and Perrimon, 1993; St Johnston, 2002).

For most of our studies, we made use of an engineered *btl*-Gal4 driver line generated by the group of Shigeo Hayashi (Shiga et al., 1996).

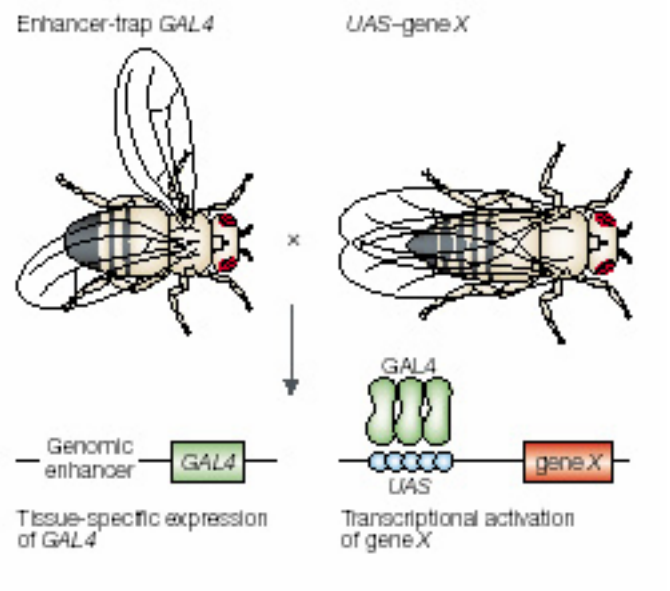


Figure 1: Directed gene expression in *Drosophila*. Transgenic lines were generated in which the GAL4 gene was inserted randomly into the genome driving GAL4 expression from specific genomic enhancers. A GAL4-dependent target gene could then be constructed by subcloning the sequence of interest behind the GAL4 binding sites. To activate the target gene, flies carrying the target (UAS gene X) were crossed to flies expressing GAL4 (enhancer trap GAL4). In the progeny of this cross, the effect of the directed misexpression could be observed (Brand and Perrimon, 1993; St Johnston, 2002).

II. Localization of Breathless in the outgrowing tracheal branches

First, we were interested in Btl localization in migrating tracheal cells. Directional cell movement requires a defined cell polarity in which cytoskeletal components are differentially localized at two poles of the cell. This polarization can be an intrinsic property of the cell or can arise in response to directional signals. The question arises whether in migrating tracheal tip cells Btl accumulates at the leading edge, or whether it is distributed uniformly throughout the plasmamembrane and only receptors in close proximity to the spatially controlled ligand get activated. Experiments have shown that Bnl signalling induces long filopodia in direction of the guidance cue in the tip cells of the outgrowing primary branches (Ribeiro et al., 2002).

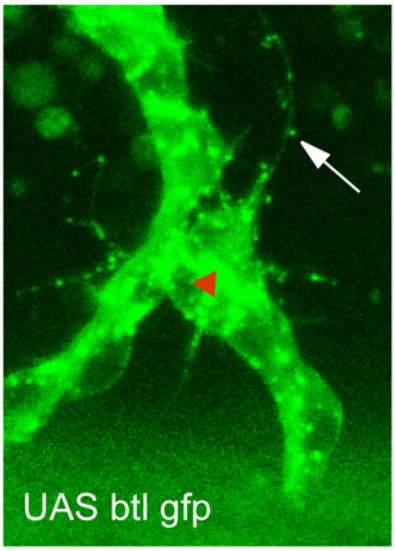


Figure 2: Localization of Btl-GFP. View on tip cell of an outgrowing dorsal branch (red arrowhead). Expression of the fusion protein Btl-GFP was regulated by *btl*-GAL4. Btl-GFP accumulated in bright spots within the cytoplasma and along the filopodial extensions (white arrow).

A *btl-GFP* fusion construct was expressed specifically in the tracheal system by the *btl*-Gal4 driver. Btl was linked to the fluorescent tag called green fluorescent protein (GFP) allowing the visualization of the location and trafficking of the protein of interest in vivo.

We observed Btl-GFP concentrated in bright spots in the cytpoplasma of all tracheal cells and along the filopodial extensions (Fig.2, white arrow). The tip cell of an outgrowing dorsal branch is shown with long filopodia (Fig.2, red arrowhead) in direction of the ligand source. This phenomenon was also

observed in another context (filopodia of tracheoblasts in airbags) by Sato and Kornberg (Sato and Kornberg, 2002). Interestingly, the filopodia may have the capability to sense Bnl and activate the signal by presenting the receptor as close as possible to the ligand source. But a more likely explanation is that, due to overexpression of the *btl-gfp* transgene by the Gal4-UAS system, these bright spots are vesicle structures, internalizing and downregulating the receptors.

Acknowledgements:
Thomas B. Kornberg kindly provided the UAS btl-gfp line.

III. Publication: Specificity of FGF signalling in cell migration in *Drosophila*

Caroline Dossenbach, Salome Röck and Markus Affolter
Development 128, 4563-4572 (2001)

A biological response is determined by the combination of pathways transducing a signal from a cell surface RTK receptor to a spectrum of signalling molecules present in a given cell. Specificity can be achieved by a dedicated factor acting downstream of a receptor and by recruiting and activating novel combinations of additional signalling molecules.
Thus, the activation of tissue specific effectors, as for example Dof, can determine the unique effect of cell the migratory response upon activation of RTKs.
In the following paper, we surprisingly found that tracheal and mesodermal cells respond to a RTK signal with directed cell migration independent of the presence or absence of Dof. Thus, Dof is a specific adapter protein of FGF signalling triggering downstream cellular responses.

IV. Publication: Downstream-of-FGFR (Dof) is a FGF-specific scaffolding protein and recruits Corkscrew upon receptor activation

Valérie Petit, Ute Nussbaumer, Caroline Dossenbach and Markus Affolter
Molecular and Cellular Biology, May 2004, in press

In this paper, a functional characterisation of Dof was performed, by combining reverse genetic and biochemical approaches. It was demonstrated that Dof is a specific substrate for the two *Drosophila* FGFRs. The N-terminal region of Dof was defined to interact constitutively with the kinase domain of Btl.
Tyrosine 515 becomes phosphorylated upon receptor activation and recruits Csw, an essential step in FGF-induced cell migration and the activation of the Ras/MAPK pathway. However, the activation of Ras is not sufficient to activate the migration machinery in tracheal and mesodermal cells.

V. Functional deletion analysis of Breathless (Btl) in vivo and in vitro

Expression of a *btl* cDNA under the indirect control of the tracheal specific enhancer *btl*-Gal4 was sufficient to fully rescue the migration defects of the tracheal system in *btl* mutant embryos (Fig. 5C,C'). The rescue capacity was tested under different temperature conditions (18°C, 25°C and 29°C) but we could not observe any differences of the rescue potential of the *btl* transgene at the different temperatures. Therefore we decided to induce the rescue by the Gal4-UAS system at 25°C for all the following experiments (data not shown).
For all the experiments, we used the $btl^{H82\Delta3}$ null allele, which has been generated by imprecise excision of the lacZ-containing P element inserted upstream of the *btl* locus. Homozygous $btl^{H82\Delta3}$ mutant embryos did not show any *btl* transcription but retained βGal expression in the tracheal and midline cells (Reichman-Fried et al., 1994).
We used this rescue assay for a functional deletion analysis of Btl, since its intracellular domains have not been characterized yet.

1. Constructs

Modified UAS *btl* transgenes were expressed by the *btl*-Gal4 driver in *btl* mutant embryos in order to identify domains of Btl essential for binding proteins triggering the tracheal migratory response. For each construct (Fig.3), four transgenic lines were established and tested for their rescue capability.

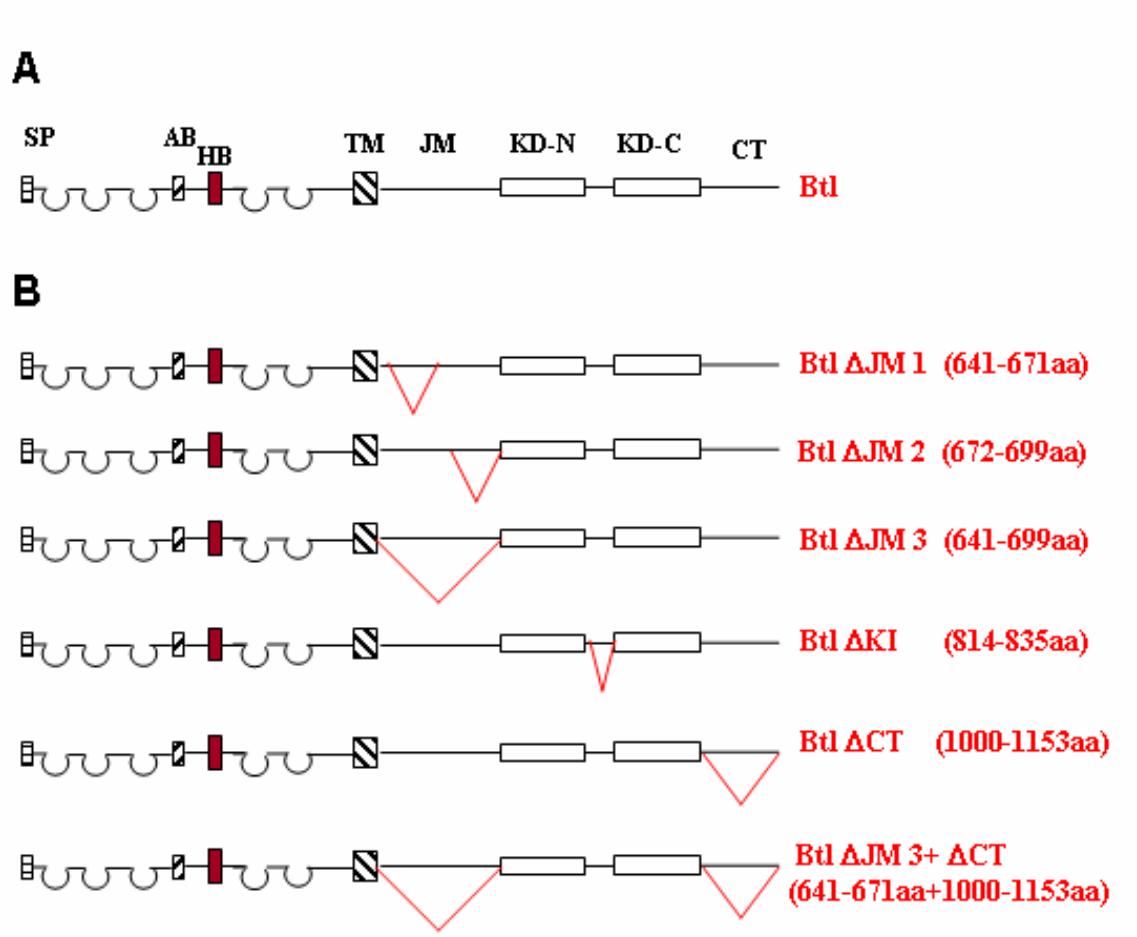


Figure 3: Schematic representation of Btl derivates tested in vivo. (A) Representation of the Btl wild-type construct. SP: signal peptide; AB: acidic box; HB: heparin binding domain; TM: transmembrane domain; JM: juxtamembrane domain; KD-N: kinase domain, N-terminal lobe; KD-C: kinase domain, C-terminal lobe; CT: C-terminus. (B) Schematic representation of the Btl deletion constructs used in the rescue assays. Deletions within the juxtamembrane domain (Btl ΔJM1 and Btl ΔJM2), deletion of the whole juxtamembrane domain (Btl ΔJM3); deletion of the kinase insert domain (Btl ΔKI); deletion of the C-terminus (Btl ΔCT); deletion of the juxtamembrane and the C-terminus (Btl ΔJM3+ΔCT). Each construct was placed downstream of UAS sequences.

First, the sequence of the juxtamembrane domain of Btl was blasted and compared to the sequence of the vertebrate Btl homologue FGFR2 to identify conserved structures of the juxtamembrane domain. This analysis was done to investigate whether Dof could be a functional homologue of the vertebrate scaffold adapter FRS2 interacting with the same domain of the receptor. Like

FRS2, Dof provides binding motifs of the Drk/SOS complex and the Shp2 homologue Csw.

The sequence homology of the two compared juxtamembrane domains is low (Fig.4). The sequence within the juxtamembrane domain of FGFR1 (but also FGFR2 and 3) required for the specific interaction of the receptor with FRS2, was identified as KRIPLRRQVTVS (421-430aa) which includes the alternatively spliced VT motif (Burgar et al., 2002). Unfortunately, this conserved VT motif (Fig.4, indicated in red) that is essential for the binding of the PTB domain of FRS2 is lacking in the Btl juxtamembrane domain.

```
Btl:    626  XXXXXXXXX--XXXIETVHQWTKKVIIYRPXXXXXXXXXXXDLQMPVIRIEKQRXXXXXXXX
                            VH+ TK++ + R            +    P++RI  +
FGFR2:  399  CRMKNTTKKPDFSSQPAVHKLTKRIPLRRQVTVSAESSSSMNSNTPLVRITTRLSSTADTPM

Btl:    686  XXXDPAQGFNEYEFPLDSNWE
                    G +EYE P D  WE
FGFR2:  362  LA-----GVSEYELPEDPKWE
```

Nevertheless, we generated three deletions within the juxtamembrane domain. The first region of 641-671aa that was deleted, corresponds to the stretch of amino acids of the juxtamembrane domain of FGFR1 FRS2 constitutively interacts with (Fig.3B, Btl ΔJM 1). The second deletion (672-699aa) contains the conserved phosphotyrosine binding motif of Crk in vertebrate FGFRs and Btl (Fig3B, Btl ΔJM 2) (Fig.4, indicated in blue). The third deletion eliminates the whole juxtamembrane domain (641-699aa) (Fig.3B, Btl ΔJM 3).

The two phosphotyrosines within the kinase insert domain of FGFR1 (Y583 and Y585) and FGFR2 have been shown to be essential for the mitogenic response in vertebrates (Wang et al., 1996). FGFR3 contains only one phosphotyrosine and is weakly mitogenic. Btl also only contains one putative phosphotyrosine within the kinase insert domain and could provide another functional binding

site. Thus, we deleted the kinase insert domain (814-835aa) of Btl (Fig.3B, Btl ΔKI).

Finally, the remaining short C-terminus of Btl has been deleted (1000-1153aa) (Fig.3B, Btl ΔCT). The C-terminus contains a phosphotyrosine motif that has been shown to interact with PLCγ. However, it has been shown that autophosphorylation on the Tyr 766 of FGFR1 is required for efficient endocytosis of the receptor, but not for mitogenesis, differentiation or chemotaxis (Mohammadi et al., 1996; Partanen et al., 1998). But interestingly, it has been demonstrated that a stretch of 15 aa c-terminal of the second kinase domain is critical for FGFR1 mediated cell migration in a modified Boyden chamber assay. The role of this stretch in regulation of migration has still to be determined (Landgren et al., 1998).

As control, a double deleted truncated receptor lacking the juxtamembrane domain and the C-terminus was generated to exclude functional redundancies between the two putative interaction domains (Fig.3B, Btl ΔJM 3+ΔCT).

2. Rescue of tracheal cell migration in *btl* mutant embryos by the truncated Btl receptors

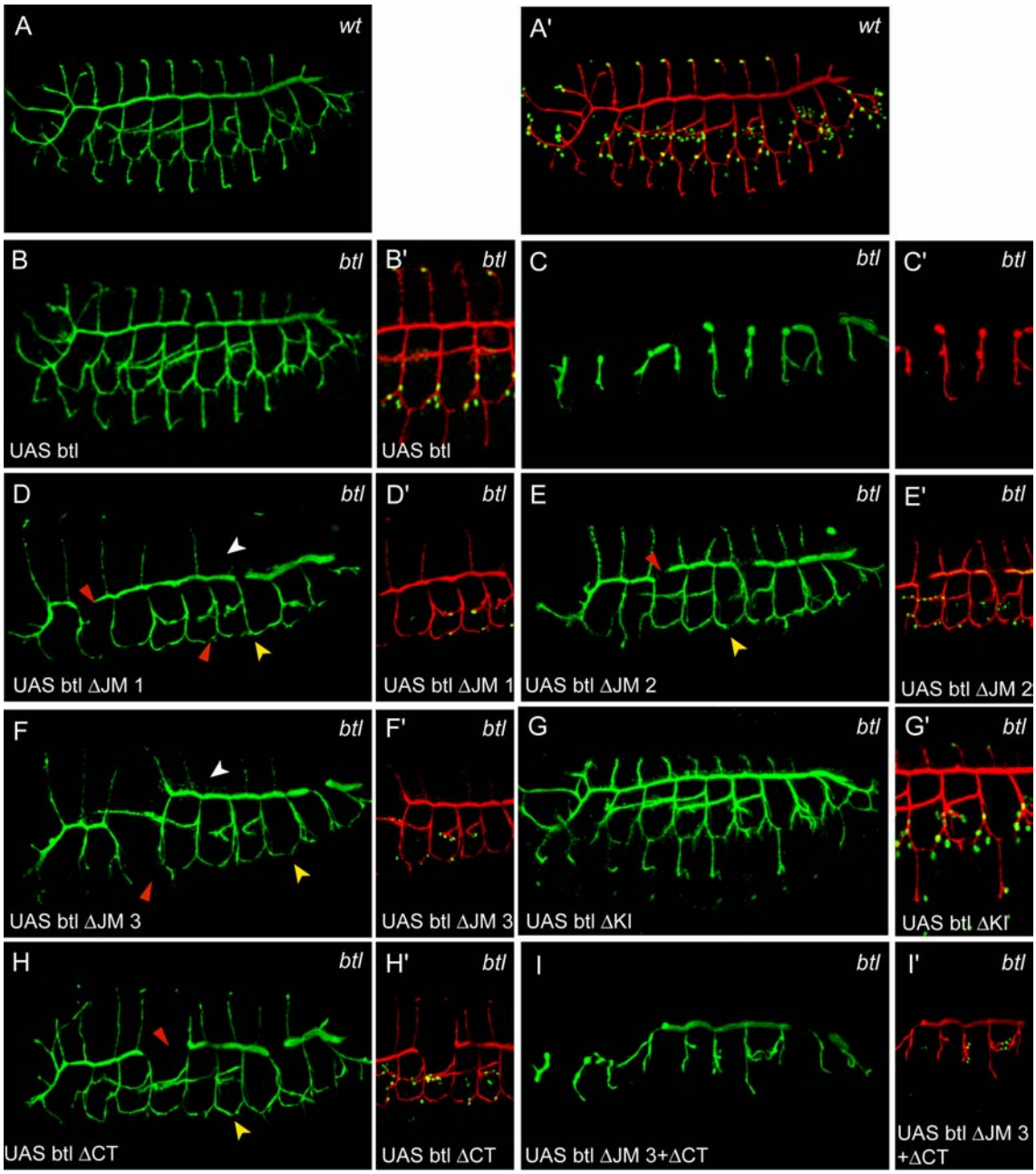


Figure 5: Rescue of tracheal cell migration by truncated versions of Btl. *Btl*$^{H82\Delta3}$ embryos expressing *btl* transgenes lacking either the juxtamembrane domain, the kinase insert domain or the C-terminus rescued tracheal cell migration and induced terminal cell fates. The lumen of the tracheal system was visualized with the 2A12 antibody (green; A-I, red; A'-I'). Terminal cells were visualized with the anti-DSRF antibody (green; A'-I'). Confocal projections of a representative embryo are shown of a (A,A') wild-type embryo, (B,B') a rescued *btl*$^{H82\Delta3}$ embryo expressing the UAS btl transgene under the control of the btl-GAL4 driver and (C,C') a *btl*$^{H82\Delta3}$ mutant embryo. Rescued *btl*$^{H82\Delta3}$ embryos expressing the following Btl derivates under the control of btl-GAL4 are shown: (D,D') UAS btl ΔJM1, (E,E') UAS btl JMΔ2, (F,F') UAS btl ΔJM3, (G,G') UAS btl ΔKI, (H,H') UAS btl ΔCT, (I,I') UAS btl ΔJM3+ΔCT. The yellow arrowhead points on the lacking ganglionic branches (D,E,F,H), the red arrowhead on dorsal trunk or lateral trunk fusion defects (D,E,F,H), and the white arrowhead on dorsal branch outgrowth defects (D,F). Abbreviations: JM: juxtamembrane domain; KI: kinase insert; CT: C-terminus.

Btl mutant embryos expressing deleted versions of Btl either lacking the first stretch of the juxtamembrane domain from amino acid 641-671 (Fig.5,D,D'), the second stretch from amino acid 672-699 (Fig.5E,E') or the whole juxtamembrane domain from amino acid 641-699 (Fig.5F,F') were able to substitute the UAS *btl*-transgene (Fig.5B,B') by rescuing tracheal cell migration almost completely.

However, the rescue capacity of the truncated receptors was less efficient than the wild type *btl* transgene (Fig.5D,E,F). In some segments, the dorsal trunk and the lateral trunk failed to fuse (red arrowheads) and the dorsal branches were rescued with various efficiency (white arrowheads). The visceral branches were misguided sometimes. Interestingly, one of the major defects was the complete lack of ganglionic branches (yellow arrowheads).

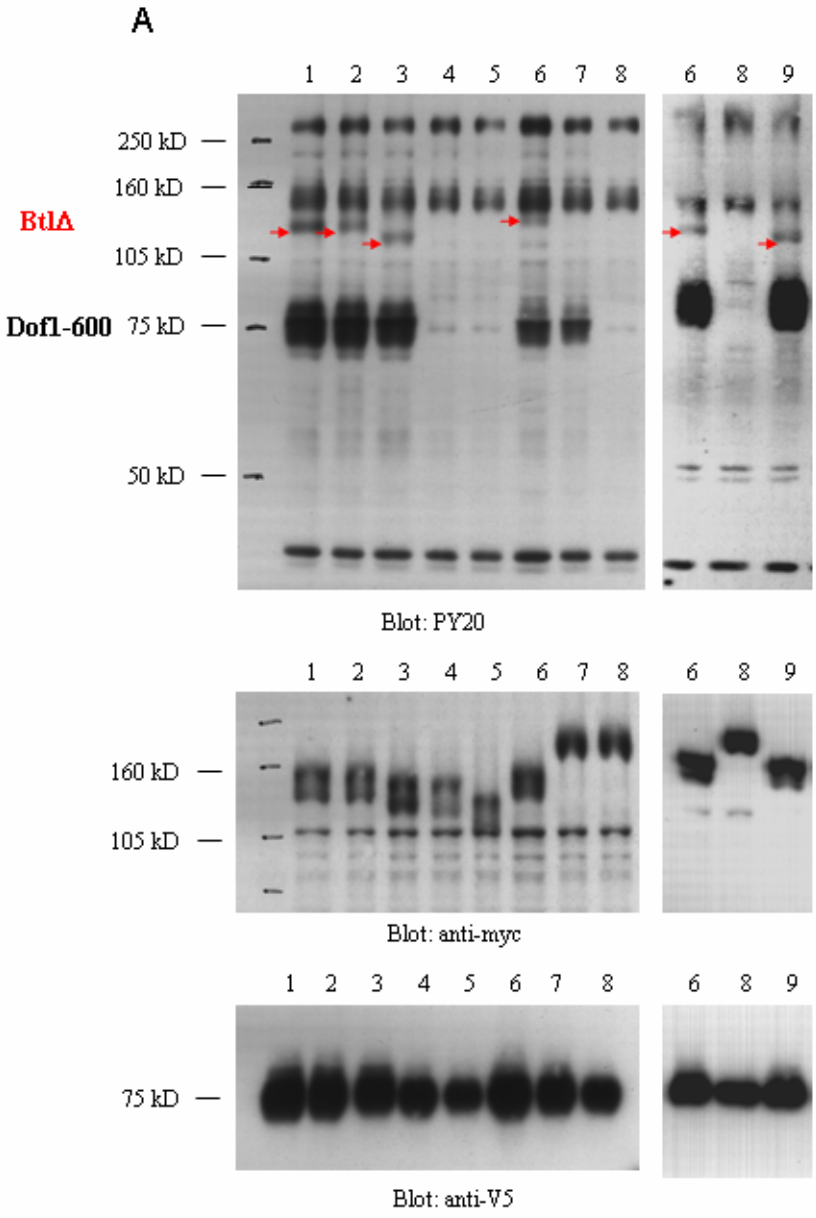
We were wondering whether these minor defects were due to a reduced efficiency of signal transmission. To investigate whether transcriptional activation is induced, we looked at the expression of the terminal cell fate marker *blistered/DSRF,* a transcriptional target of Bnl signalling and the downstream MAPK cascade (Fig.5,A'-I'). Indeed, induction of terminal cells was also reduced in the rescued tracheal systems by the transgenes UAS *btl* ΔJM 1-3 (Fig.5D',E',F'), indicating a less efficient signal propagation via the MAPK cascade (compare to the wild type tracheal system with the stereotyped pattern of terminal cells) (Fig.5A' or Fig.5B').

We observed a similar rescued tracheal system phenotype when a *btl* transgene lacking the C-terminus (1000-1153aa) was expressed in *btl* mutant embryos (Fig.5H,H'). Expression of the *btl* transgene lacking the kinase insert domain (814-835aa) completely restored the tracheal system (Fig.5G,G').
Albeit with reduced efficiency, all these transgenes were able to rescue all six primary branch outgrowth and branch fusion occurred in most segments. Unexpected and in contrast to the vertebrate FGFRs (Hadari et al., 2001; Landgren et al., 1998), the crucial link of Btl to the cytoskeleton is neither provided by the juxtamembrane domain, nor by the kinase insert domain, nor by the C-terminus.
As control, a double deletion construct consisting intracellular only of the kinase and kinase insert domain (Fig.5I,I') was tested for its rescue capacity to identify possible functional redundancies within the juxtamembrane domain and the C-terminus. This truncated receptor was not able to rescue tracheal cell migration anymore, although some dorsal branches and lateral trunk cells migrated and in some segments the dorsal trunk fused. These minor migration events were never observed in *btl* mutant embryos (Fig.5C,C'). Additionally and in contrast to *btl* mutant embryos, *blistered/DSRF* was induced (Fig.5I').
The loss of a complete rescue potential is probably due to steric defects of the dimerising receptors. The kinase domain fails to undergo proper conformational changes due to its close proximity and interactions with the plasmamembrane. Thus the kinase activity could be reduced because the tyrosines essential for kinase activity are not presented in the optimal conformational state to get trans-phosphorylated.

3. S2 assay: Molecular analysis of the Btl-Dof interaction

Previous experiments have shown that Dof is a phosphotarget of Btl and Htl (Petit et al., 2004; (Wilson et al., 2004) and that Dof is an essential and specific FGF signalling mediator for tracheal and mesodermal cell migration (Dossenbach et al., 2001; Imam et al., 1999; Michelson et al., 1998a; Vincent et al., 1998).

We addressed the question, whether rescue of cell migration in *btl* mutant embryos by the truncated receptors is due to their remaining capacity to interact with Dof thereby phosphorylating Dof. We generated myc-tagged constitutive active versions of the truncated receptors by exchanging the extracellular and transmembrane domain of Btl with the one of Torso^{4021} (mycTor^{4021}-Btl) (Reichman-Fried et al., 1994). These modified receptors were expressed in *Drosophila* Schneider cells (S2) together with Dof1-600 (V5-tagged) that has been considered as minimal Dof protein (Petit et al., 2004) under the control of a heavy metal-inducible promoter (see Materials and Methods).


A
1 2 3 4 5 6 7 8 6 8 9
250 kD
160 kD
BtlΔ
105 kD
Dof1-600 75 kD
50 kD
Blot: PY20
1 2 3 4 5 6 7 8 6 8 9
160 kD
105 kD
Blot: anti-myc
1 2 3 4 5 6 7 8 6 8 9
75 kD
Blot: anti-V5

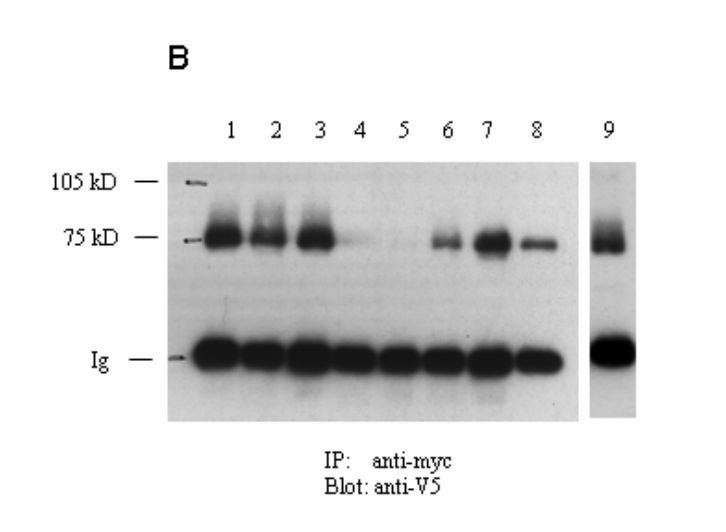


Figure 6: Dof interacts with the kinase domain of Btl and gets phosphorylated upon FGF signalling. S2 cells transfected with the following plasmids: lane 1: myc- tor^{4021}-btl ΔJM1+dof600-V5; lane 2: myc- tor^{4021}-btl ΔJM2+dof600-V5; lane 3: myc- tor^{4021}-btl ΔJM3+dof600-V5; lane 4: myc-tor^{4021}-btl ΔCT+dof600-V5; lane 5: myc- tor^{4021}-btl (ΔJM3+ΔCT)+dof600-V5; lane 6: myc- tor^{4021}-btl +dof600-V5; lane 7: myc-btl+dof600-V5; lane 8: myc-btl kinase dead+dof600-V5; lane 9: myc-tor^{4021}-btl ΔKI+dof600-V5. The cells were lysed after 20h induction of expression with 0.6mM $CuSO_4$. (A) Whole cell lysates were subjected to SDS-PAGE and tyrosine phosphorylation levels were examined by anti-phosphotyrosine immunoblotting. Protein levels were evaluated for the receptors with anti-myc antibodies and for dof600 with anti-V5 antibodies. (B) S2 cells were transfected with the indicated combinations of constructs as described in (A) lane 1-9. Whole cell lysates were immunoprecipitated with anti-Myc antibodies, subjected to SDS-PAGE and immunoblotted with anti-V5 antibodies. Ig: Immunoglobulins.

As control, we expressed the constitutive active Btl (Tor^{4021}-Btl). Co-expression of Dof1-600 resulted in strong phosphorylation of Dof detected with an anti-phospho-tyrosine antibody (Fig6A, lane6).

The non-activated receptor with the extracellular and intracellular domain of Btl was expressed as negative control. These non-activated receptors should not be able to dimerize in absence of the ligand. Unexpectedly, the receptors auto-activated their kinase activity, probably due to their overexpression in the S2 cells (Fig.6A, lane7). Consequently, we decided to generate a kinase-dead version of Btl in which the conserved lysine in the ATP binding domain was substituted with an arginine as described by T. Lee (Lee et al., 1996). Indeed, the kinase-dead receptor did not phosphorylate Dof anymore (Fig.6A, lane8).

Dof1-600 got strongly phosphorylated upon expression of the receptors lacking amino acid stretches of the juxtamembrane domain. Lane1 corresponds to Tor^{4021}-Btl ΔJM 1, lane2 to Tor^{4021}-Btl ΔJM 2 and lane 3 to Tor^{4021}-Btl ΔJM 3. Surprisingly, the receptors lacking the C-terminus (Tor^{4021}-Btl ΔCT) or both domains, the juxtamembrane domain and the C-terminus (Tor^{4021}-Btl ΔJM 3+ΔCT), were not able to phosphorylate Dof1-600 (Fig.6A, lane4 and 5). The experiment was repeated using Dof full-length to investigate whether the C-terminal part of Dof is required to interact with the C-terminus of Btl. Also Dof full-length was not phosphorylated upon expression of the c-terminal truncated receptors (data not shown).

In contrast, in vivo, Btl lacking the C-terminus almost completely rescued tracheal cell migration in *btl* mutant embryos and we know from previous experiments that tracheal cell migration is absolutely dependent on Dof (Imam et al., 1999; Vincent et al., 1998). We assume that interaction with Btl and subsequent phosphorylation of Dof is essential to recruit other molecules that are putative migratory signalling mediators (Petit et al., 2004; (Wilson et al., 2004). However, we could not even detect any phosphorylation of the c-terminal truncated receptors themselves in S2 cells (compare to the other lanes where the receptor tyrosine phosphorylation is indicated by arrows). This result indicates that the C-terminus is essential to allow the receptors to dimerize and to undergo the subsequent conformational changes, transphosphorylation and upregulation of the kinase activity. As consequence, we assume that the phosphorylation of Dof as downstream target is not possible. The receptors lacking the kinase insert domain phosphorylated Dof1-600 (Fig.6A, lane9).

From these results we can conclude that neither the juxtamembrane domain nor the kinase insert domain function as Dof interaction domains. According to the in vivo data also the C-terminus can be excluded.

Since Dof gets phosphorylated by the activated Btl receptors (including the truncated versions), we were interested to study whether Dof directly interacts with the receptor and if so, whether it interacts with the kinase domain. S2 cells were co-transfected with the myc-tagged activated version of Btl and V5-tagged

Dof1-600 and we investigated the capability of these two proteins to form a complex by co-immunoprecipitation with an anti-myc antibody from S2 cell lysates. The SDS pages were blotted with an anti-V5 antibody.

Dof1-600 was able to form a complex with the activated Btl receptor (Fig.6B, lane6). The receptors lacking parts of the juxtamembrane domain (Fig.5B, lanes1-3) and the receptor lacking the kinase insert domain (Fig.5B, lane9) also formed a complex with Dof 1-600.

Receptors lacking the C-terminus do not co-immunoprecipitate with Dof1-600, which is in line with the previous results that have shown the incapability of these receptors to phosphorylate Dof (Fig.6B, lanes4-5).

However, from the in vivo results we concluded that the C-terminus can not provide the interaction domain of Dof and we analyzed whether Dof could interact with the kinase domain of Btl and whether this interaction is dependent on the phosphorylation state of the receptor.

Interestingly, Dof co-immunoprecipitated with the kinase-dead version of Btl indicating a direct and constitutive interaction with the kinase domain of the receptor independent of the activation state (Fig.6B, lane8). Recent data confirms these results. In yeast two hybrid assays, Dof has been shown to directly interact with the kinase domain of both Btl and Htl (Battersby et al., 2003).

Acknowledgements:

The $btl^{H82\Delta3}$ was kindly provided by Ch. Klämbt and B. Shilo.

Many thanks to Ute Nussbaumer, who established the S2 cell culture system in our lab and found the optimal conditions for growing the cells. I would also thank Valérie Petit, Jorgos Pyrokowavlis and Ute Nussbaumer for introducing me into the techniques of biochemistry. B. Dickson kindly provided the hs-Tor^{4021}-Btl construct.

VI. The role of Dras, Draf and Rolled in the migratory response

Two downstream signalling components have already been identified to play an essential role in mediating the FGF signal to the migration machinery, the adapter Dof and the phosphatase Csw (Petit et al., 2004; (Imam et al., 1999; Johnson Hamlet and Perkins, 2001a; Michelson et al., 1998a; Perkins et al., 1996a; Vincent et al., 1998). Further experiments have shown that recruitment of Csw via Dof is also essential for the activation of the MAPK pathway and transcriptional activity (Petit et al., 2004; (Gabay et al., 1997a).

Either Csw or its downstream targets could provide the essential link to the cytoskeletal components. Csw has been shown to act as positive signalling transducer and activator of Ras in Torso, EGFR and Sevenless signalling pathways (Johnson Hamlet and Perkins, 2001a; Perkins et al., 1996a). Since the *csw* mutant phenotype shows impaired tracheal and mesodermal cell migration, the question is raised whether the MAPK cascade induces the migratory response or whether unidentified components activated by the phosphatase activity of Csw induce migration. Ras and the MAPK not only induce transcriptional activity but have also a wide range of substrates in the cytoplasma. In later stages of the tracheal development, the activation of the MAPK can be observed only in migrating tip cells suggesting an additional role in inducing cytoskeletal rearrangements (Gabay et al., 1997a; Sutherland et al., 1996). The following experiments try to identify the function of Ras, Raf and Rolled (MAPK) in the process of cell migration.

1. Ras effector mutants

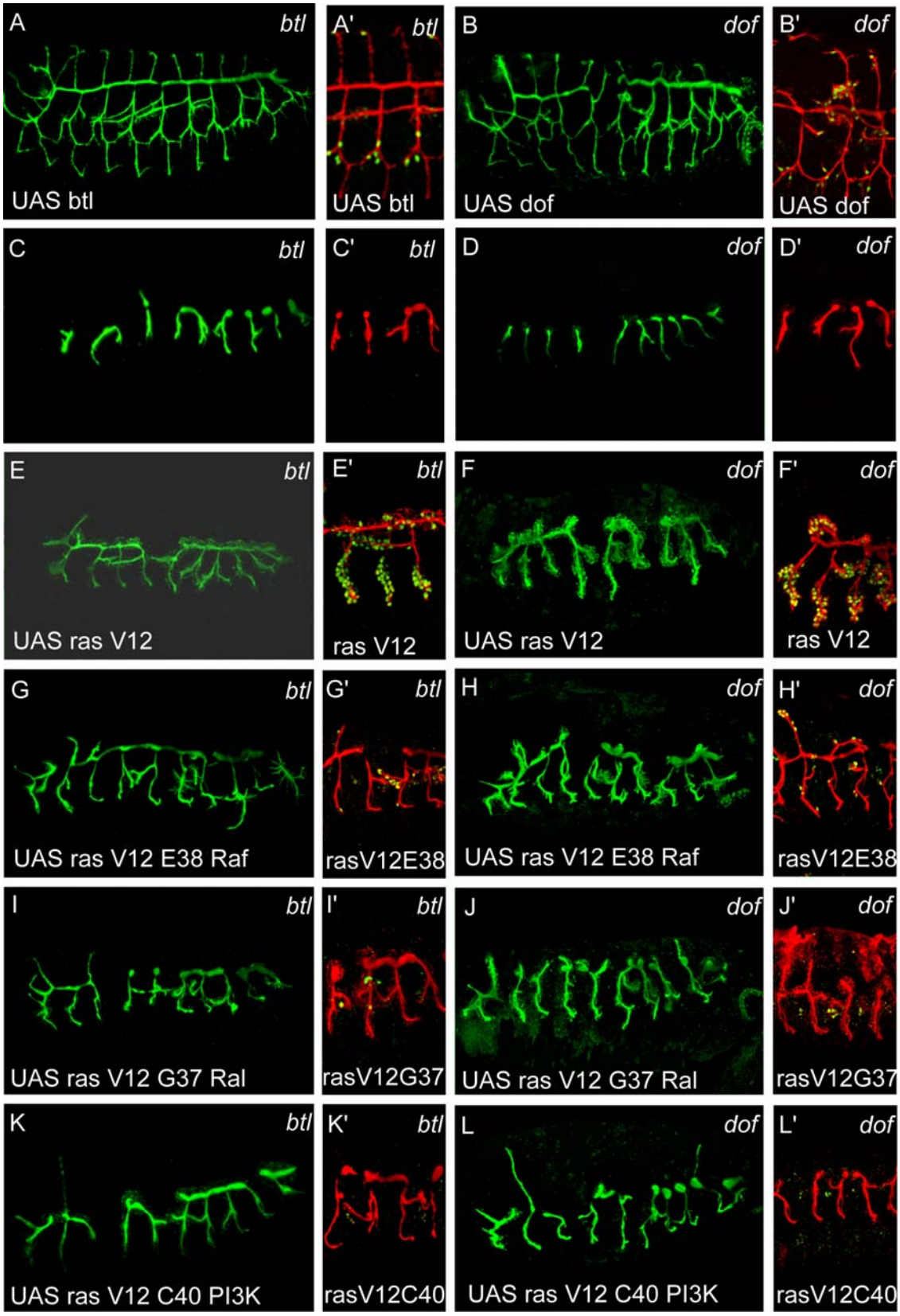
Activation of the small GTPase Ras is associated with a wide variety of cellular responses. In *Drosophila*, Ras specifies the larval head and tail structures, the ventral ectoderm fate and the dorso-ventral polarity in the eggshell. In imaginal discs, Ras is required for cell growth, differentiation of wing veins and photoreceptor cells. Since Ras is such an essential signalling component of

RTK signalling during egg and embryonic development, it is impossible to investigate Ras maternal and zygotic mutant embryos. The embryos die at early stages, because Ras null mutant cells fail to differentiate and undergo apoptosis upon exit from the cell cycle. Only effects of constitutive active (RasV12) and dominant negative Ras (RasN17) can be investigated, but overexpression of a constitutive active form of Ras does not reflect the physiological situation, especially during tracheal primary outgrowth where Ras gets locally activated in the tip cells upon Bnl signalling.

But interestingly, in cultured cells, the constitutive active Ras induces membrane ruffling due to cortical actin rearrangements.

Molecular defined partial loss of function mutations of Ras that affect specific signalling pathways downstream of Ras could provide further insights to the function of Ras. Single amino acid substitutions in the Ras effector loop result in Ras variants that interact only with one of the putative effector molecules known (Halfar et al., 2001; Rommel and Hafen, 1998).

RasV12E38 interacts with the serine-threonine kinase Raf but no longer with other effector proteins. RasV12C40 exclusively interacts with the PI3K, another downstream effector of Ras, and RasV12G37 only interacts with the small G-protein Ral. At present, the function of Ral is unknown. Recently, a protein that interacts with Ral has been identified containing a RacGAP domain (Rodriguez-Viciana et al., 1997; Rommel and Hafen, 1998) providing an interesting link to the Rho family of small GTPases that regulate cytoskeletal rearrangements.


A
btl
UAS btl
A'
btl
UAS btl
B
dof
UAS dof
B'
dof
UAS dof
C
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C'
btl
D
dof
D'
dof
E
btl
UAS ras V12
E'
btl
ras V12
F
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G
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UAS ras V12 C40 PI3K
K'
btl
rasV12C40
L
dof
UAS ras V12 C40 PI3K
L'
dof
rasV12C40

Figure 7: Ectopic activation of Ras is required but not sufficient to rescue tracheal cell migration. No rescue by expression of partial loss of function mutations of Ras that affect specific signalling pathways downstream of Ras. The lumen of the tracheal system was visualized with the 2A12 antibody (green; A-L, red; A'-L'). Terminal cells were visualized with the anti-DSRF antibody (green; A'-L'). A) Rescued $btl^{H82\Delta3}$ embryos expressing a btl wild-type transgene under the control of btl-GAL4. B) Rescued dof^{1740} embryo expressing a *dof* wild-type transgene. The irregular pattern of the tracheal system is due to mesodermal defects in dof^{1740} embryos. (C) $Btl^{H82\Delta3}$ embryo. (D) Dof^{1740} embryo. Rescued $btl^{H82\Delta3}$ and dof^{1740} embryos expressing the following Ras constructs under the control of btl-GAL4: (E,F) UAS ras V12, constitutive active Ras; (E',F') Ectopic terminal cells in embryos expressing UAS ras V12; (G,H) UAS ras V12 E38, target specific Ras mutation exclusively activating Raf; (I,J) UAS ras V12 G37, target specific Ras mutation exclusively activating Ral; (K,L) UAS ras V12 C40, target specific Ras mutation exclusively activating PI3K.

To address the question whether Ras is able to rescue tracheal cell migration defects, we expressed the constitutive active form of Ras (UAS rasV12) in $btl^{H82\Delta3}$ (Fig.7C) and dof^{p1740} (Fig.7D) (Vincent et al., 1998) null mutant embryos under the control of *btl*-Gal4.

As controls, we showed the complete rescue of tracheal cell migration by expression of the UAS btl transgene in a *btl* mutant embryo (Fig.7A) and the rescue of an UAS dof transgene in a *dof* mutant embryo (Fig.7B).

As shown in Fig.7E and F, the transgene UAS rasV12 was able to rescue modestly tracheal cell migration, either in *btl* or in *dof* mutant embryos. Ectopic activation of the transcriptional target *blistered/DSRF*, which resulted in additional terminal cells, could also be observed (Fig.7E',F').

Expression of the target specific Ras mutation activating only Raf seemed to be sufficient to rescue minor cell migration and dorsal trunk fusion in *btl* and *dof* mutant embryos (Fig.7,G,H). It activated also transcription of *blistered/DSRF* albeit reduced compared to RasV12 (Fig.7,G',H').

The specific activation of the effectors Ral and PI3K neither rescued tracheal cell migration nor induced dorsal trunk fusion in *btl* and *dof* mutant embryos (Fig.7I,I',J,J',K,K',L,L').

In general, we could observe a reduction in transcriptional activity by all the target specific mutations of Ras.

From these results, we conclude that ectopic activation of Ras in the tracheal cells is required but not sufficient to rescue tracheal cell migration.

Acknowledgements:
I would like to thank the group of E. Hafen for providing the Ras effector mutant lines.

2. Local activation of Raf

Since Ras is ectopically expressed and constitutive active in all tracheal cells, the question arises, whether local activation of Ras would be sufficient to rescue completely directional tracheal cell migration upon Bnl signalling.
Experimentally, it is difficult to activate a small GTPase in a distinct subset of tracheal cells. Therefore, we decided to activate the downstream serine-threonine kinase Raf locally.
A chimeric UAS torso-raf construct has already been generated by B. Dickson, where the kinase domain of Raf was fused to the extracellular and transmembrane domain of Tor (Dickson et al., 1992).
We adapted this construct and generated a fusion protein with the extracellular and transmembrane domain of Btl fused to the kinase domain of Raf (330-740aa). With the extracellular domain of Btl, we ensured the correct spatial and temporal activation of the construct UAS btl-raf by Bnl in *btl* and *btl,dof* double mutant embryos.

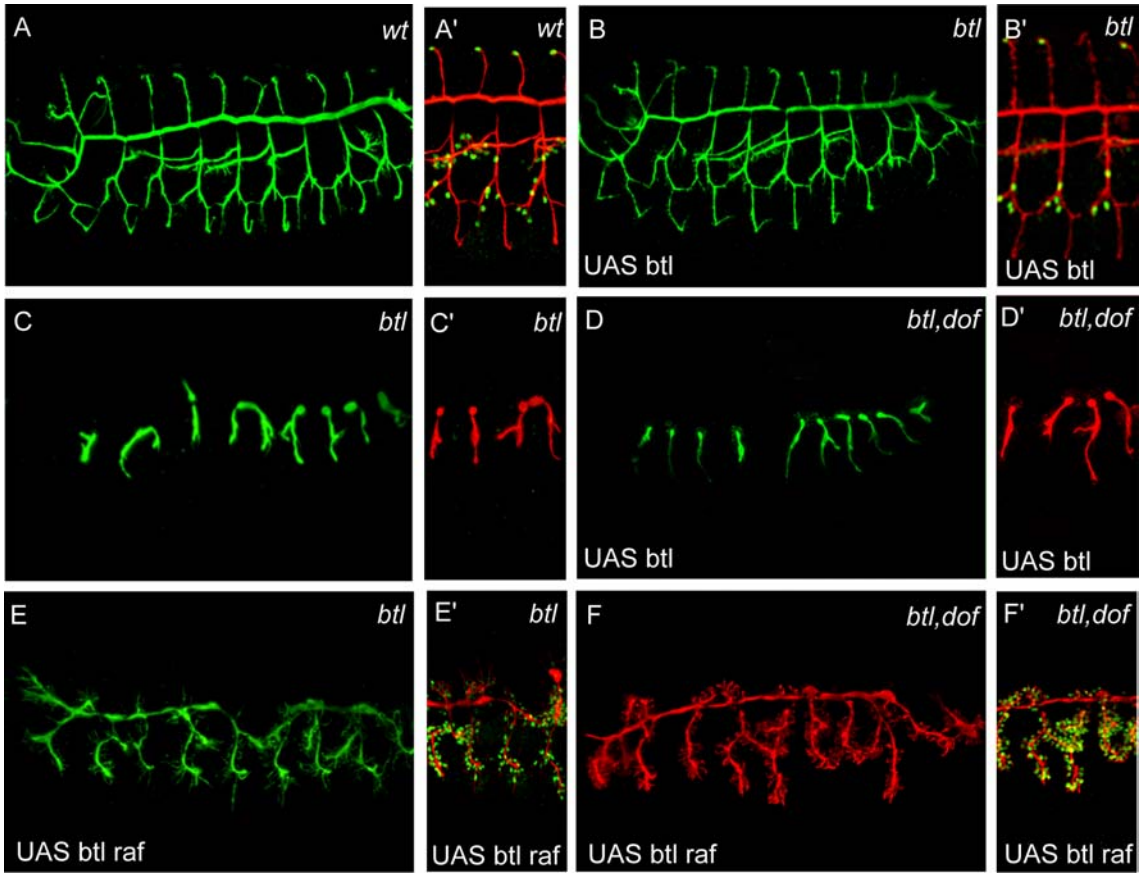

Figure 8: Local activation of Raf. The lumen of the tracheal system was visualized with the 2A12 antibody (green; A-F, red; A'-F'). Terminal cells were visualized with the anti-DSRF antibody (green; A'-F'). (A) Wild-type embryo. (B) *btl*$^{H82\Delta3}$ embryo expressing a *btl* wild-type construct under the control of btl-GAL4. (C) *btl*$^{H82\Delta3}$ embryo. (D) *btl*$^{H82\Delta3}$, *dof*^{1740} double mutant embryo expressing a *btl* wild-type construct. In the absence of Dof there is no rescue of tracheal cell migration. (E) *btl*$^{H82\Delta3}$embryo expressing the Btl-Raf fusion protein under the control of btl-GAL4. (E') Ectopic terminal cells in *btl*$^{H82\Delta3}$embryos expressing UAS btl-raf. Instead of local Bnl-dependent activation, ectopic activation of the chimeric Btl-Raf fusion protein. (F) *btl*$^{H82\Delta3}$, *dof*^{1740} embryos expressing UAS btl-raf. (F') Induction of ectopic terminal cell fates in *btl*$^{H82\Delta3}$, *dof*^{1740} embryos expressing UAS btl-raf.

As control, we expressed a wild type UAS btl construct under the control of *btl*-Gal4 in *btl* and *btl,dof* mutant embryos (Fig.8B,B',D,D'). As previously shown, Btl was not able to rescue tracheal cell migration in the absence of Dof (Fig.8D) and there was no transcriptional activation of *blistered/DSRF* (Fig.8D').
Unfortunately, the rescue of tracheal cell migration by the expression of the chimeric construct UAS btl-raf was poor in *btl* and *btl,dof* mutant embryos upon the correct activation by Bnl. The rescued tracheal phenotype showed some similarity to the expression of activated Ras (UAS RasV12) in *btl* and *dof* mutant embryos (Fig.7). Expression of UAS btl-raf mainly rescued dorsal trunk fusion in *btl* and *btl,dof* mutant embryos and induced ectopic terminal cell fates (Fig.7E,E',F,F').
These results indicate a constitutive activation of the chimeric Btl-Raf protein in all tracheal cells, even in cells that do not receive Bnl signalling. Similar to RasV12, constitutive active Raf is not able to restore a wild-type tracheal system.

Acknowledgements:
B. Dickson kindly provided the cDNA of Draf.

3. MAPK activity in tracheal cells

Thus far, we could show that ectopic activation of Ras or Raf in *btl* and *dof* mutant embryos is required but not sufficient to rescue directional tracheal cell migration. Local activation of Ras or Raf in the tip cells of the outgrowing branches was experimentally not possible.
Another approach to investigate whether the Ras/MAPK signalling is linked to the cytoskeleton is the overexpression of components of the Ras/MAPK cascade in wild-type embryos. We were interested whether wild type (wt) tracheal cells form ectopic filopodia and loose polarity upon overexpression of Ras or the MAPK.

First evidence that hyper-activated Ras/MAPK signalling does not influence the polarity and the capacity of a cell to migrate was gained from the results described in chapter III. We could show, that the fusion receptor Btl-Tor completely rescued tracheal cell migration in the absence of Btl and Dof. Interestingly, rescue by the Btl-Tor receptor in *btl,dof* mutant embryos hyper-activated the Ras/MAPK pathway visualized by the ectopic expression of *blistered/DSRF* in the tracheal cells. Almost all cells adapted terminal cell fate. These results indicated that even when the Ras/MAPK cascade is hyper-activated, the cells are still able to respond to Bnl by directional cell movement.
We analyzed MAPK activity and the induction of *blistered/DSRF* expression in wt embryos expressing UAS btl-tor, UAS bnl, UAS rasV12 and the activated version of btl (UAS tor^{4021}-btl) constructs in all tracheal cells under the control of the *btl*-Gal4 driver to investigate effects on tracheal cell migration.

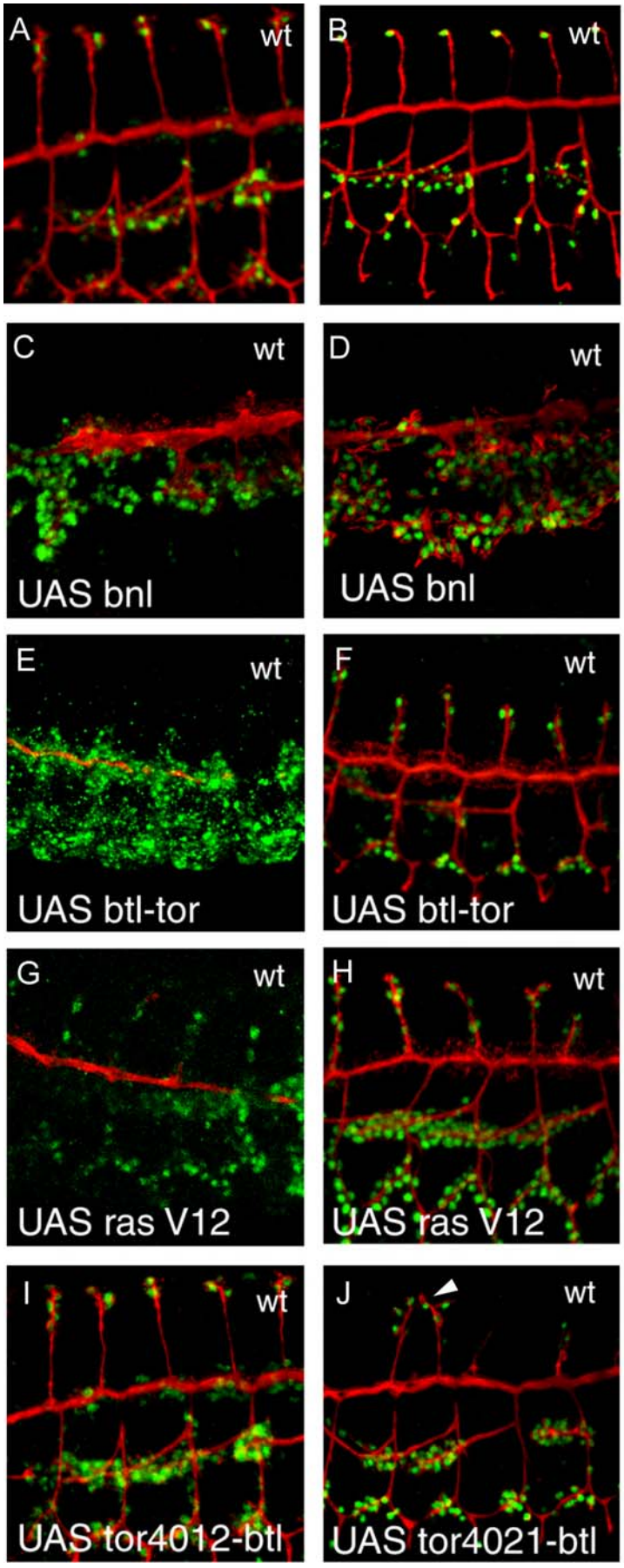

Figure 9: Hyperactivity of Ras/MAPK signalling does not influence the capacity of tracheal cells to migrate. The lumen of the tracheal system was visualized with the 2A12 antibody (red; A-J). MAPK activity was visualized with anti-dpERK antibodies recognizing the tyrosine-threonine phosphorylated MAPK (dpERK) (green; A,C,E,G,I) and terminal cells with the anti-DSRF antibody (green; B,D,F,H,J). (A) In wild-type embryos (stage 14-16), the MAPK activity is restricted to the tip cells of the outgrowing branches. (B) DSRF expression is restricted to cells with terminal cell fate. Confocal projections are shown for wild-type embryos (stage 16) expressing the following constructs under the control of btl-GAL4: (C,D) UAS bnl. (E,F) UAS btl-tor, stage 14 embryo, ectopic MAPK activation in all tracheal cells. (G,H) UAS ras V12, stage 14 embryo, ectopic MAPK activation in all tracheal cells. (I,J) UAS tor^{4021}-btl.

In wt embryos, the MAPK is activated in the outgrowing tip cells of the dorsal branch, the visceral branches, the lateral trunk anterior and posterior and the ganglionic branches (Fig.9A) (Gabay et al., 1997a; Ohshiro et al., 2002). MAPK activity was visualized by the anti-dpERK antibody that recognizes the tyrosine-threonine phosphorylated MAPK (dpERK) (Fig.9A,C,E,G,I). At the same time, terminal cells were highlighted by the expression of *blistered/DSRF* (Fig.9B,D,F,H,J).

Under conditions in which wt tracheal cells sustain high levels of Ras activity upon RasV12 overexpression, cells were not disturbed in their migration behaviour (Fig.9G,H).

In contrast, ectopic expression of the ligand Bnl completely blocked directional primary branch outgrowth. The MAPK was ectopically activated which resulted in ramifying ectopic terminal branches (Fig.9C,D).

Expression of the chimeric receptor Btl-Tor did not influence the direction of migration of wt tracheal cells although the MAPK was activated in all cells at stages where usually MAPK activity is restricted to the tip cells (Fig.9E,F).

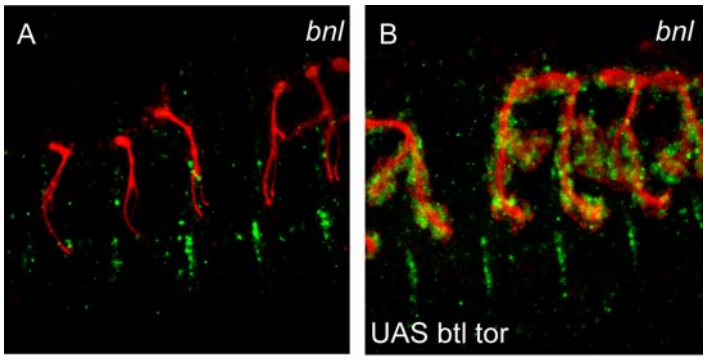

Figure 10: In the absence of Bnl, tracheal cell migration is not rescued, but the MAPK is activated by Btl-Tor. The lumen of the tracheal system was visualized with the 2A12 antibody (red; A,B). MAPK activity was visualized with anti-dpERK (green; A,B). (A) bnl^{P1} embryos do not show MAPK activity. (B) bnl^{P1} embryos expressing the UAS btl-tor construct. No rescue of cell migration but ectopic activation of the MAPK.

Interestingly, in the absence of Bnl, the chimeric Btl-Tor receptor was not able to rescue cell migration anymore but still constitutively activated the MAPK (Fig.10B) compared to a *bnl*^{P1} null mutant embryo, which lacked Bnl-dependent MAPK activity at late stages (Fig.10A).

Constitutive activation of Btl in all tracheal cells by expression of the UAS Tor^{4021}-Btl construct did not disturb tracheal cell migration in wt embryos (Fig.9I,J), only bifurcated dorsal branches could be observed (Fig.9J, arrowhead).

Previous experiments showed that the chimeric Tor^{4021}-Btl receptor did not provide a strong kinase activity since tyrosine phosphorylation was reduced. Another activated Btl receptor (λBtl) was expressed in wt cells resulting in disrupted primary branch outgrowth, incomplete dorsal trunk and lateral trunk fusion, extra-branching and misguidance of the dorsal and ganglionic branches (Lee et al., 1996).

These results suggest that local activation of the Bnl/Btl signalling, or local activation of the chimeric Btl-Tor receptor, is essential for directional outgrowth of the tracheal cells to form a functional respiratory system. Ectopic activation of either the ligand or the receptor impairs tracheal cell migration. In contrast, ectopic activation of either Ras or the MAPK in the tracheal cells does not influence the capacity of the cells to move toward the Bnl guiding cue.

Acknowledgements:

The UAS tor^{4021} btl flies were kindly provided by S. Leevers and E. Hafen.

VII. The Rho family of small GTPases

The small GTPases of the Rho family have been shown to regulate cytoskeletal reorganization, motility and adhesion. In cultured fibroblasts, RhoA is implicated in the formation of actin stress fibres and focal adhesions, Rac1 stimulates membrane ruffling, lamellipodia, focal complex formation and is present at cell junctions, and Cdc42 participate in the formation of filopodia, cell rounding and the loss of actin stress fibres. The precise mechanism by which Rho proteins affect cytoskeletal changes is not yet understood and even less is known about the upstream signalling pathways that lead to the activation of the Rho GTPases.

In *Drosophila*, six closely related homologues of the mammalian Rho, Rac and Cdc42 proteins have been identified thus far. These include Rho1, RhoL, Dcdc42 and three Rac homologues, Drac1, Drac2, and Mig-2-like (Mtl, the ortholog of *C. elegans* Mig-2). They are 70-90% conserved to their mammalian counterparts.

The Rho small GTPases are essential proteins functional throughout *Drosophila* embryogenesis in many tissues. Some of them show a more restricted expression in the mesoderm, gut and nervous system later in development.

Several experiments have already shown that also in *Drosophila* the small GTPases may be implicated in the regulation of cytoskeletal reorganizations. Dominant negative Drac1 and activated Drac1 have been shown to block completely border cell migration during oogenesis without affecting other follicle cells (Duchek and Rorth, 2001; Duchek et al., 2001; Murphy and Montell, 1996). Drac1 and Dcdc42 are also involved in the stretching of the epidermal cells required for dorsal closure. Further, Drac has a specific role in axon outgrowth initiation and elongation and activated Dcdc42 inhibits axon and dendrite outgrowth. And finally, dominant negative Drac affects myoblast fusion (reviewed in (Lu and Settleman, 1999).

The RhoGEF2 has been shown to be essential for cell shape changes during gastrulation (Barrett et al., 1997).

Since the small GTPases are essential in early embryonic developmental processes, we investigated the effect on tracheal cell migration by the expression of activated or dominant interfering alleles of Drac, Dcdc42 and Drho.

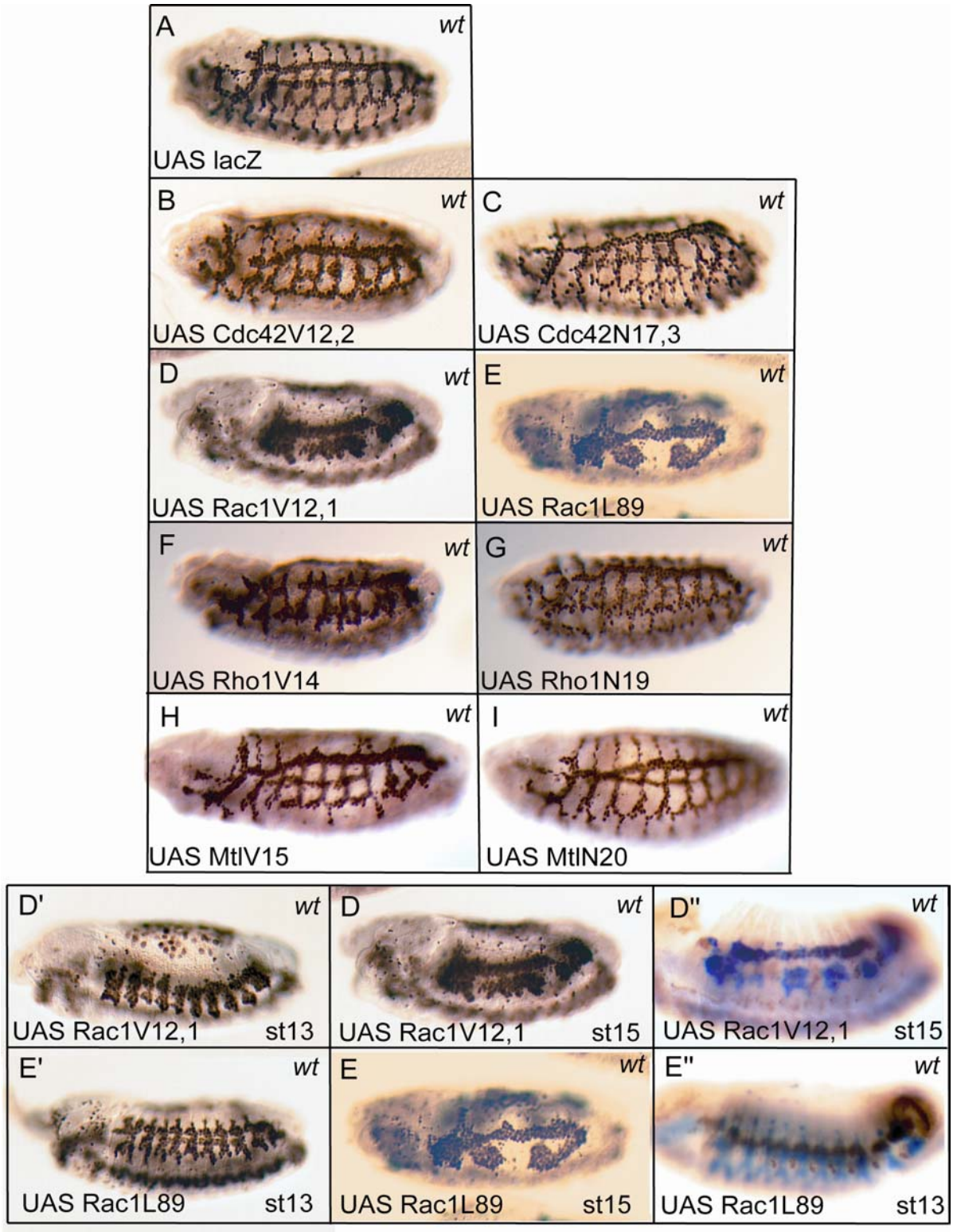

Figure 11: The small GTPases of the Rho family regulate tracheal cell-cell adhesion and actin cytoskeleton reorganizations. Tracheal cells were visualized by anti-βGalactosidase antibodies (brown; A-I). (A) Confocal projection of a representative wild-type embryo expressing the UAS lacZ construct. (B,D,F,H) Wild-type embryos expressing constitutive activated versions of the small GTPases Cdc42, Rac, Rho and Mtl were crossed to a btl-GAL4 carrying additionally UAS.GFPN.lacZ. (C,E,G,I) represent embryos expressing dominant negative versions of the small GTPases Cdc42, Rac, Rho and Mtl. (D) Stage 15 embryo expressing the constitutive active form of Rac. (D') Stage 13 embryo expressing the constitutive active form of Rac. Already at early stages, primary branch outgrowth is affected. (D'') Sal expression is visualized with an anti Sal-antibody (Boube et al.). Dorsal trunk cell fate specification was normal. Tracheal cells visualized with anti-β Galactosidase antibody (blue). (E) Stage 15 embryo expressing the dominant negative form of Rac. (E') In stage 13 embryo expressing the dominant negative Rac primary branch outgrowth is normal. Only at later stages, the tracheal cells lost polarity. (E'') Embryos expressing dominant negative Rac showed normal dorsal trunk cell fate specification visualized by the anti Sal-antibody.

To visualize the tracheal system, we expressed an UAS lacZ construct specifically in the tracheal cells under the control of the *btl*Gal4 (Fig.11A). Expression of activated Dcdc42 (DcdcV12,2) or dominant negative Dcdc42 (Dcdc42N17,3) in wild-type embryos did not affect tracheal cell migration dramatically (Fig.11B,C). Embryos expressing activated Dcdc42 lacked dorsal and ganglionic branches in few segments (Fig.11B).

A very dramatic effect could be observed in embryos carrying an activated UAS Drac1V12,1 (Fig.11D) or the dominant negative UAS Drac1L89 construct (Fig.11E). Already at early stages (Fig.11D', stage13) primary branch outgrowth was stalled. From stage14 on, the epithelial tracheal cells expressing UAS Drac1V12 seemed unable to downregulate cell-cell adhesion contacts. Instead, the mutant cells intermingled and retained clustered to end up in heaps. The cell number remained constant.

Usually, during development of a wild-type tracheal system, the epithelial cells must transiently rearrange their polarity and move without loosing but rearranging cell-cell adhesions and change their relative positions to intercalate subsequently forming unicellular tubes. Careful regulation of adherens junctions is thus required.

Time lapse movies of Marc Neumann have shown that cells expressing activated Drac1,V12,1 were not able to move dorsally and he observed ectopic filopodia extensions in all tracheal cells including the dorsal trunk.

Other experiments have shown that RacV12 induced reorganization of cell-cell junctions in MDCK cells such that the contacts were not restricted to the apical side anymore but extended over the entire lateral membranes (Rosario and Bircmeier, 2003).

In contrast, early stage embryos expressing DracL89 showed normal primary branch outgrowth (Fig.11E'). But with time, the cells seemed to loose polarity. F-actin was expressed all over the cells, forming ectopic filopodia in the dorsal branches (data from Marc Neumann and Fig.11E).

Since the cells form like two dorsal trunk-like structures, we essayed for dorsal trunk cell fate specification by using an anti-Sal antibody. From stage 13 until stage 15 only dorsal trunk cells express Sal in both embryos either expressing activated or dominant negative Drac1 (Fig.11D'',E'').

Rac has been implicated in the regulation of cadherin-dependent cell adhesion and the formation of cadherin-catenin complexes at cell junctions (Chihara et al., 2003). In contrast to our results, the group of S. Hayashi showed that in zygotic null mutant *rac1, 2* embryos, E-cadherin as well as α-catenin and β-catenin, key components of adherens junctions, are broadly accumulated in the epithelial cells and change the epithelial architecture. On the other hand, they claim that hyper-activation of Rac prevents incorporation of E-Cadherin into cell junctions thereby reducing cell adhesiveness.

Embryos expressing an activated version of Drho1 showed some dorsal and lateral trunk fusion defects (Fig.11F). The tracheal system of embryos expressing the dominant negative UAS Drho1,N19 transgene looked like wild-type (Fig.11G). Also the third Rac-like GTPase Mtl did not influence tracheal cell migration neither in an activated nor in a dominant negative version and we observed only minor defects (Fig.11H,I).

None of the activated forms of the small GTPases (neither UAS Drac1V12,1 nor UAS Dcdc42V12,2 nor UAS Drho1V14, nor UAS MtlV15) were able to rescue tracheal cell migration in *btl* or in *dof* mutant embryos (data not shown).

From these results, we assume that Drac is essential to downregulate cell-cell adhesion contacts and additionally, it could play a role in the regulation of directional actin polymerization.

1. Cdc42 heteroallelic mutant embryos

The question whether endogenous small GTPases indeed are required for cytoskeletal regulation can not be answered by dominant mutant proteins. They require the phenotypic analysis of loss of function mutations of each of the endogenous small GTPase genes.

Loss of function Dcdc42 alleles, kindly provided by R. Fehon, were investigated to examine the effects on tracheal cell migration. Heteroallelic combinations of weak and strong alleles were used to produce females that, while viable, had reduced Cdc42 activity (Fehon et al., 1997; Genova et al., 2000). We examined the tracheal phenotype of the heteroallelic combination $Cdc42^{3}$/$Cdc42^{5}$ and $Cdc42^{4}$/$Cdc42^{5}$. $Cdc42^{3}$ is a lethal missense mutation, $Cdc42^{4}$ carries a lethal mutation in the splice acceptor site, while $Cdc42^{5}$ is a viable mutation which has the aspartic acid 65 mutated to asparagine.

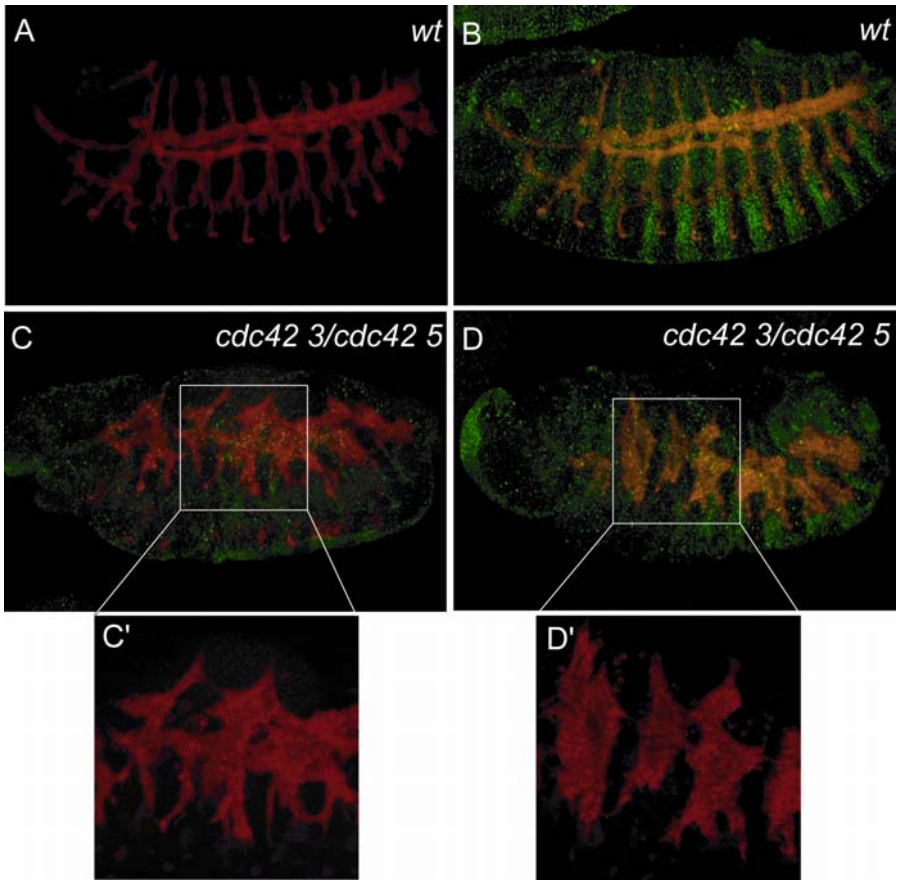

Figure 12: Cdc42 heteroallelic mutant embryos show strong primary branch outgrowth defects. (A) Confocal projection of a *trachealess* 1-eve-1 enhancer trap embryo representing the wild-type tracheal system. The tracheal cells were visualized with an anti-β Galactosidase antibody. (B) Merged image showing the wild-type tracheal system visualized with anti-β Galactosidase antibodies (red) and the epidermal derived epithelial cells with the septate junction marker anti-Fascilin 3 (green). (C,D) Two representative embryos carrying the cdc42 heteroallelic combination cdc42 3/ cdc42 5. (C',D') Higher magnification of three tracheal segments of cdc42 heteroallelic mutant embryos showing the primary branch outgrowth defects and clustering of the tracheal cells within the placodes.

The embryos were stained with the septate junction marker anti-Fasciclin 3 to highlight the ectodermally derived epithelial cells. Additionally, the *trachealess* enhancer trap marker 1-eve-1 was crossed in to visualize the tracheal cells by staining with an anti-βGal antibody (Fig.12A,B).

In $cdc42^{3}/cdc42^{5}$ mutant embryos (Fig.12C,D), several defects could be observed. Germband retraction failed to proceed to completion, leaving the embryos dorsal open. In addition holes appeared in the embryonic epidermis along the ventral midline.

Interestingly, the tracheal cells showed impaired outgrowth and clustered within the tracheal placode resulting in a strong tracheal defect phenotype.

Whether these defects were exclusively caused by reduced activity of Dcdc42 or whether this mutant phenotype was a secondary phenotype caused by truncated embryonic surrounding tissues remained an open question.

The heteroallelic combination $Cdc42^{4}/Cdc42^{5}$ unfortunately was less efficient and did not result in a mutant tracheal phenotype (data not shown).

Dcdc42 could function in collaboration with Drac in the regulation of actin dynamics.

2. Rac germline clones

S. Hakeda-Suzuki generated loss of function mutants of Drac1, Drac2 and Mtl and examined the role of Drac in axon development (Ng et al., 2002). Mushroom body (MB) axons were strongly misguided in RacJ11 mosaic mutant embryos using the MARCM (Mosaic Analysis with a Repressible Cell Marker) system (Lee and Luo, 1999). The defect increased if the neuroblast clone

additionally was homozygous for ΔRac2 and heterozygous for ΔMtl. It has been shown that different downstream effector pathways mediate axon growth, guidance and branching and require combined Rac GTPase activity.

Other developmental processes are regulated preferentially by one of the Rac's (Hakeda-Suzuki et al., 2002). Dorsal closure seems to rely more on Drac1 than on Drac2 or Mtl, whereas myoblast fusion requires either Drac1 or Drac2 but not Mtl. Longitudinal axons across the CNS midline require Mtl.

Drac1 point mutation and loss of function alleles were recovered from an ethymethane sulphonate (EMS) screen (Rac1J11). Rac1J10 is a hypomorphic allele. ΔRac2 is a null mutation but homozygous viable. Finally, ΔMtl is a null allele generated by an imprecise excision of a P-element. Each single mutation is homozygous viable, the Rac2Δ,MtlΔ double mutant is also viable but all other combinations are lethal (Hakeda-Suzuki et al., 2002).

Like described in the paper (Hakeda-Suzuki et al., 2002; Xu and Rubin, 1993), we generated germline clones, embryos lacking maternal and zygotic contribution of one or more *rac* genes, with the lines Rac1J11, Rac1J10, ΔRac2, ΔMtl in different combinations to analyse the function of the Rac's in tracheal development. Mutant females (Δ*racs*, FRT/ TM6 y+) were crossed to hs FLP; ovo^D / MKRS males according to the scheme described in the Materials and Methods. The progeny larvae of the age 24h, 48h, 72h were heat-shocked for 1-1,5h at 37°C and the larvae further developed at 25°C until adult stage. Heat-shocked virgins were collected and crossed back to heterozygous *rac* mutant males from the original stock. Eggs from the females carrying the mutation ovo^D do not develop (Pauli et al., 1995). The progeny was stained with an anti-Crumbs antibody, an apical cell marker, to visualize early tracheal development.

By using the Rac1J11 null allele in different combinations, we could not recover any germline clones.

Only few embryos could be collected that were *rac1J10,Δrac2,Δmtl* triple mutant and they did not develop further than blastoderm stage. Germline clones that were *rac1J10,Δrac2* mutant either showed a wild type tracheal system or no anti-Crumbs staining. In parallel germline clones that were *rac1J10,Δmtl* mutant

were either completely deformed and did not show any anti-Crumbs staining, or we could observe embryos with dorsal closure defects, but normal primary branch outgrowth of the tracheal system (data not shown).

It is difficult to evaluate the data of the experiments of the small GTPases. Drac has been shown to regulate epithelial cell adhesion and also Dcdc42 seems to be required for directional tracheal cell migration. But how the small GTPases are regulated by Bnl signalling has to be elucidated. At the moment, at least 22 Rho family GTPase exchange factors are encoded by the *Drosophila* genome that requires further investigation.

Acknowledgements:
I would like to thank Valérie Petit and Marc Neumann for the collaboration in this project. R. Fehon kindly provided the *Dcdc42* mutant alleles and S. Hakeda-Suzuki provided all *rac* mutant alleles and was very helpful giving me advise to keep the stocks. I would also like to thank Anna Jazwinzka for explaining how to generate germline clones at the best conditions.

VIII. Comparison to vertebrate FGF signaling

Interestingly, the vertebrate FGF signalling adapter protein FRS2 and the *Drosophila* adapter Dof are divergent in the amino acid sequence and they do not share functional protein-protein interaction domains and bind to different stretches of the FGFRs. *Dof* could be an example of a fast evolving gene used exclusively for FGF signalling in *Drosophila*, which has acquired novel functions in vertebrate immune system signalling. Nevertheless, FRS2 and Dof seem to be functional homologues, since both are adapters that bind to the FGFR constitutively and get tyrosine phosphorylated upon receptor activation. Both recruit the Grb2 (Drk)/SOS complexes and the phosphatase Shp2 (Csw) that are essential for MAPK activation and the induction of the migratory response.

We were interested to elucidate whether FRS2 could compensate for the function of Dof in *Drosophila* embryos and in S2 cells and vice versa.

One of the main differences is that FRS2 contains a non classical PTB domain, which is missing in Dof. Unlike known PTB domains that end with a C-terminal α-helix, FRS2 contains an additional β-strand (β8) that is essential for the constitutive interaction with the 22 aa stretch of the juxtamembrane domain of FGFR1 (409-430 aa) (Dhalluin et al., 2000). Only the valine427 and threonine 428 splice variants of the FGFR juxtamembrane (VT+) are able to interact with FRS2. The motif for the specific interaction was identified as KRIPLRRQVTVS including the VT motif (as it was already shown in Fig.4). Studies in transfected mammalian cells have shown that only the VT+ FGFR variant but not the VT- could activate the MAPK. And interestingly, both VT isoforms are co-expressed in the same organs in mice in vivo. However, differential expression of VT+ and VT- isoforms of FGFR1 have been identified during mesoderm formation in *Xenopus* embryos (Burgar et al., 2002).

First, we were interested to study whether Dof acts as functional homologue of FRS2 and is able to interact with the FGFR2 to trigger downstream signalling cascades. The kinase domains of human FGFRs and Btl are conserved to about 60% and could provide enough sequence similarity to serve as interaction domain for Dof.

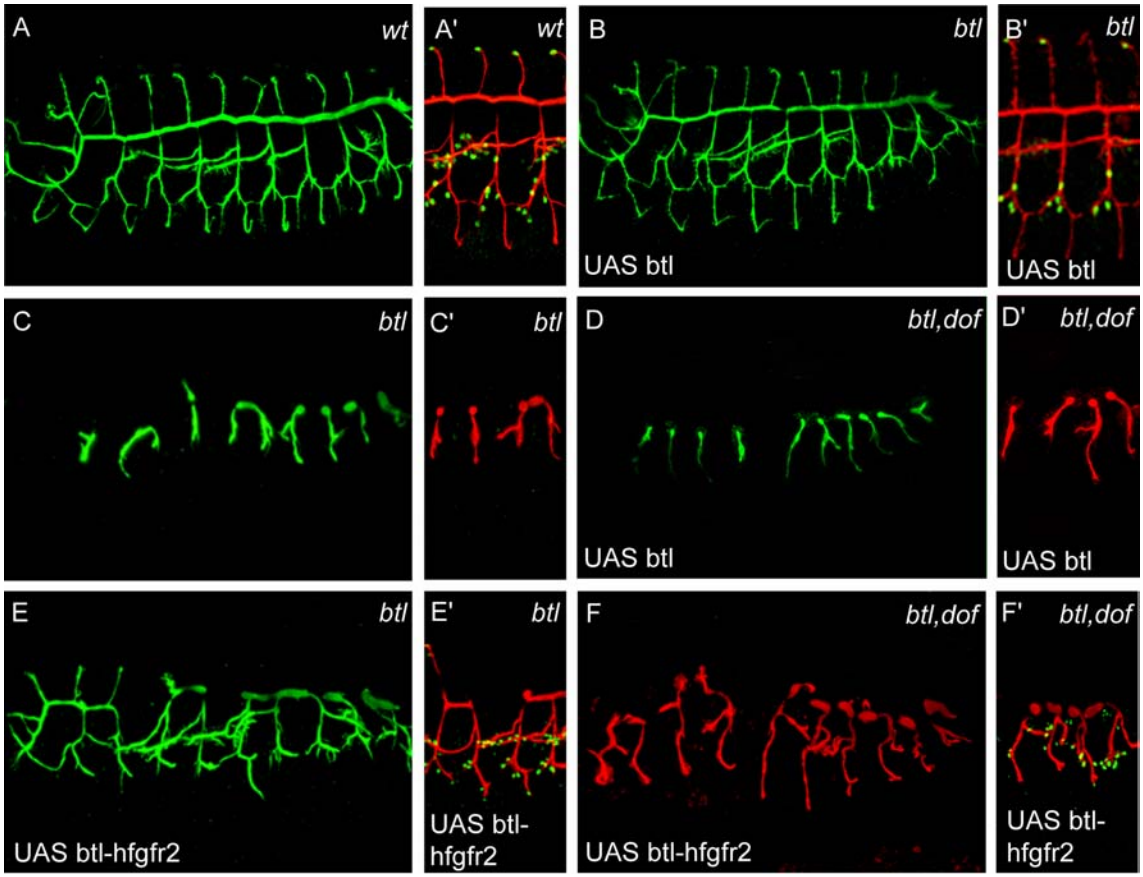

Figure 13: The chimeric Btl-FGFR2 receptor does not rescue cell migration defects, but activates transcriptional targets in the absence of Dof. The lumen of the tracheal system was visualized with the 2A12 antibody (green; A-F, red; A'-F'). Terminal cells were visualized with the anti-DSRF antibody (green; A'-F'). (A) Wild-type embryo. (B) Tracheal cell migration defects of $btl^{H82\Delta3}$ embryos are fully rescued by expression of a btl wild-type transgene under the control of btl-GAL4. (C) $btl^{H82\Delta3}$ embryo. (D) In $btl^{H82\Delta3}$, dof^{1740} mutant embryos, expression of Btl does not rescue primary branch outgrowth defects. (D') There is no transcriptional activation of blistered/DSRF. (E,E') Expression of a chimeric receptor, which consists of the intracellular domain of the human FGFR2, partially rescues primary branch outgrowth and induces terminal cell fates in $btl^{H82\Delta3}$ embryos. (D,D') In the absence of Dof, expression of the chimeric Btl-FGFR2 receptor does not rescue cell migration but induces transcription of blistered/DSRF.

We expressed a chimeric Btl-FGFR2 receptor construct under the control of *btl*-Gal4 in *btl* and *btl,dof* mutant embryos (Fig.13E,E',F,F') and compared tracheal cell migration to the wild type tracheal system (Fig.13A,A') and the rescue by the UAS *btl* transgene in *btl* and *btl,dof* mutant embryos (Fig.13B,B',D,D'). We

could observe partial rescue of tracheal cell migration in the presence of Dof (Fig.13E). Some dorsal branches grew out, the visceral branches were formed and one or two ganglionic branch grew toward the central nervous system, although it seemed to be stalled half-way. The dorsal trunk and the lateral trunk did not fuse properly. Also transcriptional activation of *blistered/DSRF* was induced by Btl-hFGFR2 (Fig.13E') in contrast to *btl* mutant embryos (Fig.13C). In contrast to the deletion receptors in the previous deletion analysis, this chimeric receptor induced terminal cell fates in all the primary branches that grew out, including the tip cell of the dorsal and ganglionic branches. These results indicate that the kinase domain of the human FGFR provides enough sequence homology to interact with Dof, although the interaction might be weak.

In *btl,dof* double mutant embryos UAS btl-fgfr2 was not able to rescue cell migration effectively anymore (Fig.13F). Strikingly, the chimeric receptor activated transcription of *blistered/DSRF* (Fig.13F'). We assume that Btl-FGFR2 is able recruit other unknown adapters in the absence of Dof that are able to substitute for the function of Dof and activate the MAPK cascade as for example Shc. Shc has been shown to recruit the Grb2/SOS complex (Luschnig et al., 2000). Nevertheless, Dof seems to be essential for the interaction with FGFR2 to trigger the migratory response at least partially and FGFR2 can not fully substitute the activity of Btl.

The sequence analysis of the two FGFRs Btl and FGFR2 showed that Btl does not contain a VT+ motif in the juxtamembrane domain, and we assume that FRS2 is not able to interact with Btl.

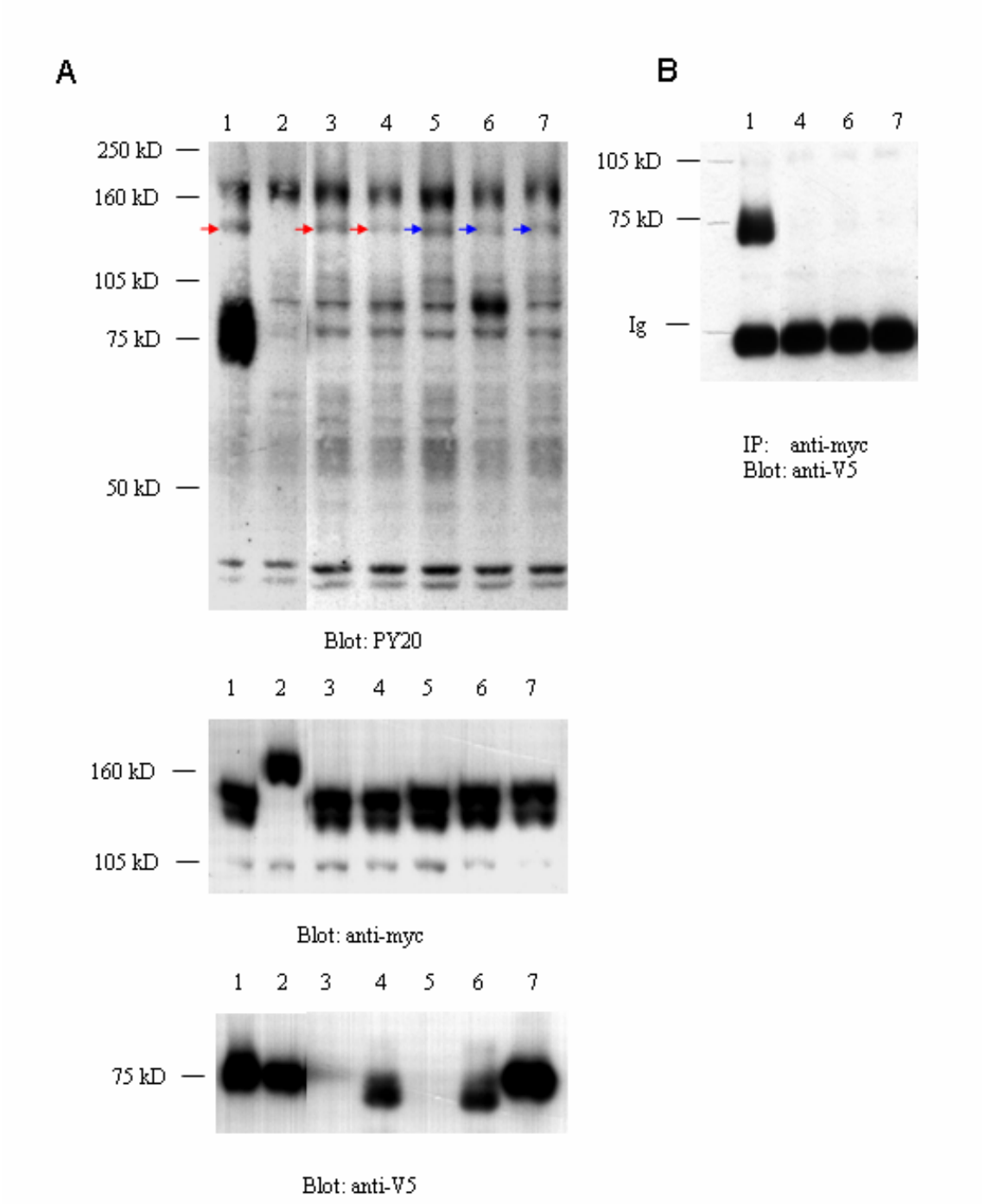

Figure 14: In vitro assay to investigate the interaction and phosphorylation of the adapters Dof and FRS2 by Btl and FGFR2. S2 cells transfected with the following plasmids: lane 1: myc- tor^{4021}-btl +dof600-V5; lane 2: myc- tor^{4021}-btl kinase dead+dof600-V5; lane 3: myc-tor^{4021}-btl; lane 4: myc- tor^{4021}-btl +frs2-V5; lane 5: myc- tor^{4021}-fgfr2; lane 6: myc- tor^{4021}-fgfr2 +frs2-V5; lane 7: myc-tor^{4021}-fgfr2+dof600-V5. The cells were lysed after 20h induction of expression with 0.6mM $CuSO_4$. (A) Whole cell lysates were subjected to SDS-PAGE and tyrosine phosphorylation levels were examined by anti-phosphotyrosine immunoblotting. Protein levels were evaluated for the receptors with anti-myc antibodies and for dof600 and frs2 with anti-V5 antibodies. (B) S2 cells were transfected with the indicated combinations of constructs as described in (A) lane 1,4,6,7. Whole cell lysates were immunoprecipitated with anti-myc antibodies, subjected to SDS-PAGE and immunoblotted with anti-V5 antibodies. Ig: Immunoglobulins.

However, we tested whether the activated version of Btl was able to phosphorylate FRS2 in S2 cell culture experiments. In addition, we were interested whether the activated FGFR2 receptor phosphorylates Dof1-600.
FRS2 (V5-tagged) is a protein of 509 aa and should run between 50 and 75 kDa. Fig.14A shows SDS pages where the subjected lysates were detected with anti-phospho tyrosine antibodies. The receptors again are myc-tagged.
Lane1 shows the positive control with the activated Btl receptor (Tor^{4021}-Btl) (red arrows) strongly phosphorylating Dof1-600. The negative control receptor Btl kinase-dead does not phosphorylate Dof1-600 (lane2).
As expected, the activated Btl receptor was not able to phosphorylate FRS2 (lane4). But somewhat surprising, the chimeric Tor^{4021}-FGFR2 (blue arrows) also did not phosphorylate FRS2 in S2 cells (lane6). An additional unexpected result was, when compared to the in vivo rescue experiments, that Tor^{4021}-hFGFR2 did not phosphorylate Dof1-600 (lane7). Lane 3 and 5 show receptor expression alone as additional control.
Fig.14B shows a co-immunoprecipitation experiment with an antibody against anti-myc to analyze complex formation between activated Btl, FGFR2 and the adapters Dof or FRS2. The immunoprecipitates were analyzed by SDS page followed by immunoblotting with an anti-V5 to visualize Dof1-600 and FRS2. Corresponding to the previous results obtained, we could not observe any complex formation neither between Tor^{4021}-Btl and FRS2 (lane4), nor between Tor^{4021}-hFGFR2 and FRS2 (lane6), nor between Tor^{4021}-hFGFR2 and Dof1-600 (lane7).
We can not explain the failing interaction between hFGFR2 and FRS2 since the VT+ spliced variant of the human receptor was used for the experiment.
The *Drosophila* S2 cell maybe miss stabilizing molecules of mammalian cells to form a constitutive complex between the human FGFR and FRS2. Depending on the mammalian cell types used for co-immunoprecipitation experiments, the complex formation of FGFR1 and FRS2 has also shown either to be weak or could not be detected at all. We assume that some cells types provide additional components to stabilize the constitutive interaction between the FGFRs and FRS2 (Cross et al., 2002; Ong et al., 2000b).

Acknowledgements:
I would like to thank Ute Nussbaumer for doing the S2 culture experiments in parallel as control to verify the results I obtained. J. Xu and M. Goldfarb kindly provided the cDNAs of hFGFR2, hFGFR1 and FRS2.

IX. Btl mutant mosaic clones in the tracheal system

Finally, we were interested in the question whether Bnl signalling induces the migratory response only in the tip cell of the outgrowing primary branches and the stalk cells would be pulled behind the migrating tip cells by cell-cell adhesion forces.

Or all the tracheal cells have the capacity to move according to their developmental history and Bnl signalling determines the tip cells as leading cells followed by the other migrating stalk cells.

We prefer the first model. Experiments have shown that *btl* expression declines in later stages of tracheal development and gets restricted to the tips of the outgrowing branches (Ohshiro et al., 2002). It has also been shown, as the migration of the tracheal tips begins, the pattern of dp-ERK dependent on FGF signalling refines and can be observed within each branch only in the migrating tip cells (Gabay et al., 1997b). Therefore, we assume that Bnl signalling activates not only the MAPK cascade but also cytoskeletal rearrangements exclusively in the tip cells.

To determine in which tracheal cells Bnl signalling induces the capability to move, we carried out a genetic mosaic analysis using the FLP recombination system as it was already performed for $spry^{-/-}$ mosaic mutant cells in the tracheal system (Hacohen et al., 1998).

The FLP recombination system induces high frequency mosaicism for a particular gene by the use of the FRT sequence from yeast. The FLP recombinase induces mitotic recombination between the FRT sites located on homologous chromosome arms. The FRT sites have to be located closer to the centromere than the gene of interest (Xu and Rubin, 1993).

To confirm our model, a *btl* mutant tip cell does not respond to the ligand Bnl anymore and is not able to migrate. Subsequently, the stalk cells should stay at their original position within the placode.

If the second model would be true, the nearby stalk cells could compensate for the function of the *btl* mutant tip cell and would be able to respond to Bnl

signalling at early stages of tracheal development by moving toward the ligand source.

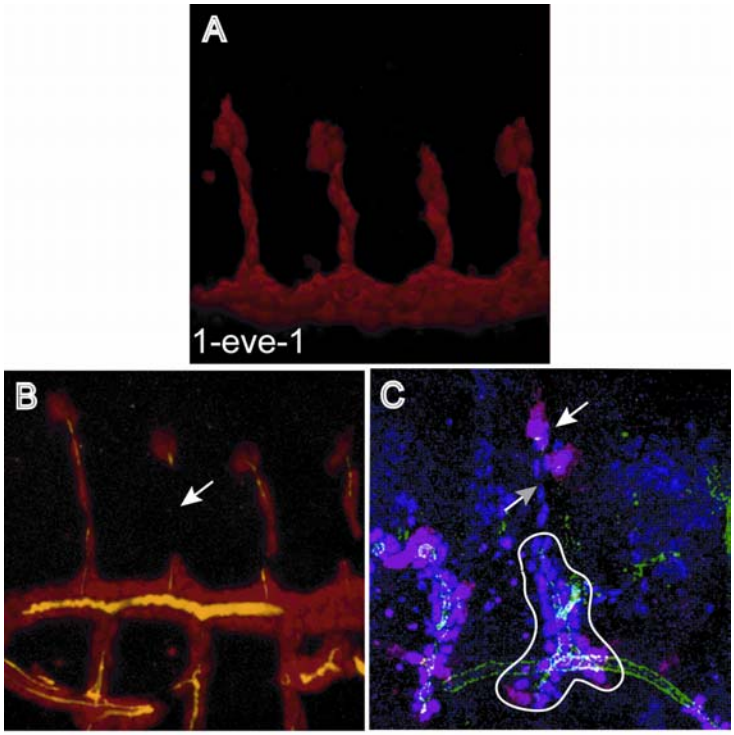

Figure 15: Bnl signalling induces the migratory response exclusively in the tip cells. (A) View on wild-type dorsal branches of a 1-eve-1 enhancer trap embryo. The trachea cells are visualized with an anti-β Galactosidase antibody (red). (B) Embryo with the genotype hsFLP; *btl*$^{H82\Delta3}$,FRT/ 1-eve-1, FRT. To induce btl mutant mosaic clones, 6h old embryos were heat-shocked for 20 min. at 37°C. The tracheal cells are visualized by anti-β Galactosidase antibodies (red) and the lumen of the tracheal system with the 2A12 marker. The arrow points on a btl mutant clone within the dorsal branch. (C) Single cell rescue experiment. Flies with the genotype btl<y+>Gal4, UAS actin-GFP/ Cyo; *btl*$^{H82\Delta3}$ / TM3 were crossed to hsFLP/ hsFLP;; UAS btl, *btl*$^{H82\Delta3}$ / TM3 flies. The 6h old progeny was heat-shocked for 30 min. at 37°C. Btl mutant show nuclear lacZ expression that was visualized by antiβ-Galactosidase antibodies (blue). Anti-Crumbs antibodies outline the apical cell surfaces (green). Single rescued cells expressing the btl transgene are labelled with anti-GFP antibodies (red). The white arrow points on two rescued tip cells pulling the btl mutant stalk cells to form a dorsal branch (grey arrow).

Recombinant lines were generated using the FRT line w;P[w+]70C, P[ry+;hs-neo;FRT] 80B and *btl*$^{H82\Delta3}$. Additional recombinants with the same FRT line and the 1-eve-1 marker were generated. (Fig.15A, view on dorsal branches). Homozygous mutant *btl*$^{H82\Delta3}$ clones were generated by FLP-mediated

recombination in heterozygous embryos of the genotype hsFLP; *btl*$^{H82\Delta3}$,FRT/ 1-eve-1, FRT. The heterozygous embryos were collected after 6h developing on grape juice plates, heat-shocked for 20 minutes at 37°C to induce mitotic recombination and further developed at 25°C for another 10h (according to the protocol in (Hacohen et al., 1998).

Additionally to βGal staining (Fig.15B, red), the tracheal system was stained with the luminal marker 2A12 (Fig.15B, green) to visualize the lumen of the homozygous *btl*$^{H82\Delta3}$ mutant cells that have lost the *btl*$^{+}$ chromosome carrying the 1-eve-1 tracheal lacZ marker.

The problem was that the *btl*$^{H82\Delta3}$ mutant cells also showed nuclear βGal staining (see chapter V) what made it very difficult to distinguish the *btl*$^{H82\Delta3}$ mutant clone cells from the *btl*$^{+}$ cells.

We screened several hsFLP; *btl*$^{H82\Delta3}$,FRT/ 1-eve-1, FRT recombinant lines for *btl* mutant clones. The results were difficult to interpret. Fig.15B shows dorsal branches of a mosaic clone embryo. Either some *btl* mutant stalk cells were not able to follow the tip cells retaining nearby the dorsal trunk (Fig.15B, arrow pointing out no βGal staining and no lumen). The other dorsal branch cells, which seemed to be *btl*+ and show cytoplasmic βGal staining migrated dorsally. These results suggest that Bnl signalling regulates cell-cell adhesion contacts which allows the epithelial stalk cells to arrange from multicellular complexes to unicellular tubes and to follow the tip cells. In the *btl* mutant stalk cells, the cell-cell adhesion contacts probably are not downregulated anymore.

Another explanation could be, that the tip cells were mutant and retained nearby the dorsal trunk unable to move (arrows). The stalk cells responded to the Bnl signal by directed cell migration compensating for the function of the tip cells and moved along the dorsal branch migratory route.

However, we could not find an answer whether only the tip cells are able to migrate or whether all tracheal cell have the capacity to move.

Marc Neumann established an experiment, which allowed to express genes of interest in single tracheal cells of *btl* mutant embryos. He generated a line with a flipout cassette allowing specific expression of a gene in tracheal cells after

heat-shock induction (btl<y+>Gal4, UAS actin-GFP/ Cyo; *btl*$^{H82\Delta3}$ / TM3). Additionally, I generated the following recombinant line hsFLP/ hsFLP;; UAS btl, *btl*$^{H82\Delta3}$ / TM3 and crossed the two described transgenic lines together. The progeny was heat-shocked after 6h at 37°C for 30 minutes and the embryos developed further at 25°C for another 10h. The FLP recombinase induced homologous recombination at the FRT sequences (symbolized here as <>) and flipped out the y+ containing cassette thereby bringing the *btl*-enhancer in proximity of the open reading frame of Gal4 and activates expression of Gal4 and the subsequent expression of Btl in some of the tracheal cells.

Interestingly, a dorsal branch grew out from a placode (surrounded by a white line) (Fig.15C). We could observe two rescued leading tip cells expressing Btl and GFP at the same time (white arrow) and the *btl* mutant stalk cells (grey arrow) were pulled by the migrating tip cells along the migratory axes and formed an unicellular branch.

This result is more convincing than the previous ones and a first indication, that induction of migration in the tip cells by Bnl signalling is sufficient to form the primary branches. The stalk cells do not require Bnl signalling and are pulled dorsally by cell-cell adhesion contacts.

Acknowledgements:

B. Müller from the lab of E. Hafen kindly provided the FRT-line: w;P[w+]70C, P[ry+;hs-neo;FRT] 80B for this experiment.

I would like to thank especially Marc Neumann for establishing the great “single cell rescue system”, for providing the line btl<y+>Gal4, UAS actin-GFP/ Cyo; btl^{LG19}/ TM3 and his collaboration to address the question in which tracheal cells FGF signalling is essential to induce cell migration.

Discussion

In this thesis we performed a functional analysis of the *Drosophila* FGF receptor homologue Breathless with the aim to identify domains specifically interacting with Dof or other putative adapter proteins that are essential to trigger the cell migration response. Furthermore, we were interested to identify downstream targets of Dof providing a direct link to the cytoskeleton.
And finally, another intention was to gain more information about the specificity of the FGFRs compared to other RTKs with regard to cell migration.
As a model system to study cell movement, we investigated the development of the tracheal system in *Drosophila melanogaster*. This respiratory organ is, after cell fate determination and few cycles of cell divisions, only established by directional cell migration, cell differentiation and cell fusion processes without any further cell divisions.
Bnl/FGF signalling has been shown to act as chemoattractand and guides the outgrowing tracheal buds (Sutherland et al., 1996). In contrast, during mouse lung development, FGF signalling not only induces chemoattraction but also proliferation of the epithelial cell sheet also at later stages (Cardoso, 2001; Warburton et al., 2000).

I. Tracheal and mesodermal cells respond to functionally distinct RTKs with directed migration

Previous experiments have already addressed signalling specificity in tracheal cell migration (Reichman-Fried et al., 1994). Chimeric receptors were generated with the extracellular and transmembrane domains of the dominant Tor (Tor^{4021}) fused to the intracellular domains of the following RTKs: Tor, EGFR and Sevenless. A minimal rescue of tracheal migration in *btl* mutant embryos under the control of an hsp70 promoter could be observed. The chimeric receptors were ubiquitously expressed and activated in all tracheal cells. Dorsal trunk formation was mainly rescued in contrast to dorsal and ganglionic branches. Lateral trunk fusion could not be rescued under these assay conditions.

Experiments have shown that overexpression of Bnl or an activated form of Btl (λ-Btl) in all tracheal cells disrupted or even completely impaired primary branch outgrowth (see chapterVI,3, (Lee et al., 1996; Sutherland et al., 1996).
Since local activation of Btl by the spatially restricted ligand Bnl is essential for guiding the tracheal cells in six stereotyped directions, we thought to generate chimeric receptor proteins with the extracellular and transmembrane domain of Btl. Thereby we ensured that the transmembrane receptor proteins were activated at the correct time and in the correct local position mimicking wild type situation as closely as possible.
Interestingly, we found that a chimeric receptor with the extracellular domain of Btl and the intracellular domain of Tor could functionally substitute for wild-type Btl and almost completely rescued tracheal cell migration in *btl* mutant embryos. Also the chimeric Btl-EGFR could rescue tracheal cell migration, albeit less efficiently.

II. Dof acts specifically in the FGF signalling pathway

We were interested whether the previously identified adapter protein Dof is specifically required to trigger the migratory response and whether it interacts with distinct RTKs. In *dof* mutant embryos, tracheal and mesodermal cell migration is completely impaired. Surprisingly, the chimeric Btl-Tor and Btl-EGFR were able to rescue cell migration in the absence of Btl and Dof. In addition, both fusion receptors efficiently activated the transcriptional targets of the tracheal secondary and terminal cell differentiation program.
The results were confirmed by S2 cell culture assays, which showed that Dof is exclusively a phospho-target of FGFRs and is not a substrate of Tor (Petit et al., 2004).
These results demonstrated that tracheal and mesodermal cells respond to RTK signalling with directed cell migration and the induction of the differentiation program in the absence or presence of Dof. Thus, Dof acts specifically in the FGF signalling pathway (Dossenbach et al., 2001; Vincent et al., 1998).

Interestingly, in another model system used to study cell migration in *Drosophila*, namely border cell migration during oogenesis, guidance seems to be receptor specific. The border cells are specified somatic follicle cells that migrate through the nurse cells toward the oocyte. They turn at the oocyte and continue to migrate dorsally. EGFR signalling guides the border cells dorsally and interestingly, this migration is EGFR specific and not dependent on MAPK activation. In contrast to our results, an activated form of Htl could not substitute for the function of the EGFR (Duchek and Rorth, 2001).

1. Dof provides a functional homologue of FRS2 in *Drosophila*

The question rose whether RTKs produce an intrinsic generic signal differently interpreted by different cell types. We wanted to investigate whether conserved components of RTK signalling as for example Ras, Raf and the MAPK are putative inducers of the migratory response or whether FGF signalling specific components as for example Dof (or the functional adapter homologue FRS2 in vertebrates) provide the essential link to the cytoskeleton.

Dof mutant embryos show strong tracheal and mesodermal migration defects. Also FRS2$\alpha^{-/-}$ knockout mice die at early embryonic stages because of severe gastrulation migratory defects (Hadari et al., 2001).

Although there is no sequence homology between the vertebrate and the *Drosophila* adapter protein and they contain different protein-protein interaction domains, they can be considered as functional homologues. Both adapters recruit the Grb2/SOS complexes thereby providing a link to the MAPK cascade and subsequent transcriptional activation. In addition, both recruit the phosphatase Shp2/Csw upon tyrosine-phosphorylation of FGFRs and activate the PI3K pathway (Petit et al., 2004; (Hadari et al., 2001; Xu and Goldfarb, 2001; Xu et al., 1998).

Dof belongs to a small family of proteins including BCAP and BANK, two vertebrate scaffolding proteins that have recently been identified (Okada et al., 2000; Yokoyama et al., 2002). However, unlike Dof they are not implicated in FGF signalling, but in downstream signalling of the B-cell antigen receptor. Both proteins regulate the release of calcium within B cells. BANK interacts directly

with the IP-3 receptor, whilst BCAP is thought to be an adapter protein that mediates the activation of the PI3K and phospholipase Cγ2 upon B-cell antigen receptor stimulation. Thus, Dof, BCAP and BANK are all involved in signalling, although they appear to have undergone rapid change during the course of evolution and may have evolved in parallel with FRS2.

Our in vivo experiments have shown that Dof partially substituted for the function of FRS2 when a chimeric Btl-FGFR2 receptor was expressed in *btl* mutant embryos. In fact, we observed a quite effective rescue of cell migration. Interestingly, this chimeric receptor is not able to trigger cell migration anymore in the absence of Dof, but still induces transcriptional activation via the MAPK cascade. In contrast to Btl and Htl, which are absolutely dependent on Dof to induce cell migration but also to activate transcriptional targets, FGFR2 seems to be able to interact with other adapter proteins in the absence of Dof to activate transcription. Shc provides a good candidate to link the FGFR2 to the downstream MAPK cascade, since it has been shown to recruit the Grb2/SOS complex upon phosphorylation by RTKs (Luschnig et al., 2000).

We assume that in the opposite situation, FRS2 is not able to interact with Btl to fulfil the function of Dof, because the sequence homology of the juxtamembrane domains of Btl and the FGFR2 is poor. FRS2 binds with its PTB domain to a highly conserved stretch of amino acids within the vertebrate FGFRs containing the differentially spliced VT motif. Btl lacks this essential VT motif. Thus, it does not provide a binding site for FRS2.

In our cell culture assay, we observed neither phosphorylation of FRS2 by an activated version of FGFR2 nor complex formation between FRS2 with FGFR2. Furthermore, Dof became not phosphorylated upon FGFR2 activity and an activated version of Btl did not phosphorylate FRS2.

This experiments should be repeated in mammalian cells, as for example Cos cells. In mammalian cells, additional components might be provided for a stable constitutive interaction of FRS2 with the FGFRs.

Co-immunoprecipitation experiments in different mammalian cell lines have shown that complex formation between the FGFRs and FRS2 was weak or

could not be detected at all depending on the cell line (Cross et al., 2002; Ong et al., 2000b).

2. Dof probably interacts constitutively with the kinase domain of Btl

Our co-immunoprecipitation experiments have shown that Dof interacts independent of the activity state of Btl with the kinase domain of the receptor (see chapter V,1-3). Furthermore, yeast two hybrid assays proposed that Dof interacts with the C-terminal-lobe of the kinase domain (Battersby et al., 2003).
A conserved domain within the N-terminus of Dof is required for the interaction with the receptor (Petit et al., 2004; (Wilson et al., 2004).
The functional deletion analysis of Btl is in line with these results. Deletion of all putative interaction domains for adapter proteins, either the juxtamembrane domain, the kinase insert domain or the C-terminus of Btl, did not affect the capacity of Btl to rescue tracheal cell migration in *btl* mutant embryos and terminal cell fate differentiation was induced.
To define the exact sequence stretch of the interaction domain Btl with Dof, point mutations have to be introduced within the C-terminal lobe of the kinase domain without affecting the essential and highly conserved autophosphorylation sites required for the kinase activity.
The S2 cell culture assays revealed that Dof is able to form a complex with Btl and becomes phosphorylated upon Btl activation. Also receptors either lacking the juxtamembrane domain or the kinase insert domain phosphorylated Dof. Surprisingly, in S2 cells, Btl receptors lacking the C-terminus did not auto-phosphorylate. Consequently, we did not observe any phosphorylation of Dof. We assume, that the short C-terminus is required for conformational changes of the kinase activation loop upon dimerization of the receptors to enable trans-phosphorylation. In vivo, probably a mechanism or chaperone proteins exist substituting for the function of the C-terminus. The S2 cells apparently lack this mechanism or proteins.

III. Csw provides the essential link to the cytoskeleton

Finding that mutations in *csw* produce the same tracheal phenotype, as *bnl*, *btl* and *dof* embryos, in which tracheal cell migration is completely blocked, indicate an essential role of Csw in the induction of cell locomotion (Perkins et al., 1992). Also the vertebrate homologue Shp2 has been shown to be essential for EMT at initial steps of gastrulation, which includes cellular differentiation processes as cell shape changes, adhesion or migration (Saxton and Pawson, 1999).

In addition, Shp2 was shown to be required for tubulogenesis by another chemotactic factor, the Hepatocyte Growth Factor/ Scatter Factor (HGF/SF). HGF signalling has been implicated in stimulating tubulogenesis during angiogenesis and during the development of the lung. Shp2 regulated cytoskeletal structures and cellular adhesion, inhibited the Rho GTPase and promoted cellular motility downstream of Met, the RTK activated by HGF. Further experiments have shown that Shp2 was essential for sustained activation of the MAPK and for the induction of branching morphogenesis of MDCK cells. Interestingly, sustained activation of the MAPK through different adapters (Grb2, Shc, or Shp2) was not required for cellular motility (compare also to chapter VI,3; reviewed in (Rosario and Bircmeier, 2003).

The group of L. Perkins already performed a yeast two-hybrid screen to identify proteins potentially interacting with Csw. They used the C-terminus of Csw as bait and screened *Drosophila* ovary and imaginal disc yeast fusion libraries. Thereby they identified the *Drosophila* homologue of Importin-7 (DIM-7). DIM-7 associated with phosphorylated D-ERK and was required for the nuclear import of D-ERK (Lorenzen et al., 2001).

Unfortunately, L. Perkins did not publish other Csw interacting factors. It would be interesting to know whether one of the 22 Rho family GEFs, identified in the *Drosophila* genome, interact with Csw. It has been shown that Rac1, Rac2 and Mtl have largely overlapping functions and probably share a common set of effectors. Thus, the activators of the Rac proteins (RacGEFs) seem to be essential for a limited activation of the Racs resulting in a specific cellular response (Hakeda-Suzuki et al., 2002).

We assume that Csw recognizes and recruits activated and phosphorylated proteins and subsequently dephosphorylates these proteins to transduce a specific cellular response.

Co-immunoprecipitation experiments or GST pull-downs in the S2 cell culture system would provide more information about complex formation of Csw upon Btl activation with different candidate interaction partners. An interaction between the phosphorylated version of Dof and Csw has already been shown by co-immunoprecipitation experiments (Petit et al., 2004).

Another possibility to identify new Csw interaction partners involved in cell motility would be to use S2 cells in a different context. Under routine culture conditions, S2 cells display a spherical morphology and are not motile. The cells undergo a dramatic change of morphology when they are plated on glass coverslips coated with the lectin concanavalin A by forming actin containing membrane ruffles. Some cells do not possess this kind of lamella but rather possess dynamic filopodia.

RNAi studies were performed to identify proteins involved in lamella formation. 19 proteins were identified as candidates and implicated in aspects of actin function or in cell motility. Most candidates were direct actin-binding proteins, like Profilin, CAP and Cofilin. Also the small GTPase Cdc42 was identified to prevent normal lamella formation when inhibited by RNAi. Interestingly, Rac1, Rac2 and Mtl did not prevent lamella formation. Only when additionally the SH2-SH3 adapter protein Nck was inhibited, lamella defects could be observed indicating that Rac-like proteins and Nck were partially redundant in activating the WASP/SCAR complex (Rogers et al., 2003).

Slingshot, a phosphatase that activates the actin severing activity of Cofilin could be another interesting candidate to test.

If proper cues are provided to S2 cells, probably other aspects of the actin cytoskeleton such as filopodia formation, cell migration and cell polarity could be investigated.

Other candidates that would be interesting to investigate have been proposed by the following experiments in mammals. Experiments with isolated fibroblasts of $Shp2^{-/-}$ mutant mice have shown that Shp2 promoted Src family kinases

(SFK) activation by an indirect mechanism. Shp2 regulated the phosphorylation of the Csk regulator PAG/Cbp, thereby controlling Csk access to SFKs. SFKs have been shown to activate the p190RhoGAP subsequently providing a link to stress fibres and focal adhesion regulation.

Impaired spreading of $Shp2^{-/-}$ cells might also reflect hyperphosphorylation of Vav2, a Rho GTPase GEF that has been shown to be recruited to the tips of filopodia in living cells (Kranewitter et al., 2001). It would be interesting to investigate whether *Drosophila* homologues of SFKs, Csk, PAG/Cbp, p190RhoGAP and Vav2 exist.

IV. Ras is not sufficient to elicit the migratory response

Experiments have shown that recruitment of Csw via Dof is essential for the activation of the Ras/MAPK pathway (Petit et al., 2004). We investigated whether activation of Ras in the tracheal tip cells is sufficient to trigger the migratory response upon Bnl signalling. The results we obtained make us believe that Ras activation is required but not sufficient to activate this response.

Expression of a constitutive active form of Ras (RasV12) specifically in tracheal cells of *btl* and *dof* mutant embryos partially rescued primary branch outgrowth, mainly dorsal trunk fusion occurred. Other experiments have shown that expression of RasV12 in mesodermal cells of *htl* mutant embryos resulted in a certain rescue of mesodermal cell migration (chapterVI,1 and (Michelson et al., 1998a).

However, when we tried to disturb cell migration under conditions, where all tracheal cells of wild-type embryos sustained high levels of Ras activation by expressing RasV12, tracheal cell migration and primary branch formation were not affected (chapterVI,1-3). Also when the chimeric receptor Btl-Tor was expressed in the tracheal cells, the Ras/MAPK pathway got hyper-activated, but directional outgrowth of the tracheal branches was normal (chapterVI,3).

Additional experiments of Marc Neumann indicated that Ras was not sufficient to regulate cytoskeletal reorganizations. He could show that overexpression of RasV12 in wild type embryos did not produce ectopic filopodia in dorsal trunk cells.

In sharp contrast, ectopic activation of Bnl or λBtl, a constitutive active version of Btl, completely impaired tracheal cell migration and ectopic filopodia formation could be observed (chapterVI,3 and Marc Neumann).

Also during border cell migration in oogenesis, expression of RasV12 or RasN17 in the border cells only moderately affected their migration (Duchek and Rorth, 2001).

1. Local activation of Raf

We were interested to study whether spatial correct activation of the Ras/MAPK cascade would be sufficient to fully rescue tracheal cell migration in *btl* or *dof* mutant embryos. Experimentally, it is difficult to activate the small GTPase Ras at the correct local position.

Thus, we tried to activate the downstream target of Ras, the serine-threonine kinase Raf, at the correct local position of the outgrowing branches by generating a chimeric Btl-Raf protein. The kinase domain of Raf was fused to the extracellular domain of Btl. Unfortunately, we were not able to mimic the physiological more relevant situation activating the construct by the spatially restricted ligand Bnl because the chimeric Btl-Raf construct auto-activated itself even in cells that did not receive Bnl.

Indeed, previous experiments have shown that expression of both, Tor-Raf and Tor^{4021}-Raf fusion constructs in the eye resulted in a dominant rough eye phenotype indicating that even in absence of a ligand, the Tor-Raf fusion protein was constitutive active. In the eye, Tor-Raf activation was sufficient to rescue the R7 precursors from adopting the cone cell fate in the absence of *sev* function (Dickson et al., 1992).

Further evidence that even local activation of Ras is not sufficient to induce a migratory response has been provided by Valérie Petit. She generated a minimal Dof (Dof1-600) construct lacking the ankyrin-repeats. This truncated

Dof construct was not able to rescue cell migration anymore in contrast to the minimal Dof protein (Dof1-600) in *dof* mutant embryos but it still activated Ras and downstream transcriptional targets (Petit et al., 2004).

2. Drac and Dcdc42 are involved in the regulation of actin dynamics

The small GTPases of the Rho family have been shown to regulate cytoskeletal rearrangements. Unfortunately, it is very difficult to address genetically the importance of the small GTPases. Therefore, we precisely examined the effect of constitutive active and dominant negative versions of these proteins. Additionally, we made use of the Rac mutations (*rac1J10, Δrac2, Δmtl*) generated by the group of B. Dickson (Hakeda-Suzuki et al., 2002) and investigated the tracheal development of mutant germline clones. Unfortunately, we could not draw any conclusions from these experiments, since triple *rac* mutant embryos stopped developing at blastodermal stage. Combinations of double *rac* mutant embryos (either *rac1J10,Δrac2* or *rac1J10,Δmtl*) also lacked development of a tracheal system probably due to severe defects raised already during early embryonic development.

In contrast, genetic analysis of *cdc42* mutant embryos by generation of heterozygous embryos carrying a lethal *cdc42* allele over a *cdc42* viable allele, clearly showed strong defects in primary branch outgrowth.

According to the analysis of the effects of activated and dominant negative versions of Rac expressed in wild-type embryos, Drac has been proposed to downregulate cell-cell adhesion contacts and to regulate directional actin dynamics probably in collaboration with Dcdc42.

None of the small GTPases of the Rho family rescued cell migration in *dof* mutant embryos probably due to the fact that at least two or three members of the small GTPases are essential to rescue cell migration.

V. Mitotic clones to identify new targets involved in cell migration

Previous conclusions have suggested that Shp2/Csw is the key molecule in FGF signalling regulating directed cell migration and tubulogenesis. Additional downstream targets of Csw have to be identified as it has been outlined before. We assume, that these downstream targets activate the small GTPases and regulate subsequent cytoskeletal reorganizations.

Since most of the potential downstream Csw candidates identified by screens so far are maternally contributed and essential during oogenesis or early embryonic development, we tried to establish a genetic mosaic analysis using the FLP recombination system to test potential downstream candidates of Csw.

1. Single mutant tracheal cells

We wanted to analyze single mutant tracheal cells and the effect of these mutant cells on their neighbours with regard to migration.

First, we investigated the effect of single *btl* mutant cells and their environment to establish the experiment. We addressed the following questions: Is primary branch outgrowth completely blocked when the tip cells lack Btl? Are *btl* mutant stalk cells pulled by cell-cell adhesion contacts by the wild-type tip cells or do they stop moving?

To answer these questions, single mutant *btl* cells were generated as it was already described by the group of M.A. Krasnow (Hacohen et al., 1998). $Spry^{-/-}$ mosaic mutant cells were performed in the tracheal system and the mutant cells were recognized by negatively labelling the cells. The mutant cells lost the tracheal cell marker 1-eve-1 lacZ upon mitotic recombination (Hacohen et al., 1998).

The problem with this approach was that the tracheal cells undergo only about two cycles of cell divisions previous to invagination, and we observed only single cell clones that hardly could be recognized by negative labelling, since they still showed some lacZ expression due to the $btl^{H82\Delta 3}$ allele (imprecise

excision of the P-element containing lacZ). Therefore we were not able to draw any conclusions from this experiment.

The system would also not be ideal to investigate candidate genes acting downstream of Csw. Despite the fact that the single mutant cell clones should not show any lacZ expression anymore, it is difficult to visualize negatively labelled clones.

Consequently, we thought to label the single mutant cells positively.

A very nice system was developed to label mitotic clones positively, the MARCM system (described in detail in (Lee and Luo, 1999). Gal80 antagonizes Gal4 activity thereby repressing the expression of the marker protein with UAS sequences introduced upstream of the gene. Only mitotic clones loosing Gal80 induce the positive marker (GFP for example) upon activation by Gal4. At the same time, these clones are homozygous mutant for the gene of interest.

Unfortunately, the MARCM system cannot be used to induce mitotic clones in the tracheal system. The Gal80 protein expressed under the control of a tubulin-promoter for example is stable for 48h until it gets degraded in the cells. Since tracheal development starts from 6h after egg lying and is finished after 18h, the Gal80 protein is still present even in cells that have lost the gene due to mitotic recombination.

Since the MARCM system is not feasible in the embryonic tracheal system, again, we had to find another system to induce mitotic clones. A very promising system to identify downstream components of Csw and candidates of directional cell motility are the air sacs in larvae (Sato and Kornberg, 2002).

Clemens Cabernard established this project.

2. Larval air sac development is promising to identify regulators of cell migration

In the developing air sacs adepithelial cells with tracheal identity (tracheoblasts) extended long cytoneme-like filopodia, migrated and proliferated in response to Bnl. At third larval instar, *btl* expressing adepithelial cells budded from the tracheal branch, increased in cell number and expanded posterior toward the region of greatest *bnl* expression.

In contrast to the tracheal cells during embryonic development, the tracheoblast during air sac formation in larvae undergo several cycles of cell division, therefore providing better conditions to induce mitotic clones by the MARCM system. Additionally, we assume that air sacs not only develop by cell proliferation but also by migration of the cells. Sato and Kornberg showed in their experiments that indeed, the tracheoblast extend long filopodia extensions toward ectopic Bnl expressing clones in the wing disc.

From vertebrate FGF signalling we know that FGFs not only induced migratory responses but also mitogenic responses. We do not know whether in air sac development Bnl signalling induces both, the mitogenic and the migratory response. Dissecting the two downstream cascades remains challenging.

This approach is the most effective way to identify putative interacting molecules of Csw to provide the link to the small GTPases and other direct actin binding molecules.

First, *btl, dof* and *csw* mutant clones should be generated as control. Further candidates to test are Ras, Raf and Rolled, the small GTPases and actin binding proteins, which are also strong maternally contributed. Also interesting candidates to investigate would be Shc, the *Drosophila* homologues of FAK and Src.

Using the NCBI Blast program one putative *Drosophila* homologue of FAK, the FAK-like protein CG10023 that shows 38% sequence identity to the vertebrate FAK, could be identified. Another very interesting protein to test is the *Drosophila* Src42A that has been shown to genetically interact with Csw, Ras, Pole hole (Raf), Dsor1 (MEK) and SOS. Unfortunately the Blast results did not show any homologue of the vertebrate adapter protein Shb that has been shown to form a complex with Shp2, Src and FAK in mammalian cells.

And finally, the p21-activated kinase (Pak) that is known to act as a mediator of the activity of the Rac GTPase has to be tested. In *pak* mutants, similar defects has been described as those to zygotically null *rac1,2* mutants (Chihara et al., 2003).

3. Single cell rescue experiment using the flipout system

Marc Neumann established another approach to test signalling components and their ability to rescue migration of single cells within the tracheal system.
By using the flipout system (described in detail in (Zecca et al., 1995) we can try to rescue single cell migration in *btl* mutant embryos by expressing the genes of interest (UAS X) and actin-GFP (UAS actin-GFP) under the control of a btl<y+>Gal4 enhancer induced by hsFLP.
The preliminary results, which showed two single tip cells expressing Btl in a *btl* mutant background were a first indication that Bnl signalling induced the potential to migrate exclusively in the tracheal tip cells. The *btl* mutant stalk cells followed the rescued tip cells, intercalated and formed an unicellular dorsal branch. Furthermore, these results indicate that Bnl signalling is not required to regulate the rearrangement of the cell-cell adhesion contacts of the epithelial stalk cells.
Drac that has been shown to regulate cell-cell adhesion is probably not a downstream target of Bnl signalling as it was proposed by the group of S. Hayashi (Chihara et al., 2003). Bnl signalling also does not induce cytoskeletal reorganizations within the stalk cells and they seem to be pulled only by cell-cell adhesion forces rearranged by the leading tip cells.
In the tip cells, Bnl signalling might induce the migratory response by regulating Drac in collaboration with Dcdc42 to control F-actin reorganizations and subsequent formation of filopodia. The experiments of Marc Neumann have shown that the activated version of Rac (RacV12) induced non-orientated ectopic filopodia also in dorsal trunk cells. Cells expressing RacL89 showed ectopic filopodia in the dorsal branches and F-actin was visible all over the cells. In triple mutant *rac1J10, Δrac2, Δmtl* embryos, the embryos showed dorsal closure defects and lacked both lamellipodia and filopodia formation (Hakeda-Suzuki et al., 2002). These are further indications that Rac and Cdc42 play a role in regulating the formation of actin containing filopodia.

VI. Concluding remarks

We strongly assume that spatially and timely limited induction of FGF signalling in the tracheal tip cells is required for the formation of orientated filopodia to sense the guidance cue Bnl but also to reorganize the cytoskeleton of the cell body in order to move toward the guidance cue. This possibility is strengthened by the observation that Dof, a protein essential for tracheal cell migration, is indeed observed to be expressed stronger in the tip cells (Vincent et al., 1998). In addition, transcription of *btl* gets restricted to the tip cells during tracheal development (Ohshiro et al., 2002).

Dof links the FGFR to the downstream conserved MAPK cascade and transcriptional activation, but triggers additional cytoplasmic signalling events by recruiting Csw. Csw has been shown to activate Ras and transcriptional targets but it also elicits the migratory response. Downstream effectors of Ras seem to be required but not sufficient to induce cell migration.

Using the larval air sac system, we hope to identify soon new components essential for cell migration. Additionally, with this system, we can confirm whether the small GTPases Rac and Cdc42 indeed regulate the actin cytoskeleton of tracheal cells. Furthermore, we are able to identify downstream targets of Csw and upstream effectors of the small GTPases.

The identification of new components of FGF signalling regulating tracheal cell migration in *Drosophila* is challenging because it could help to identify new drug targets in humans against cancerogenic metastasis formation or tumour angiogenesis. Several FGFs were discovered because of their oncogenic potential.

Except the adapter proteins FRS2 and Dof, which show only functional homologies, other components of the FGF signalling pathway are highly conserved within the animal kingdom. Albeit, different migrating cell types may use different combinations of these proteins. Dissection and understanding of the *Drosophila* FGF signalling pathway in detail could provide ideas which molecules are essential linkers to the actin cytoskeleton. Furthermore, it is essential to distinguish the different downstream cellular responses elicited by

FGF, if we want to generate effective antagonists blocking the FGF signal in diverse pathogenic situations.

Materials and Methods

I. *Drosophila* strains and genetics

The generation of the transgenic UAS btl, UAS btl-htl, UAS btl-tor, UAS btl-EGFR, UAS htl, UAS htl-btl, UAS htl-tor and UAS htl-EGFR flies was described in detail in (Dossenbach et al., 2001; Vincent et al., 1998). The original UAS btl cDNA was kindly provided by B. Shilo and modified by C. Dossenbach. The UAS btl derivates (including the UAS btl deletion versions) were introduced into the *Drosophila* genome by P-element-mediated germline transformation (Rubin and Spradling, 1982). At least four independent transformant lines were analysed for each construct.

The *btl*-Gal4 line kindly provided by S. Hayashi (Shiga et al., 1996) was used to drive the UAS transgenes specifically in tracheal cells, the *twist*-Gal4 line (obtained from M. Akam) was used to drive expression in mesodermal cells.

The $btl^{H82\Delta 3}$ null allele was generated by imprecise excision of the lacZ-containing P-element inserted upstream of the *btl* locus. Homozygous $btl^{H82\Delta 3}$ mutant embryos did not show any *btl* transcription but retained βGal expression in the tracheal and midline cells (Reichman-Fried et al., 1994). The *dof* mutant (dof^{P1740}) is a lethal P-element enhancer trap insertion in 88C (Vincent et al., 1998). Using the mentioned *btl* and *dof* alleles, a *btl,dof* double mutant chromosome was generated. Bnl^{P1} contains a P[lacZ,ry+] element and corresponds to the line l(3)0857 from A. Spradling (Sutherland et al., 1996). For the crosses, we additionally used the following marker lines: +/+; 73B/ TM2 Blue[69] (provided by M. Bienz) and L/Cyo; rf10/ TM3 (Ubx lacZ) (a gift from L. Keegan).

Thomas B. Kornberg kindly provided the UAS btl-gfp line (Sato and Kornberg, 2002). The group of E. Hafen provided the Ras effector mutant lines (Halfar et al., 2001; Rommel and Hafen, 1998). B. Dickson kindly provided the cDNA of Draf to generate the UAS btl-raf construct. The UAS torso^{4021}-btl (UASbtl^{act}) flies were provided by S. Leevers and E. Hafen (derived from the hs-torso^{4021}-

FGFR1 construct described in (Reichman-Fried et al., 1994)). UAS bnl was kindly provided by the group of M. Krasnow to ectopically activate FGF signalling. The *trachealess* enhancer trap line 1-eve-1 was used to visualize the tracheal cells (Perrimon et al., 1991). UAS rasV12 was provided by E. Hafen and N. Perrimon. R. Fehon kindly provided the *cdc42* mutant alleles (Fehon et al., 1997; Genova et al., 2000) and S. Hakeda-Suzuki provided all *rac* mutant alleles (Hakeda-Suzuki et al., 2002). The following lines are listed in the stocks of U. Nussbaumer without further references: #218 UAS dcdc42N17,3, #478 UAS dracV12,1, #479 UAS dcdc42V12,2, #480 UAS dracL89. UAS rhoV14 and UAS rhoN19 were kindly provided by M. Mlodzik and UAS mtlV15 and UAS mtlN20 by B. Dickson. J. Xu and M. Goldfarb kindly provided the cDNAs of hFGFR2, hFGFR1 and FRS2 (Ong et al., 2000a; Xu et al., 1998). B. Müller from the lab of E. Hafen kindly provided the FRT-line: w;P[w+]70C, P[ry+;hs-neo;FRT] 80B to establish *btl* mutant mosaic clones. And finally, M. Neumann kindly provided the line btl<y+>Gal4, UAS actin-GFP/ Cyo; $btl^{H82\Delta3}$/ TM3 for the single cell rescue experiment.

II. Rescue assays

To test whether the 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 Btl derivates (UAS c; 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 +/ +; $btl^{H82\Delta3}$/ TM2 (Blue[69]) flies. Subsequently, *btl*-Gal4/ +; $btl^{H82\Delta3}$/ TM3 (Ubx *lacZ*) and UAS c/ +; $btl^{H82\Delta3}$/ TM3 (Ubx *lacZ*) flies were crossed and the progeny was 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 $btl^{H82\Delta3}$ mutant embryos. The crosses were repeated using a *btl*,*dof* chromosome +/ +; $btl^{H82\Delta3}$,dof^{P1740}/ TM2 (Blue[69]) in order to test whether the constructs were able to rescue

tracheal defects in *btl,dof* double mutant embryos under the control of the *btl*-Gal4 driver.

We tested the mesodermal rescue capacity of the UAS constructs following the above described strategy. To test whether mesodermal defects could also be rescued in *dof* mutant embryos, we crossed dof^{P1740} mutant flies to either UAS gene c transgenic flies or to *twist*-Gal4 transgenic flies: UAS c/ +; dof^{P1740}/ + flies were further crossed to *twist*-Gal4/ +; dof^{P1740}/ + flies as it was described previously in (Vincent et al., 1998). Embryos were collected at 25°C for 15 hours. Homozygous dof^{P1740} mutant embryos were identified either by the lack of Eve-expressing cells at the dorsal midline, which indicate defects in mesoderm spreading (*btl*-Gal4 crosses), or by the lack of tracheal cell migration (*twi*-Gal4 crosses). Rescue of mesodermal cell migration was scored with the Evenskipped antibody (see main text).

1. Schematic representation of the crosses

IA)

btl GAL-4 + L rf10 btl GAL-4 +
_____;_____ X _____;_____ → _____;_____
btl GAL-4 + Cyo TM3 (Ubx lacZ) Cyo TM3 (Ubx lacZ)

IB)

UAS c + L rf10 UAS c +
_____;_____ X _____;_____ → _____;_____
UAS c + Cyo TM3 (Ubx lacZ) Cyo TM3 (Ubx lacZ)

IIA)

btl GAL-4 + + $btl^{H82\Delta3}$ btl GAL-4 $btl^{H82\Delta3}$
_____;_____ X _____;_____ → _____;_____
Cyo TM3 (Ubx lacZ) + TM2 $Blue^{69}$ + TM3 (Ubx lacZ)

IIB)

UAS c + + $btl^{H82\Delta3}$ UAS c $btl^{H82\Delta3}$
_____;_____ X _____;_____ → _____;_____
Cyo TM3 (Ubx lacZ) + TM2 $Blue^{69}$ + TM3 (Ubx lacZ)

III)

UAS c $btl^{H82\Delta3}$ / + TM3 (Ubx lacZ) X btl GAL-4 $btl^{H82\Delta3}$ / + TM3 (Ubx lacZ) → **UAS c $btl^{H82\Delta3}$ / btl GAL-4 $btl^{H82\Delta3}$**

UAS c dof^{P1740} / + + X btl GAL-4 dof^{P1740} / + + → **btl GAL-4 dof^{P1740} / UAS c dof^{P1740}**

III. Genetic mosaic analysis using the FLP/FRT system

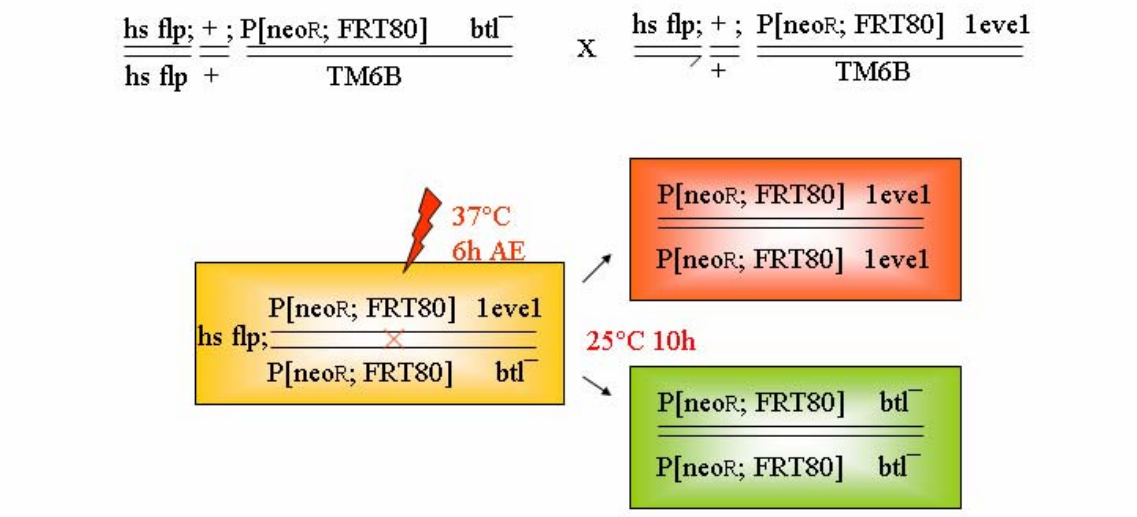

Figure 1: The genetic mosaic analysis using the FLP recombination system was carried out as it was already performed for *spry*$^{-/-}$ mosaic mutant cells in the tracheal system (Hacohen et al., 1998). Instead of *spry*$^{-/-}$, *btl*$^{H82\Delta3}$ was recombined onto the FRT80 chromosome.

The genetic mosaic analysis using the FLP recombination system was carried out as it was already performed for *spry*$^{-/-}$ mosaic mutant cells in the tracheal system (Hacohen et al., 1998).

The FLP recombination system induces high frequency mosaicism for a particular gene by the use of the FRT sequence from yeast. The FLP recombinase induces mitotic recombination between the FRT sites located on homologous chromosome arms. The FRT sites have to be located closer to the centromere than the gene of interest (Xu and Rubin, 1993)

Recombinant lines were generated using the FRT line w;P[w+]70C, P[ry+;hs-neo;FRT] 80B and *btl*$^{H82\Delta3}$. Additional recombinants using the same FRT line and the 1-eve-1 marker were generated. Flies carrying the P[ry+; hs-neo, FRT] element were selected by their resistance to G418 (Geneticin, GIBCO laboratories). G418-containing medium was made as follows: a few holes were made in the fly medium with toothpicks that was previously heated up in the microwave for a few seconds, and 0.2-0.3ml of 25mg/ml freshly prepared G418 solution was added and mixed. The vials were allowed to dry over night at 18°C.

Additionally, the progeny of the recombinant stocks were stained with an anti β-Gal antibody to visualize the remaining nuclear β-Gal staining of the *btl*$^{H82\Delta3}$ allele and the cytoplasmic β-Gal of the enhancer trap line 1-eve-1. Homozygous mutant *btl*$^{H82\Delta3}$ clones were generated by FLP-mediated recombination in heterozygous embryos of the genotype hsFLP; *btl*$^{H82\Delta3}$,FRT/ 1-eve-1, FRT. The heterozygous embryos were collected after 6h developing on grape juice plates, heat-shocked for 20 minutes at 37°C to induce mitotic recombination and further developed at 25°C for another 10h (according to the protocol in (Hacohen et al., 1998).

IV. Rac germline clones

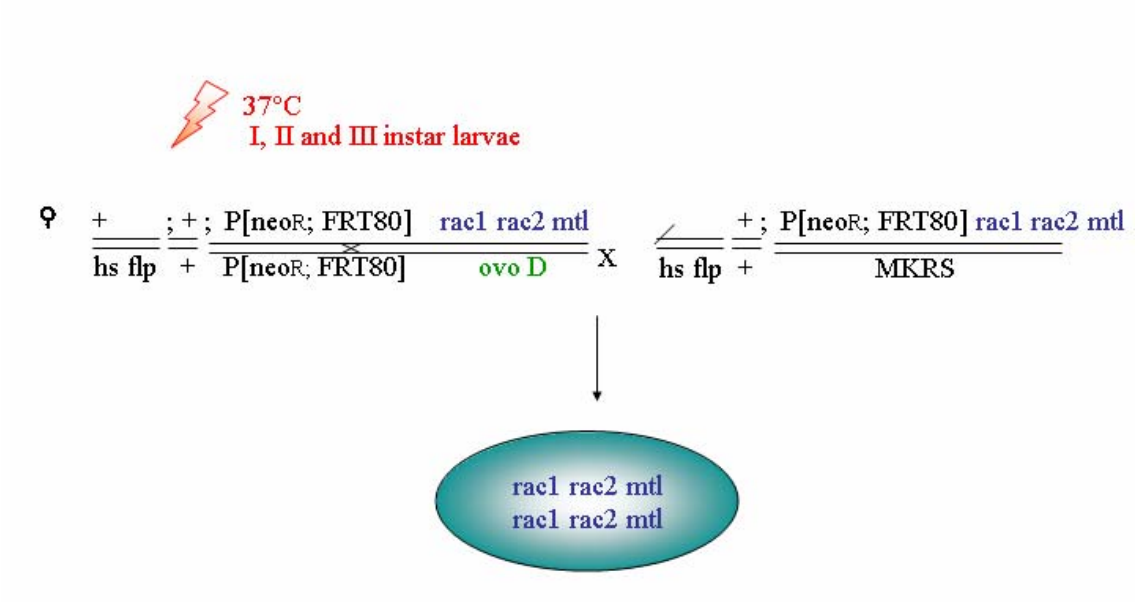

Figure 2: **Schematic representation of the cross to generate germline clones, which are triple mutant for *rac1,rac2 and mtl*.** Mutant females (Δ*racs*, FRT/ TM6 y+) were crossed to hs FLP; ovo^{D} / MKRS males. The progeny larvae of the age 24h, 48h, 72h were heat-shocked for 1-1,5h at 37°C and the larvae further developed at 25°C until adult stage. The developing heat-shocked virgins were collected and crossed back to heterozygous *rac* mutant males from the original stocks (see Fig.2). Eggs from the females carrying the mutation ovo^{D} do not develop (Pauli et al., 1995).

As described in the papers (Hakeda-Suzuki et al., 2002; Xu and Rubin, 1993), germline clones were generated with the mutant lines *rac1J11, rac1J10, ⊿rac2, ⊿mtl* in different combinations (single, double or triple *rac* mutant embryos). Mutant females (Δ*racs*, FRT/ TM6 y+) were crossed to hs FLP; ovo^{D} / MKRS males. The progeny larvae of the age 24h, 48h, 72h were heat-shocked for 1-1,5h at 37°C and the larvae further developed at 25°C until adult stage. The developing heat-shocked virgins were collected and crossed back to heterozygous *rac* mutant males from the original stocks (see Fig.2). Eggs from the females carrying the mutation ovo^{D} do not develop (Pauli et al., 1995).

V. Immunohistochemistry

1. Embryo collection and fixation

Embryos were collected overnight at 25°C for approximately 16h on grape juice plates. After collection and dechorionation in 4% chlorax solution they were washed with 120mM NaCl, 0.03% TritonX-100 solution and subsequently fixed in 9.6% formaldehyde/heptan solution (1:1) for 15 minutes. The formaldehyde solution was replaced by 700µl MeOH and the embryos were heavily shacked for 2 min. to remove the viteline membrane. Finally, after removing the Heptane solution, the embryos were washed with MeOH several times and stored in MeOH at -20°C.

2. Protocol of fluorescent antibody stainings

After replacing the MeOH with PBST (1,3M NaCl, 0.03M Di-Natriumhydrogenphosphat, 10% Tween-20), the embryos were blocked with

400µl 10% BSA in PBST for 45 min. on the slow rotor. In PBST diluted 1° antibody was added and incubated 2h at room temperature or at 4°C overnight. After washing the embryos 3x 15 min. with PBST, in PBST diluted 2° antibody (Peroxidase conjugated antibody usually diluted 1:500) was added and incubated for 1h at room temperature. The 2° antibodies were preincubated at 4°C overnight using wild-type embryos in order to reduce background fluorescence.

Again the embryos were washed 3x 15 min. with PBST. For confocal analysis the signal was amplified. 300µl of biotinylated tyramide (NEN Life Science Product) (stock solution diluted 1:50 in amplification diluent solution) was added and incubated for 10 min. on the slow rotor. Finally, after washing the embryos several times, 300µl of Streptavidin conjugated antibody (for example SA-Fluorescein diluted 1:300) was added and incubated at room temperature for 30 min. The embryos were washed a last time 3x 15 min. in PBST and mounted directly in Vectashield and the coverslip was sealed with nail polish.

Pay attention when performing fluorescent immunostainings using 2A12. IgG antibodies have to be hybridized before the 2A12 IgM antibody.

Fluorescent images were captured using confocal microscopy (Leica TCS SP2) and processed using the Leica TSC NT 1.6 program and Photoshop 7.0 (Adobe). All images represent projections of sections on one focal plane.

3. Antibodies

The following antibodies were used: mouse mAB 2A12 against the lumen of the trachea (dilution 1:10; provided by N. Patel), rabbit anti β-galactosidase (dilution 1:500; Cappel), mouse anti-DSRF (dilution 1:50; Guillemin et al., 1996) and mouse anti-Evenskipped (both have to be diluted 1:50; provided by M. Frasch). Anti-Fasciclin 3 7910 and mouse anti Crumbs Cq4 (dilution 1:20; Developmental studies of Hybridoma Bank, University of Iowa), anti GFP (dilution 1:500; MBL) and anti-dpERK (dilution 1:200; SIGMA).

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. For the other stainings, we used as secondary antibodies biotinylated anti-mouse IgG and biotinylated anti-mouse IgM, which were revealed by using the biotinylated horseradish peroxidase ABC kit (Vectastain) or by staining for alkaline phosphatase activity.

VI. Molecular biology

Standard molecular protocols are described in detail in Maniatis (Sambrook et al., 1989). The constructs prepared for the rescue assay were generated by PCR using the appropriate oligonucleotides and subcloned into the pUASt vector to generate transgenic fly lines. For expression in Schneider (S2) cells, the cDNAs were cloned into the pMT/V5-HisA vector (Invitrogen) containing the heavy metal methalothionein promoter. Commercial kits were used for extraction and purification of the plasmid DNA from E. coli (Quiagen or SIGMA). All constructs were verified by sequencing (ABI PRIM 310 Genetic Analyzer) before embryo injection or before transfection of S2 cells.

1. Cloning

1.1 Btl derivates to generate transgenic flies

Btl derivates were generated by polymerase chain reaction (PCR) using the appropriate oligonucleotides. The strategy and oligonucleotides used to generate the Btl and Htl chimeric fusion proteins are described in detail in (Dossenbach et al., 2001; Vincent et al., 1998).

Internal deletions of Btl were generated by PCR removing those amino acids, which have to be annotated to be part of the domain to be removed. The UAS btl construct (kindly provided by B. Shilo and modified by C. Dossenbach) was used as template. In a first PCR reaction, a fragment was created with a primer containing the Bsu36I restriction site within the *btl* sequence in 5'-3' direction and a primer designed at the position, where the deletion starts, in 3'-5' direction. A second fragment was generated with the primer designed at the

position, where the deletion stops, in 5'-3' direction, and a primer containing the Asp718I site of pUASt in 3'-5' direction. The primers creating the deletion should have an overlap of 40°C for proper annealing and fusion of the two fragments in the second PCR round.

UAS btl ΔJM 1:	deletion from bp 2049-2139
UAS btl ΔJM 2:	deletion from bp 2139-2223
UAS btl ΔJM 3:	deletion from bp 2049-2223
UAS btl ΔKI:	deletion from bp 2566-2631
UAS btl ΔCT:	deletion from bp 3128 on

Primers:

Bsu36 I d	CAGTGTACCTTAGGGTAGTG
Asp718 c	CGGGGTACCCTCGAGCC
UAS btlΔJM 1:	
De 2049 c	CGATCCTAACCGTTTCGATGC
De 2139 d	AACGGTTAGGATCGAGAAGC
UAS btlΔJM 2:	
De 2139 c	CAGCGGTATCACTGGCATCTG
De 2223 d	CAGTGATACCGCTGGACTCAAAC
UAS btlΔJM 3:	
De 2223 db	AACGGTTCCGCTGGACTCAAAC
De 2049 cb	CAGCGGAACCGTTTCGATGC
UAS btlΔKI:	
Btl ki c	CGCCAAGACCAGGTCGATTCTG
Btl ki d	CCTGGTCTTGGCGAAAAGGAG

Deletion of the C-terminus of the *btl* gene was generated by PCR with the primer designed of the XhoI site of *btl* in 5'-3' direction and a primer introducing a stop codon CAG-›TAG at position bp 3128 containing a XhoI site in 3'-5' direction.

XhoI d	CAAATCGCTCGAGGAATGGAG
De 3128 c	CCGCTCGAG**CTA**CAGGATCCCATCG

UAS btlΔJM 3+ΔCT was generated by introducing a deletion of the C-terminus using the previously prepared UAS btlΔJM3 construct as template.

To generate the UAS btl-raf construct, we used the following primers:

Btl EcoRI d	CGGAATTCGACCGCAACTGTAAC
Raf XbaI c	CTAGTCTAGA**CTA**GATATTCCCAGC
Btl raf c	GTCGATGCAGCATGAACGTTATG
Btl raf d	CGTTCATGCTGCATCGACAGGGTCG

And finally, to generate the UAS btl-fgfr2 construct, we used the following primers:

Btl EcoRI d	CGGAATTCGACCGCAACTGTAAC
Fgfr2 XbaI c	GCTCTAGA**TCA**TGTTTTAACAC
Btl fgfr2 c	CATTCGCAGCATGAACGTTATG
Btl fgfr2 d	CATGCTGCGAATGAAGAACAC

1.2 Btl derivates to transfect S2 cells

Ute Nussbaumer subcloned the constitutive active version of Btl in which the extracellular and transmembrane domain of *torso*^{4021} (Reichman-Fried et al., 1994) was fused to the intracellular domain of *btl* derived from the hs *tor4021-btl* cDNA (kindly provided by E. Hafen) into the PMT/V5-HisA vector. This construct was used as template for the S2 cell culture assays. The same internal primers creating the deletions previously described were used but instead of Bsu36I d and Asp 718 c, the following primers were generated.:

Tor4021 DraIII d	TACAGGCACACGGTGGAC

Btl AvrII c GTGGACTCCTAGGGAGAC

For the deletion of the C-terminus:

De 3128 NotI c TTGCGGCCGCCTACAGGATCCCATCGAAACTC

For generation of the myc-tagged Btl construct, the following strategy was used: PUASt carrying the original *btl* gene was digested with EcoRI and SspI and the *btl* fragment was isolated (bp 421-3316). This insert was triple ligated with a PCR amplified fragment containing the Asp 718 site of pUASt, the myc Tag and the amino acid sequence of *btl* until the SspI site and a PMT/V5-HisA vector fragment that has been previously digested with Asp 718I and EcoRI (ratio vector: insert1: insert2; 1:2:2).

The following primers were used:

Myc Asp718 d CGGGTACC**ATG**TTCAAGATCCTG

Btl SspI c CAGGGAAATATTGTACCATCTG

Myc btl c ACTTTTGCGGCGCCCGTTC

Myc btl d GGCGCCGCAAAAGTGCCGATC

The kinase dead version of *myc-btl* was generated by the following strategy using the primers described in (Lee et al., 1996): The PMT/V5-HisA vector carrying the myc-tagged *btl* gene was digested with Asp718I and XhoI; the isolated vector fragment was subsequently ligated with an insert fragment containing part of the *btl* sequence flanked with the Asp718I and Asp700I sites and a PCR fragment that was performed to mutate the highly conserved lysine residue within the ATP binding domain to arginine. Unfortunately, the *btl* sequence contained an additional internal XhoI site at position bp 2673 (aa 748) and the sequence between the XhoI site of *btl* and the XhoI site of PMT/V5-HisA was still missing. Thus the isolated vector construct containing part of the *btl* sequence was recut with XhoI and a small fragment flanked with XhoI sites isolated from PMT/V5-HisA *myc-btl* was subcloned into the vector construct.

Btl Asp700 d CTCCGCATCGAAACGGTTCATCAGTGG

Btl XhoI c	GGTACTCCATTCCTCGAGCGATTTGAA
Btl K748 c	CTCCTTGACCATCCGGACGGCCACGAT
Btl K748 d	ATCGTGGCCGTCCGGATGGTCAAGGAG

To generate the PMT/V5-HisA myc-tor^{4021}-fgfr2 construct and the PMT/V5-HisA FRS2 construct, we used the following primers:

Tor4021 DraIII d	TACAGGCACACGGTGGAC
Fgfr2 NotI c	TTTATAGCGGCCGC**TCA**TGTTTTAACACTGC
Tor fgfr2 c	CTTCATTCGGCAGAACGTCAGGG
Tor fgfr2 d	TCTGCCGAATGAAGAACACG
FRS2 EcoRI d	CGGAATTC**ATG**GGTAGCTGTTG
FRS2 XhoI c	CCGCTCGAGCATGGGCAGATCAG

2. The polymerase chain reaction (PCR)

PCR reactions were carried out in a thermocycler (Techne Thermal Cycler). A 50μl reaction contained 1μl of each primer (10μM), 10μl polymerase buffer (Roche), 200μM dNTP mix, 1μl Pfu Turbo polymerase (Stratagene) or Dynazyme (Bioconcepts) and variable amounts of template (100μg/μl). The following PCR cycles were applied:

94.5°C	1'	
80°C	1' (add Pfu)	
94.5°C	30"	
35°C	30"	2 cycles
72°C	(60'' per 1kb cDNA)	
94.5°C	30"	
50°C	30"	20 cycles
72°C	(60'' per 1kb cDNA)	

72°C 10'

For fragments longer than 1000 bp, the Expand™ High Fidelity PCR Kit was used (according to the protocol from Boehringer Mannheim).
The PCR reactions were purified (QIAEX gel extraction kit or QIAQUICK PCR purification kit from QIAGEN) for further use.

3. Restriction digests

4μg of vector was digested overnight at 37°C in a volume of 50μl and subsequently dephosphorylated by the alkaline phosphatase (ROCHE) for 1h at 37°C. The PCR products were digested in a volume of 50μl for 1h at 37°C. The cDNAs were purified through agarose gel electrophoresis.

4. Ligation and bacterial transformation

Ligation (T4 DNA ligase, Biolabs) and bacterial transformations were carried out using standard protocols. All vectors carried an ampicillin resistance for bacterial selection. E. coli XL1 blue bacteria were used for plasmid amplification. The bacterial colonies were amplified in an overnight culture (3ml L. Broth medium containing 100μg/μl ampicillin) and the plasmids were isolated using a Miniprep kit (Sigma). The presence of an insert was verified by restriction digest before isolating large amounts of plasmids (30ml L. Broth medium and 100μg/μl ampicillin) following the protocol of the Midiprep kit (QIAGEN).

VII. S2 cell culture assay

1. Cell transfection

Drosophila Schneider (S2) cells were cultured at 25°C in Schneider's *Drosophila* medium (GIBCO) supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin (complete medium). For transfection experiments, $3{,}5x10^6$ S2 cells were plated per 35mm-

diameter dish in complete medium. Cells were transiently transfected with different combinations of plasmids (0.6 μg total) using the Effectene reagent following the manufacturer's instructions (QIAGEN). Protein expression was induced 20 hours after transfection by addition of 0.6 mM $CuSO_4$.

2. Cell lysates

Cells were lysed in modified RIPA buffer (50 mM Tris pH8, 150 mM NaCl, 1% Nonidet P-40, 0.5% Deoxycholate, 0.1% SDS, 0.4 mM EDTA, 10% glycerol, 0.2 mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride (PMSF) and Protease Inhibitors Cocktail (ROCHE).

3. Immunoprecipitation

For immunoprecipitation assays, cell lysates were precleared with protein G-Sepharose beads (Amersham Pharmacia) for 3 hours at 4°C on a rocking platform and then incubated with the primary antibody overnight at 4°C on a rocking platform. Protein-antibody complexes were recovered by incubation with protein G-Sepharose beads for 1 hour at 4°C. Bead-bound complexes were washed four times with cold lysis buffer and boiled with 2X SDS sample buffer.

4. Western blots

For Western blot analysis of total cell lysates, equal amounts of protein were separated by SDS-PAGE under reducing conditions, transferred on nitrocellulose or PVDF Immobilon (Millipore) membranes and probed using appropriate antibodies in 5% dry milk in TBS-T (20 mM Tris-HCl pH 7,6; 150 mM NaCl, 0.1 % Tween 20). Proteins were visualized using enhanced chemiluminescence (ECL kit; Amersham Corp.) following the manufacturer's instructions. The following primary antibodies were used for immunoprecipitation and Western blot experiments: anti-Myc 9B11 (dilution 1:1000; Cell Signalling), anti-V5 (dilution 1:5000; Invitrogen), anti-dpERK (dilution 1:2000; Sigma), anti-PY20 (dilution1:2000; Transduction Laboratories), as secondary antibody we used the anti-mouse HRP P0260 (dilution 1:2000; Dako).

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Appendix

I. Publications

Caroline Dossenbach, Salome Röck, Markus Affolter,
Development 128 (2001), 4563-4572,
"Specificity of FGF signaling in cell migration in *Drosophila*."

Valérie Petit, Ute Nussbaumer, Caroline Dossenbach, Markus Affolter,
Molecular and Cellular Biology, (2004), in press,
“Downstream-of-FGFR is a FGF-specific scaffolding protein and recruits Corkscrew upon receptor activation.”

II. Participation at following congresses

2001 “14th International Congress of Developmental Biology", Kyoto, Japan
-Research poster presentation
-Invited speaker of Prof. K. Furukubo-Tokunaga at the „Institute of Biological Sciences”, University of Tsukuba, Tsukuba, Japan

2001 "Swiss Drosophila meeting", Fribourg, Switzerland
-Oral presentation during a scientific session

2003 "44th Annual Drosophila Research Conference", Chicago, USA
-Research poster presentation

III. Tutorial responsibilities

2001 Introduction in Biology for Biology students
(Management Prof. H. Riezman)
-Teaching 1st semester students the basics of Biology including: Genetics, Developmental Biology, Ecology and Evolution

2002 The “Basics of Ethics” for Biology and Pharmacy students
(Management Prof. A. Seelig and Prof. Ch. Rehmann-Sutter)
-Edition of the chapter “Ethics in the history of Philosophy” in the Ethics curriculum
-Supervision of 2 student groups

	-Discussion leader -Setting and correcting written essays about a chosen ethical topic (“stem cell research” and “human dignity”)
2002	Superervision of „Schweizer Jugend forscht“ students
2003	The “Basics of Ethics” for Biology and Pharmacy students (Management Prof. A. Seelig und Prof. Ch. Rehmann-Sutter) -Discussion leader and group advisor

IV. Lectures

Molecular Mechanisms of Development	Prof. M. Affolter Prof. W.J. Gehring
Marine Biology Course Banyuls	Prof. W.J. Gehring
Cellular Signalling	Prof. A.N. Eberle Prof. K. Ballmer-Hofer
Genes, Brain and Behaviour	Prof. P. Caroni Prof. J. Kapfhammer
Mutation and Evolution	Prof. T. Bickle

V. Eid

Ich erkläre, dass ich die Dissertation, „Functional dissection of the *Drosophila melanogaster* Fibroblast Growth Factor signalling pathway in branching morphogenesis of the developing tracheal system“, nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Fakultät eingereicht habe.

Basel, den 18. April 2004 Caroline Dossenbach