Int. J. Dev. Biol. 46: 125-132 (2002)

Regulation of cell migration during tracheal development in *Drosophila melanogaster*

VALÉRIE PETIT, CARLOS RIBEIRO, ANDREAS EBNER and MARKUS AFFOLTER*

Abteilung Zellbiologie, Biozentrum der Universität Basel, Basel, Switzerland

ABSTRACT Most of the knowledge concerning the intracellular mechanisms involved in cell locomotion have been obtained from *in vitro* studies of cells in culture. Many of the concepts derived from these studies have been partially confirmed in *in vivo* systems but numerous questions regarding the developmental control of cell migration remain to be addressed. Tracheal morphogenesis in *Drosophila melanogaster* embryos represents an *in vivo* model system to study the genetic control of cell migration. We review what is known about tracheal development and regulation of tracheal cell migration. We try to link these *in vivo* studies and the movement of cells over two dimensional substrates and elaborate on important questions which remain to be addressed in the future.

KEY WORDS: *actin, branching morphogenesis, cell migration, signaling, trachea, transcription*

Cell migration plays a fundamental role in numerous normal and pathological processes, including embryonic development, wound healing, inflammation and metastasis of tumor cells. Much of the current understanding of the mechanisms controlling cell migration comes from *in vitro* studies of cells in culture. Notably, the nature of the molecular machinery producing the force to drive cell locomotion has been elucidated starting from *in vitro* systems. Recent studies have identified many genes involved in the regulation of guided cell migration *in vivo*. We will try to compare the results of these two fields, using tracheal cell migration in *Drosophila melanogaster* as an *in vivo* model system, and discuss some of the directions and questions of future studies.

Current View of Individual Cell Migration Over a Two Dimensional Substrate

Cell locomotion involves a succession of adhesions (formation of cell-substratum contact sites) and de-adhesions (disassembly of contacts) of the cell to the underlying substrate, accompanied by a net forward movement of the cell (for reviews see Lauffenburger and Horwitz, 1996; Sheetz, *et al.*, 1999). At the macroscopic level, a succession of several steps leading to cell locomotion can be distinguished: formation of membrane protrusions, establishment of stable contacts between the cell and the substratum, cytoskeletal contraction to move the cell body forward, release of adhesions at the rear of the cell and recycling of membrane components from the back to the front of the cell (see Fig. 1).

A number of molecular components have been identified that play important roles in the steps mentioned above. The motile cell first extends membrane processes, such as filopodia or lamellipodia, from the leading edge in the direction of movement. These protrusive extensions are produced by local actin polymerization. Actin filaments grow at their barbed end toward the leading edge of the cell, which provides the force for membrane protrusion (for a review see Machesky and Insall, 1999). New barbed ends originate either from nucleation of new filaments from G-actin pools, or from uncapping, severing or branching of existing filaments. Numerous reports have now identified the Arp2/3 complex and members of the WASP (Wiskott-Aldrich Syndrome protein) family as important initiators of actin filament nucleation and branching at the leading edge of motile cells (Machesky, *et al.*, 1994; Machesky and Gould, 1999; Pantaloni, *et al.*, 2000; Zigmond, 2000; Machesky and May, 2001). Additional proteins play important roles in the dynamics of actin filaments by capping their ends (capping protein, gelsolin), severing them (gelsolin, ADF/cofilin), crosslinking them (α-actinin, fascin, filamin), sequestering G-actin subunits (β-thymosins, profilin), recruiting actin filaments to the surface (Ena/Mena/VASP family) and promoting pointed end depolymerization to provide G-actin monomers for addition at the barbed ends (ADF/cofilin) (for reviews see Cooper and Schafer, 2000; Pantaloni, *et al.*, 2001).

Abbreviations used in this paper: Bnl, Branchless; Btl, Breathless; Dpp, Decapentaplegic; ECM, extracellular matrix; Kni, Knirps; Rho, Ras homologous; Trh, Trachealess; WASP, Wiskott-Aldrich Syndrome protein.

***Address correspondence to:** Prof. Markus Affolter. Abteilung Zellbiologie, Biozentrum der Universität Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland. Fax: +41-6-1267-2078. e-mail: markus.affolter@unibas.ch

0214-6282/2002/$25.00

Printed in Spain
www.ijdb.ehu.es

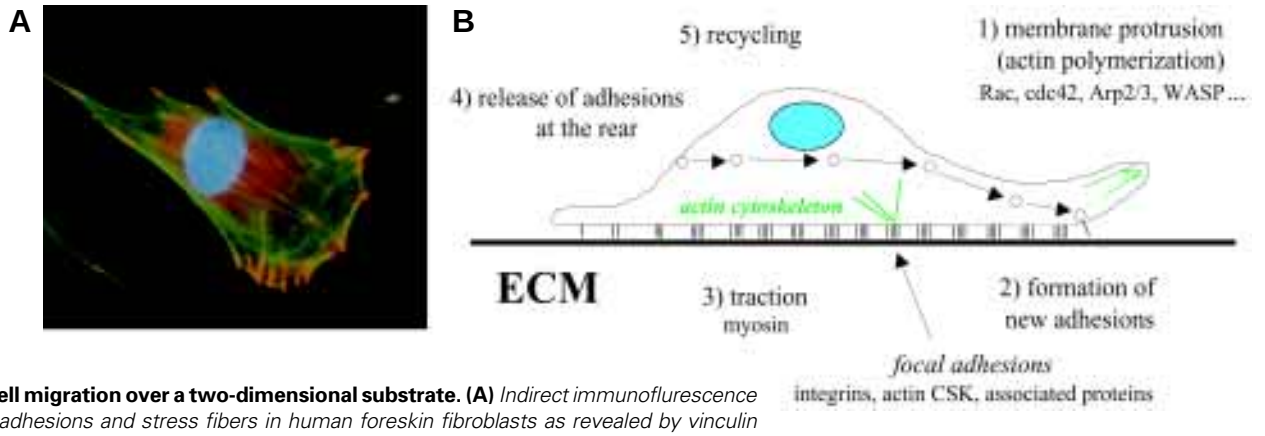

Fig. 1. Cell migration over a two-dimensional substrate. (A) *Indirect immunoflurescence of focal adhesions and stress fibers in human foreskin fibroblasts as revealed by vinculin staining in red and F-actin staining in green. The nucleus is revealed by DAPI staining. (Courtesy of S. Dufour).* **(B)** *Illustration of a migrating cell and the five steps involved in the locomotion process. ECM, extracellular matrix; CSK, cytoskeleton. (Adapted from Lauffenburger and Horwitz, 1996).*

Numerous reports indicate that quantitative alteration of some of these proteins affects the speed of cell migration. In addition, the function of most of these proteins is affected by the presence of PIP2 (phosphatidylinositol-4,5-biphosphate) (Toker, 1998).

Membrane extension at the front of the cell eventually lead to the formation of new attachments to the substratum. Specialized structures are formed at this contact point, termed focal adhesions, where transmembrane adhesion receptors, mainly of the integrin family, provide a structural link between the actin cytoskeleton and extracellular matrix (ECM) components (for reviews see Jockusch*, et al.*, 1995; Critchley, 2000; Petit and Thiery, 2000). Various cytoskeletal and signaling proteins assemble at the cytoplasmic face of focal adhesions and serve as signal transducers between the ECM and the actin cytoskeleton (Yamada and Geiger, 1997; Schoenwaelder and Burridge, 1999). Quantitative changes of focal adhesion components, such as the adhesive receptors (integrins), cytoskeletal components (*i.e.* Focal adhesion kinase (FAK), Src kinase, paxillin, vinculin or talin) or changes in integrin/ECM ligand affinity can alter the adhesive strength of the cell to the substratum and hence the speed of cell migration. Maximal cell migration occurs at intermediate adhesive strength when cytoskeletal forces are in balance with adhesion (Huttenlocher*, et al.*, 1996; Palecek*, et al.*, 1997).

To move the cell body forward, intracellular contractile forces depending on myosin motor activity are generated at these cell-substratum contacts. 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. Mechanisms that allow the release of cytoskeletal connections at the rear of the cell involve the coordinated regulation of physical and biochemical processes (Palecek*, et al.*, 1996; Palecek*, et al.*, 1998). As a last step, the motile cell has to recycle membrane and associated adhesion receptors to the front of the cell in order to recruit them to the extension process. Membrane is internalized at the rear of the cell and delivered through the endocytic pathway to the sites of protrusion in migratory cells (Bretscher and Aguado-Velasco, 1998; de Curtis, 2001).

Regulation of Motility of Cells in Culture

Various factors in the cellular microenvironment participate in the regulation of local actin polymerization and cell migration. These include a number of soluble growth factors, chemotactic factors and ECM proteins, the pivotal role of which has been clearly established using *in vivo* and *in vitro* model systems. These different ligands bind to their appropriate receptors on the cell surface and trigger signaling pathways that impinge on the reorganization of the cytoskeleton, thereby regulating actin polymerization/depolymerization and the state of adhesion site assembly and disassembly, ultimately allowing the cell to migrate. In the following, we will briefly outline how a signal from outside of the cell can be converted into a migratory response and what is known at present about the regulation of this complex process.

An Important Role for Small Rho GTPases in Cytoskeletal Reorganization Required for Cell Migration

Members of the Rho (Ras homologous) family of GTPases are major elements regulating changes in cell morphology and reorganization of the cell cytoskeleton in response to external stimuli. Similar to Ras, Rho family GTPases cycle between inactive GDP-bound and active GTP-bound forms. This 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. The balance of GEF and GAP activity toward Rho proteins determines their level of activity in the cell. Members of the Rho family have been identified in organisms ranging from yeast to humans, including for example RhoA-E, G, H, Rac1-3, Cdc42, G25K, TC10 and Rnd1-3 in mammals (for a review see Hall, 1994). In fibroblasts, RhoA is implicated in the formation of actin stress fibers and focal adhesions (Ridley and Hall, 1992; Hotchin and Hall, 1995), whereas Rac1 and Cdc42 participate in the formation of lamellipodia and filopodia, respectively, as well as in the regulation of associated focal complexes (Ridley and Hall, 1992; Nobes and Hall, 1995). Although the role of Rho family GTPases is not fully understood, numerous studies using mutants as well as dominant negative and constitutively activated forms of these GTPases support their importance in cell migration *in vitro* and *in vivo* (Murphy and Montell, 1996; Shaw*, et al.*, 1997; Keely*, et al.*, 1997; Nobes and Hall, 1999).

Downstream Effectors of Small G Proteins in Cell Migration

In the GTP-bound form, Rho GTPases interact with effector proteins in order to elicit a downstream response. Identification of specific targets has lead to a better understanding of how Rho

proteins regulate different cellular processes (for reviews see Van Aelst and D'Souza-Schorey, 1997; Bishop and Hall, 2000). We will focus on the targets that control the reorganization of the cytoskeleton required for cell migration.

Some effectors act rather directly on the actin polymerization process. This is the case for phosphatidylinositol-4-phosphate 5-kinase (PIP 5-kinase), a target of Rac. Through the increase of PIP2 levels, PIP5-kinase regulates the function of many actin-associated proteins (for example gelsolin, profilin and vinculin). This is also the case for WASP, an exclusive target of Cdc42 (Rohatgi, *et al.*, 1999). WASP contains multiple domains that interact with different signaling molecules, phosphoinositides and components of the machinery required for actin polymerization, such as actin monomers and the Arp2/3 complex (for a review see Zigmond, 2000). In its inactive state, WASP adopts an auto-inhibitory conformation, in which the C-terminus interacts with the central part of the molecule. Upon activation by GTP-Cdc42, which competes with the C-terminus for the same binding site, this intramolecular inhibition is released and permits the binding of WASP to the Arp2/3 complex (Kim, *et al.*, 2000). Binding of WASP to the Arp2/3 complex activates the actin-nucleating function of the latter and thereby locally increases actin polymerization.

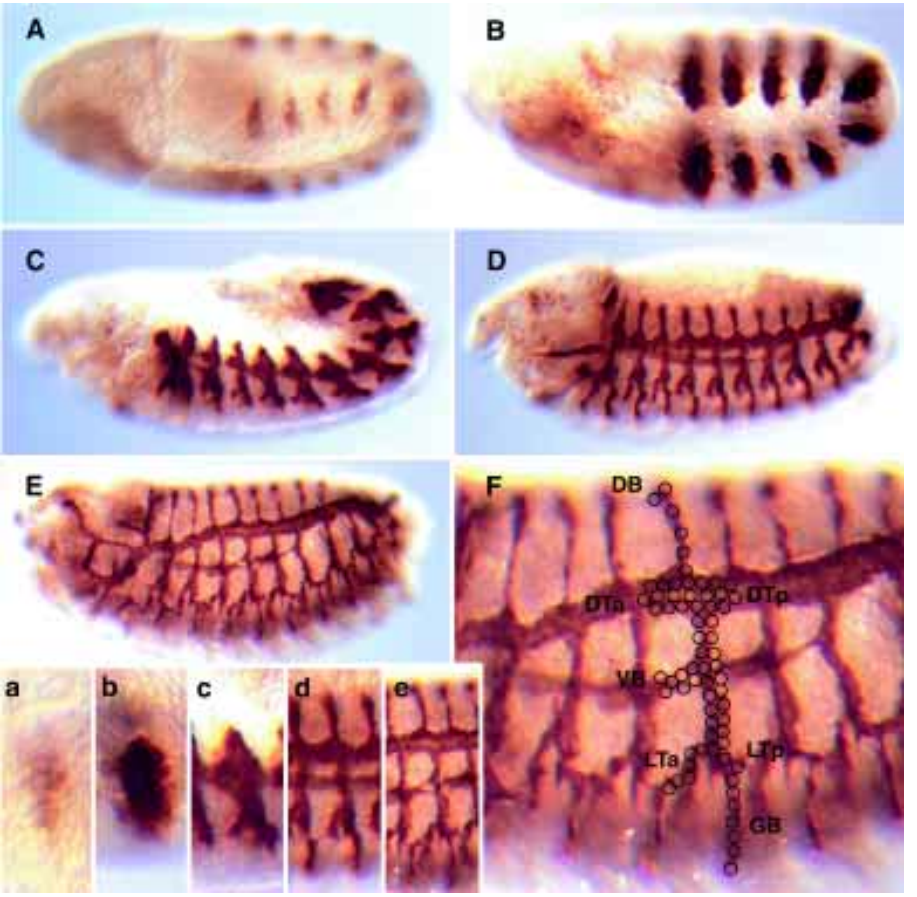

Fig. 2. Embryonic development of the tracheal system. *(A-E) Tracheal cells are visualized by anti-β-Gal staining of the 1-*eve*-1 enhancer trap line. This line has a P-element inserted in the 5 prime region of the* trh *gene (Perrimon* et al.*, 1991; Wilk* et al.*, 1996). Expression is first detected at late stage 10* **(A)** *in the ectoderm and outlines the tracheal pits at stage 11* **(B)**. *Outgrowth of primary branches at stage 12* **(C)** *and stage 14* **(D)**. *At stage 16* **(E)**, *dorsal and lateral trunks are fused. Nomenclature of the tracheal branches* **(F)**: *dorsal branch (DB, 5-7 cells); dorsal trunk anterior and posterior (DTa/p, 19-21 cells); visceral branch (18-20, not all cells are shown); lateral trunk anterior (LTa, 7-10); lateral trunk posterior (LTp, 4), and ganglionic branch (GB, 6-8). Cell numbers are according to Samakovlis* et al.*, 1996.* **(a-e)** *Enlargements of the corresponding stages in A to E.*

As already mentioned, cell migration involves a succession of adhesion and de-adhesion of the cell to the substrate, which implies a tight regulation of the assembly and disassembly of focal adhesions and their associated stress fibers. Phosphorylation of myosin light chain (MLC) is required for its association with actin, which leads to contraction and stabilization of stress fibers. MLC phosphorylation is regulated by the opposing effect of MLC-kinase (MLCK) and MLC-phosphatase. Some targets of Rho proteins exert their effects by regulating the phosphorylation state of MLC, and in this manner affect the adhesive state of the cell. This is the case for the kinase Pak (p21-activated kinase), a target of Cdc42 and Rac. Paks are serine/threonine kinases that contain a Cdc42/Rac interaction motif called the CRIB (Cdc42/Rac-interactive binding) site (for a review see Bagrodia and Cerione, 1999). Expression of different mutant forms of Pak1 showed that it induces two types of effects on cell morphology, one related to its protein kinase activity, which is essential for the disassembly of focal adhesions, and one that is kinase-independent and promotes lamellipodia formation and membrane ruffling (Manser, *et al.*, 1997; Sells, *et al.*, 1997; Obermeier, *et al.*, 1998; Zhao, *et al.*, 1998). Moreover, Pak activation leads to a decrease in MLC phosphorylation through phosphorylation of MLCK, thus destabilizing actin stress fibers (Sanders, *et al.*, 1999).

In contrast to Pak, the Rho-associated serine/threonine kinases ROKα/β, which are targets of the Rho GTPase, phosphorylate MLC and inactivate the MLC-phosphatase thus increasing actomyosin assembly (Kimura, *et al.*, 1996; Amano, *et al.*, 1996). Therefore, activation of these effectors by Cdc42/Rac or Rho provides a molecular control of the level of MLC phosphorylation and hence the adhesive strength of a cell to the substrate.

Regulation of G Protein Activity by Extracellular Ligands

A number of soluble growth factors and ECM proteins have been reported to induce cell migration by interacting with their appropriate receptors, ultimately regulating GTPase activity. For many of these ligands, the molecules linking the activated receptors to the Rho-family GTPases have not been identified yet. A nice example describing the isolation of molecules regulating Rac GTPase *in vivo* has recently been reported in studies aimed at a better understanding of axon guidance in the visual system of *Drosophila* (for a review see Lin and Greenberg, 2000). In that particular case, the activated guidance receptor(s) appears to directly recruit the SH2-SH3 adaptor Dreadlocks (Dock)/Nck, which in turn binds to the Pak kinase

mentioned above (Hing*, et al.*, 1999). In parallel, Trio, a GEF for Rac, is also activated by the guidance signal, pushing the equilibrium of Rac to GTP-Rac, which in turn promotes Pak kinase activation (Newsome*, et al.*, 2000). These studies thus propose that distinct signals transduced via Trio and Dock act combinatorially to activate Pak in spatially restricted domains within the growth cone, thereby controlling the direction of axon extension.

Since directed cell migration and axon guidance both occur through regulated actin polymerization/depolymerization, the genetic isolation of genes involved in axon guidance might identify components more generally implicated in actin metabolism, which might therefore also play a role in cell migration.

Regulation of Migration *In Vivo*

Most of the knowledge summarized above concerning the basic steps involved in cell migration have been derived from studying cells in culture. Many of the concepts derived from these studies have been partially confirmed in *in vivo* systems but numerous questions regarding the developmental control of cell motility remain. Tracheal morphogenesis in the embryo of *Drosophila melanogaster* has been used as a model system to study the genetic control of cell migration in a shaping organism. We will first describe what is known about tracheal development and present a conceptual framework for the regulation of tracheal cell migration as derived from these studies. We then try to span links between these *in vivo* studies and the movement of cells over two dimensional substrates and elaborate on important questions that remain to be addressed in the future.

The Tracheal System of Drosophila melanogaster

The tracheal system of *Drosophila* consists of a branched network of epithelial tubes that provides oxygen from the environment to all tissues of the body. The interconnected network develops from individual clusters of ectodermal cells that invaginate into the underlying mesoderm and form 10 sacs on both sides of the embryo, each containing about 80 cells (for reviews see Manning and Krasnow, 1993; Shilo*, et al.*, 1997; Metzger and Krasnow, 1999; Affolter and Shilo, 2000). Without further cell divisions, each sac forms five to six primary branches (dorsal branch, dorsal trunk anterior and posterior, lateral branch anterior and posterior, and visceral branch) by stereotypical, directed cell migration (see Fig. 2). Each of these branches has a defined identity that specifies tube size and the subsequent determination of specialized cell fates at precise positions and in the appropriate number. Most branches differentiate a number of terminal cells, which form fine cytoplasmic extensions through which gas is exchanged with the target tissues. In addition, fusion cells at the extremity of dorsal and lateral branches and the dorsal trunk allow the interconnection of adjacent tracheal metameres, leading to the formation of a continuous luminal network.

Tracheal Cell Fate Determination

The determination of tracheal identity in clusters of cells, the tracheal placode, is achieved in part by the local expression of the Trachealess (Trh) and the Drifter/Ventral veinless (Dfr/Vvl) transcription factors (de Celis*, et al.*, 1995; Anderson*, et al.*, 1995; Wilk*, et al.*, 1996; Isaac and Andrew, 1996; Zelzer and Shilo, 2000). *trh* encodes a basic helix-loop-helix (bHLH)-PAS-domain protein which forms a complex with Tango (Tgo), a broadly expressed bHLH-PAS protein, whereas *drf/vvl* encodes a POU-domain DNA binding protein. The expression of numerous genes crucial for tracheal development is dependent on *trh*, *vvl* or the cooperation of both; these genes encode, for example, the FGF receptor Breathless (Btl), the FGF signal transduction component Downstream of FGF-R (Dof), the Dpp type I receptor Thick veins (Tkv) and the EGF signaling component Rhomboid (Rho) (Boube*, et al.*, 2000).

After their determination, the placodes invaginate in a concerted manner and form a tracheal sac. This process generates a lumen, from which tubular branches subsequently bud off in five to six directions. Recent studies suggest that EGF receptor and Hedgehog transduction pathways might contribute to the process of tracheal invagination (Llimargas and Casanova, 1999; Glazer and Shilo, 2001).

Subdivision of the Tracheal Placode

In the early tracheal placode, the fate of cells with respect to their future position in the tracheal tree is not specified (Samakovlis*, et al.*, 1996). Positional cues are provided by nearby cells, which induce specific tracheal subfates within the tracheal fields and thereby assign cells to the future branches prior to the initiation of migration. The Decapentaplegic (Dpp) signaling pathway specifies the fate of the tracheal branches that will bud from the dorsal and ventral part of the tracheal placode. The Dpp ligand, a member of the TGFβ family, is expressed in ectodermal cells positioned dorsally and ventrally to the invaginated placode (Vincent*, et al.*, 1997; Wappner*, et al.*, 1997). In the absence of Dpp signaling, the dorsal branches do not form and the lateral trunk and ganglionic branches show severe defects. When Dpp signaling is activated in all tracheal cells, prospective dorsal trunk cells migrate in dorsal direction instead of migrating along the anteroposterior axis. Dpp signaling activates the expression of the zinc finger proteins Knirps (Kni) and Knirps-related (Knrl) in responding tracheal cells (Chen*, et al.*, 1998). Activation of Kni/Knrl is not only essential to determine the correct number of cells in dorsal and ventral branches, but is also critical in allowing cells to respond to the chemoattractant Bnl (see below), to control subsequent branch patterning events, and to determine the size of the tube to be formed during morphogenesis (Chen*, et al.*, 1998; Beitel and Krasnow, 2000).

Two other signaling pathways have recently been described that play similar roles in subdividing the tracheal placode. The wingless (Wg)/WNT pathway is required for the formation of the dorsal trunk by activating the expression of the transcription factor *spalt* (*sal*) (Llimargas, 2000; Chihara and Hayashi, 2000). When Wg signaling is activated in all tracheal cells, visceral branch cells turn on *sal* expression and migrate as dorsal trunk cells. On the other hand, the dorsal trunk is missing in mutants affecting the function of proteins of the Wg/WNT pathway (*armadillo, porpucine, dishevelled, pangolin/dTCF*). Wg protein is expressed by ectodermal cells on the anterior and posterior side of each tracheal placode, but it appears that other DWnt ligands also act on tracheal development. A recent study describes a role for the Hedgehog (Hh) signaling pathway in tracheal branch patterning (Glazer and Shilo, 2001). Hh protein is expressed in segmental ectodermal stripes abutting the anterior border of the tracheal placodes, and induces expression of target genes such as *patched* in anterior tracheal cells. In addition to defects observed in invagination of the placode, cells in many tracheal branches fail to migrate properly in *hh* mutants.

Although target genes for the above pathways have been identified in tracheal cells, how these signaling pathways are

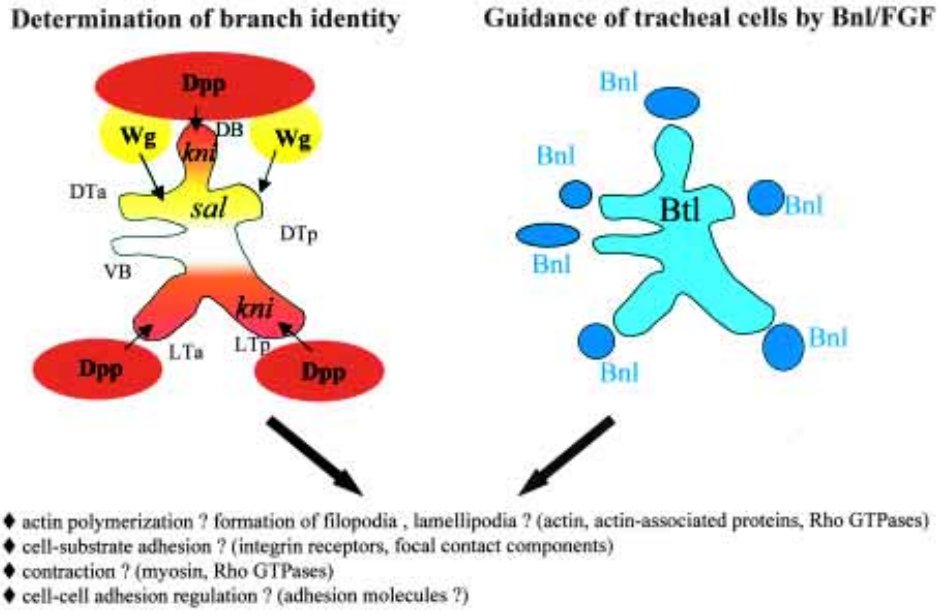

Fig. 3. Illustration of a tracheal placode and the signaling pathways involved in branch fate determination and guidance of tracheal cells. *The six primary branches are represented: DB, dorsal branch; DTa and DTp, dorsal trunk anterior and posterior; LTa and LTp, lateral trunk anterior and posterior; VB, visceral branch. The* sal *expression domain is outlined in yellow, whereas the* kni *expression domain that depends on Dpp signaling is represented in orange. Tracheal integration of these signaling pathways eventually leads to cell migration in the proper direction by mechanisms that remain to be determined. Bnl, Branchless; Btl, Breathless; Dpp, Decapentaplegic;* kni, knirps; sal, spalt; *Wg, Wingless. A comprehensive list of genes involved in tracheal cell migration can be found at* http://www.bioz.unibas.ch/affolter/trachea

interpreted and specify cell migration remains elusive. An attractive hypothesis is that these pathways activate the expression of distinct cell adhesion molecules in each branch leading to the cell sorting of the different tracheal cells.

Guided Migration of Primary Tracheal Cells

Although Dpp, Wnt and Hh signaling defects result in the absence of cell migration in distinct directions and despite the fact that the corresponding ligands are expressed in non-tracheal cells around the placode, none of these signaling molecules appears to act as a chemoattractant. Until now, the only known chemoattractant for tracheal cells is the Fibroblast Growth Factor (FGF)–like protein encoded by the *branchless* (*bnl*) gene (Sutherland*, et al.*, 1996). *bnl* is expressed dynamically in groups of non-tracheal cells around the invaginated placode and prefigures the direction in which the six primary branches will grow out. The *breathless (btl)* FGF-R gene is expressed on the surface of all tracheal cells and mediates the effect of Bnl in the tracheal system (Glazer and Shilo, 1991; Klambt*, et al.*, 1992; Reichman-Fried*, et al.*, 1994). In *bnl* and *btl* mutants, the specification of tracheal cells is normal and the placodes invaginate but primary branches fail to migrate. In contrast, ectopic Bnl can redirect tracheal cell migration to new sites of expression, thus demonstrating its role as a chemoattractant (Sutherland*, et al.*, 1996).

Signal transduction through the vertebrate FGF-R requires association of the FGF-ligand with its receptor as well as with heparan sulfate proteoglycans (HSPGs) in order to form an active signaling complex (Schlessinger*, et al.*, 2000). Recent studies have identified enzymes required for the biosynthesis and modification of HSPGs which are essential for signaling by Btl during *Drosophila* tracheal morphogenesis. Indeed, mutations in *sugarless* and *sulfateless*, which encode the homologues of UDP-D-glucose dehydrogenase and heparan sulfate N-deacetylase/N-sulfotransferase, respectively, result in defects in the migration of tracheal cells similar to those observed in the absence of the Bnl/FGF ligand or receptor (Lin*, et al.*, 1999).

Once activated, the FGF receptor signaling complex signals through the mitogen activated protein kinase (MAPK) cascade, which is a signal transduction pathway common to many receptor tyrosine kinases (RTKs). A novel component of the FGF-R signaling cascade in *Drosophila*, which acts specifically in the FGF-R and not in other RTK signaling pathways, has been identified. This gene, named *downstream of FGF-R (dof)*, is essential for the FGF-mediated activation of the MAPK cascade and for tracheal cell migration as well as for mesoderm development (Vincent *et al.*, 1998); mutations allelic to *dof* have been described and the corresponding genes called *heartbroken* or *stumps* (Michelson*, et al.*, 1998; Imam*, et al.*, 1999). Dof is present exclusively in cells that express FGF receptors and represents a novel cytoplasmic protein containing putative ankyrin-repeats and a coiled-coil domain. *dof* mutant embryos show the same defects in tracheal migration as *bnl* and *btl* mutant embryos, as well as defects in mesodermal migration similar to those seen in embryos carrying mutations in the gene *heartless (htl)*, which encodes the second *Drosophila* FGF-R. Dof has been shown to act downstream of both FGF-Rs and upstream of Ras in the activation of the MAPK cascade, but its precise role in conveying the chemotactic response in tracheal cells remains to be elucidated. No Dof homologs have been identified so far in other organisms.

Although localized *bnl* expression directs the budding of all primary branches, tight spatial control of *bnl* does not appear to be essential for the formation of dorsal trunk branches (Sutherland*, et al.*, 1996). Dorsal trunk formation thus appears to rely on additional guidance cues. Wolf and Schuh recently identified a mesodermal cell, named bridge cell, located at the posterior edge of each dorsal trunk bud and expressing the transcription factor *hunchback* (*hb*) (Wolf and Schuh, 2000). In *hb* mutants, dorsal trunk branches fail to complete migration and subsequently fail to fuse; all the other branches seem to migrate properly, suggesting that the *hb*-expressing bridge cell is essential for dorsal trunk formation (Wolf and Schuh, 2000). The precise function of the bridge cell and molecular targets of *hb* remain to be elucidated.

Additional Substrates for Migration

Little is known about the substrates supporting tracheal cells during the locomotion process. A recent study identified and described the different cellular contexts encountered by each branch of the tracheal system during its formation (Franch-Marro and Casanova, 2000). Tracheal cells that form the dorsal branches migrate in preexisting grooves between muscle precursors of adjacent metameres, whereas cells that form the dorsal trunk and ventral branches migrate across or along mesodermal cells. Visceral branch migration has been studied in more detail and cell surface receptors of the integrin family have been implicated in the migration process (Boube*, et al.*, 2001). The αPS1 integrin encoded by the *multiple edematous wings (mew)* gene is specifically expressed in the visceral branch cells under the control of the transcription factors *kni/knr/knrl* (Boube*, et al.*, 2001). In *mew* mutants visceral branches migrate normally out from the placode and toward the visceral mesoderm but fail to migrate along this substrate upon contact (Boube*, et al.*, 2001). These results indicate that the αPS1 subunit is required for migration over the mesoderm, stimulating motility rather than guiding it. Additional cues, possibly Bnl itself, regulate the initial guided migration from the placode to the visceral mesoderm and presumably support integrin-mediated migration over the mesoderm. This report is the first identification of an adhesion molecule whose expression is restricted to a subset of tracheal cells under the dependence of the transcription factors that initially subdivide the tracheal placode (see above). It will be interesting to find out whether other branches also express distinct adhesion molecules to allow for their migration along distinct pathways.

Although the development of the tracheal system and genes controlling this process have been investigated for a number of years in several laboratories, many questions concerning the migration of tracheal cells remain unanswered. How is the motile state specifically induced in tracheal cells at the appropriate time? How is cell movement directed by the Bnl/FGF chemoattractant? How are the additional, branch-specific signaling systems interpreted? What are the molecular links between the guidance cues and the cellular machinery required for migration? And how is migration arrested at the correct destination? In the last chapter, we will compare the *in vivo* and the cell culture experiments and briefly comment on how some of these questions might be addressed.

Cell Migration *In Vivo*: Which Processes are Controlled by Extracellular Signals?

As outlined in the first sections, cell locomotion involves a highly regulated succession of filopodia/lamellipodia formation, adhesion and de-adhesion. Numerous molecules have been identified that are either involved as effectors (actin polymers, adhesion complexes, etc.) or as regulators (WASP, small G proteins, cell surface receptors) of the migration process. Less is known about guided cell migration events *in vivo* but genetic studies start to provide insight into the molecular control of guidance. Numerous genes have been identified that are required for tracheal development and a first picture of the branching process can be drawn. Most of the identified gene products (which are either implicated in cell signaling and/or in transcriptional regulation; see Fig. 3 and http://www.bioz.unibas.ch/affolter/trachea) are regulating the migration process, and are not part of the migration machinery as such. Why did these genetic studies only lead to the isolation of regulatory components?

Many of the proteins that play essential roles in the locomotion process as defined in cell culture studies (*i.e.* actin and actin regulatory proteins) are also required for other essential processes (*i.e.* cell polarity and cell division); therefore it might be difficult to associate these factors directly with tracheal cell migration in straightforward genetic screens. In addition, genes encoding such factors might have a strong maternal contribution, allowing a homozygous mutant embryo to use the maternally provided wild type gene product for zygotic tracheal development. The generation of homozygous mutant germ line clones will help in the identification of such factors. However, more than 40% of lethal, zygotic mutations do not complete oogenesis in homozygous mutant germ line clones, thus prohibiting the analysis of later developmental stages. Conditional mutations and reverse genetics using constructs expressing dominant active and dominant negative gene products will have to help to define the role of these generally required components.

Despite these limitations, the genetic studies on tracheal development have given insight into a directed migratory process *in vivo* and how this process might be regulated. Clearly, FGF signaling acts as a major guidance system and the local production of the Bnl/FGF ligand prefigures subsequent migration directions. The expression pattern of Bnl is extremely dynamic and presumably controlled by separate transcriptional enhancers under the control of the earlier-acting genes that specify positional cues along the anterior-posterior and dorsal-ventral body axes (Metzger and Krasnow, 1999). It will be crucial to find out how the FGF signaling pathway is connected to cytoskeletal regulation and how Dof, a novel protein, fits into this scheme. Studies at the cellular level have to address the question of whether FGF signaling induces the formation of filopodia/lamellipodia and/or regulates cell adhesion. Using GFP-tagged proteins and four-dimensional confocal microscopy in living embryos, the dynamics of the migration process will have to be addressed, both in wild type and mutant situations. It is likely that Ras, Cdc42, Rac and Rho are major targets of FGF signaling with regard to guidance but this remains to be demonstrated. Mutations in some of the small GTPases have been isolated (Strutt*, et al.*, 1997; Fehon*, et al.*, 1997) and dominant negative and constitutively active forms have been engineered; their effects on tracheal development will have to be analyzed in detail. It will also be crucial to find out in which cells of a migrating branch FGF signaling is initiated or is strongest and whether FGF signaling polarizes the responding cells.

Interestingly, and in addition to Bnl/FGF signaling, several other signaling systems (Dpp, Wnt, Hh) are crucial for the formation of distinct tracheal branches. The involved signaling molecules do not act as chemoattractants, but instruct tracheal cells at the onset of the migration process with regard to their migration directionality; for example, all tracheal cells that respond to Dpp signaling migrate along the dorsoventral axis, irrespective of where the Dpp signal comes from. Consistent with this observation, Dpp does not appear to polarize the responding cells but results in specific changes in nuclear gene expression. But what are the cellular events targeted by these signaling pathways, or in other words, what genes are transcriptionally controlled by Kni/Knrl? Is the actin polymerization machinery modified, or is cell adhesion differentially regulated by Dpp (and Wnt) signaling? If cell adhesion is regulated, is adhesion in-between tracheal cells or adhesion of tracheal cells to the migration substrate regulated? Clearly, without the information of Dpp and Wnt signaling, tracheal cells do not respond to the Bnl chemoattractant with directed outgrowth, and signaling from these two pathways has

to be integrated somewhere in the locomotion process. These examples illustrate the complexity of information that needs to be processed by migratory cells *in vivo* in order to navigate properly through a developing organism.

An interesting question to be addressed in the future concerns the genetic regulatory network governing the formation of tracheal cells as such, a process which initiates tracheogenesis. Tracheal cells respond in a certain time window to the Bnl/FGF signaling system or to other RTKs by directed migration (Dossenbach, *et al.*, 2001); most cells in the organism respond to receptor tyrosine kinase signaling with altered nuclear gene expression. What primes tracheal cells to respond in this specific fashion? Some of the selector genes under whose control epidermal cells are determined to become tracheal cells have been identified (*trh*, *tgo*, *dfr/vvl*). It is likely that some of the targets regulated by these transcription factors set the stage for the subsequent migration process, and the identification of these target genes would provide information regarding to the establishment of the "migratory-competent" state of the cell. Of course it is equally possible that the tracheal determinants repress the expression of inhibitors of cell migration. Careful comparison of the transcriptome of tracheal cells with adjacent epidermal cells using DNA chip technology and other novel, more sensitive techniques should provide insight into this question.

Acknowledgments

We thank A. Jung for critical readings of the manuscript. The presented study was supported by a grant from HFSPO and from the Swiss National Science Foundation and by the Kantons Basel-Stadt and Basel-Land.

References

AFFOLTER M. and SHILO B.Z. (2000). Genetic control of branching morphogenesis during *Drosophila* tracheal development. *Curr Opin Cell Biol* 12*:* 731-735.

AMANO M., ITO M., KIMURA K., FUKATA Y., CHIHARA K., NAKANO T., MATSUURA Y. and KAIBUCHI K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem* 271*:* 20246-20249.

ANDERSON M.G., PERKINS G.L., CHITTICK P., SHRIGLEY R.J. and JOHNSON W.A. (1995). drifter, a *Drosophila* POU-domain transcription factor, is required for correct differentiation and migration of tracheal cells and midline glia. *Genes Dev* 9*:* 123-137.

BAGRODIA S. and CERIONE R.A. (1999). Pak to the future. *Trends Cell Biol* 9*:* 350-355.

BEITEL G.J. and KRASNOW M.A. (2000). Genetic control of epithelial tube size in the *Drosophila* tracheal system. *Development* 127*:* 3271-3282.

BISHOP A.L. and HALL A. (2000). Rho GTPases and their effector proteins. *Biochem J* 348 Pt 2*:* 241-255.

BOUBE M., LLIMARGAS M. and CASANOVA J. (2000). Cross-regulatory interactions among tracheal genes support a co-operative model for the induction of tracheal fates in the *Drosophila* embryo. *Mech Dev* 91*:* 271-278.

BOUBE M., MARTIN-BERMUDO M.D., BROWN N.H. and CASANOVA J. (2001). Specific tracheal migration is mediated by complementary expression of cell surface proteins. *Genes Dev* 15*:* 1554-1562.

BRETSCHER M.S. and AGUADO-VELASCO C. (1998). Membrane traffic during cell locomotion. *Curr Opin Cell Biol* 10*:* 537-541.

CHEN C.K., KUHNLEIN R.P., EULENBERG K.G., VINCENT S., AFFOLTER M. and SCHUH R. (1998). The transcription factors KNIRPS and KNIRPS RELATED control cell migration and branch morphogenesis during *Drosophila* tracheal development. *Development* 125*:* 4959-4968.

CHIHARA T. and HAYASHI S. (2000). Control of tracheal tubulogenesis by Wingless signaling. *Development* 127*:* 4433-4442.

COOPER J.A. and SCHAFER D.A. (2000). Control of actin assembly and disassembly at filament ends. *Curr Opin Cell Biol* 12*:* 97-103.

CRITCHLEY D.R. (2000). Focal adhesions - the cytoskeletal connection. *Curr Opin Cell Biol* 12*:* 133-139.

DOSSENBACH C., ROECK S. and AFFOLTER M. (2001). Specificity of FGF signaling in cell migration in *Drosophila*. *Development* 128: 4563-4572.

DE CELIS J.F., LLIMARGAS M. and CASANOVA J. (1995). Ventral veinless, the gene encoding the Cf1a transcription factor, links positional information and cell differentiation during embryonic and imaginal development in *Drosophila* melanogaster. *Development* 121*:* 3405-3416.

DE CURTIS I. (2001). Cell migration: GAPs between membrane traffic and the cytoskeleton. *EMBO Rep* 2*:* 277-281.

FEHON R.G., OREN T., LAJEUNESSE D.R., MELBY T.E. and MCCARTNEY B.M. (1997). Isolation of mutations in the *Drosophila* homologues of the human Neurofibromatosis 2 and yeast CDC42 genes using a simple and efficient reverse-genetic method. *Genetics* 146*:* 245-252.

FRANCH-MARRO X. and CASANOVA J. (2000). The alternative migratory pathways of the *Drosophila* tracheal cells are associated with distinct subsets of mesodermal cells. *Dev Biol* 227*:* 80-90.

GLAZER L. and SHILO B.Z. (1991). The *Drosophila* FGF-R homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension. *Genes Dev* 5: 697-705.

GLAZER L. and SHILO B.Z. (2001). Hedgehog signaling patterns the tracheal branches. *Development* 128*:* 1599-1606.

HALL A. (1994). Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Annu Rev Cell Biol* 10*:* 31-54.

HING H., XIAO J., HARDEN N., LIM L. and ZIPURSKY S.L. (1999). Pak functions downstream of Dock to regulate photoreceptor axon guidance in *Drosophila*. *Cell* 97*:* 853-863.

HOTCHIN N.A. and HALL A. (1995). The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases. *J Cell Biol* 131*:* 1857-1865.

HUTTENLOCHER A., GINSBERG M.H. and HORWITZ A.F. (1996). Modulation of cell migration by integrin-mediated cytoskeletal linkages and ligand-binding affinity. *J Cell Biol* 134*:* 1551-1562.

IMAM F., SUTHERLAND D., HUANG W. and KRASNOW M.A. (1999). stumps, a *Drosophila* gene required for fibroblast growth factor (FGF)-directed migrations of tracheal and mesodermal cells. *Genetics* 152*:* 307-318.

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

JOCKUSCH B.M., BUBECK P., GIEHL K., KROEMKER M., MOSCHNER J., ROTHKEGEL M., RUDIGER M., SCHLUTER K., STANKE G. and WINKLER J. (1995). The molecular architecture of focal adhesions. *Annu Rev Cell Dev Biol* 11*:* 379-416.

KEELY P.J., WESTWICK J.K., WHITEHEAD I.P., DER C.J. and PARISE L.V. (1997). Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* 390*:* 632-636.

KIM A.S., KAKALIS L.T., ABDUL-MANAN N., LIU G.A. and ROSEN M.K. (2000). Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. *Nature* 404*:* 151-158.

KIMURA K., ITO M., AMANO M., CHIHARA K., FUKATA Y., NAKAFUKU M., YAMAMORI B., FENG J., NAKANO T., OKAWA K., IWAMATSU A. and KAIBUCHI K. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* 273*:* 245-248.

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

LAUFFENBURGER D.A. and HORWITZ A.F. (1996). Cell migration: a physically integrated molecular process. *Cell* 84*:* 359-369.

LIN M.Z. and GREENBERG M.E. (2000). Orchestral maneuvers in the axon: trio and the control of axon guidance. *Cell* 101*:* 239-242.

LIN X., BUFF E.M., PERRIMON N. and MICHELSON A.M. (1999). Heparan sulfate proteoglycans are essential for FGF receptor signaling during *Drosophila* embryonic development. *Development* 126*:* 3715-3723.

LLIMARGAS M. (2000). Wingless and its signalling pathway have common and separable functions during tracheal development. *Development* 127*:* 4407-4417.

LLIMARGAS M. and CASANOVA J. (1999). EGF signalling regulates cell invagination as well as cell migration during formation of tracheal system in *Drosophila*. *Dev Genes Evol* 209*:* 174-179.

MACHESKY L.M., ATKINSON S.J., AMPE C., VANDEKERCKHOVE J. and POLLARD T.D. (1994). Purification of a cortical complex containing two unconventional actins from Acanthamoeba by affinity chromatography on profilin-agarose. *J Cell Biol* 127*:* 107-115.

MACHESKY L.M. and GOULD K.L. (1999). The Arp2/3 complex: a multifunctional actin organizer. *Curr Opin Cell Biol* 11*:* 117-121.

MACHESKY L.M. and INSALL R.H. (1999). Signaling to actin dynamics. *J Cell Biol* 146*:* 267-272.

MACHESKY L.M. and MAY R.C. (2001). Arps: actin-related proteins. *Results Probl Cell Differ* 32*:* 213-229.

MANNING G. and KRASNOW M.A. (1993). Development of the *Drosophila* tracheal system. In: Bate M. and Martinez Arias A. (Eds.), The development of *Drosophila* melanogaster. Cold Spring Harbor Laboratory press, pp. 609-685.

MANSER E., HUANG H.Y., LOO T.H., CHEN X.Q., DONG J.M., LEUNG T. and LIM L. (1997). Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. *Mol Cell Biol* 17*:* 1129-1143.

METZGER R.J. and KRASNOW M.A. (1999). Genetic control of branching morphogenesis. *Science* 284: 1635-1639.

MICHELSON A.M., GISSELBRECHT S., BUFF E. and SKEATH J.B. (1998). Heartbroken is a specific downstream mediator of FGF receptor signalling in *Drosophila*. *Development* 125*:* 4379-4389.

MURPHY A.M. and MONTELL D.J. (1996). Cell type-specific roles for Cdc42, Rac, and RhoL in *Drosophila* oogenesis. *J Cell Biol* 133*:* 617-630.

NEWSOME T.P., SCHMIDT S., DIETZL G., KELEMAN K., ASLING B., DEBANT A. and DICKSON B.J. (2000). Trio combines with dock to regulate Pak activity during photoreceptor axon pathfinding in *Drosophila*. *Cell* 101*:* 283-294.

NOBES C.D. and HALL A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81: 53-62.

NOBES C.D. and HALL A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J Cell Biol* 144*:* 1235-1244.

OBERMEIER A., AHMED S., MANSER E., YEN S.C., HALL C. and LIM L. (1998). PAK promotes morphological changes by acting upstream of Rac. *EMBO J* 17*:* 4328-4339.

PALECEK S.P., HUTTENLOCHER A., HORWITZ A.F. and LAUFFENBURGER D.A. (1998). Physical and biochemical regulation of integrin release during rear detachment of migrating cells. *J Cell Sci* 111*:* 929-940.

PALECEK S.P., LOFTUS J.C., GINSBERG M.H., LAUFFENBURGER D.A. and HORWITZ A.F. (1997). Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature* 385*:* 537-540.

PALECEK S.P., SCHMIDT C.E., LAUFFENBURGER D.A. and HORWITZ A.F. (1996). Integrin dynamics on the tail region of migrating fibroblasts. *J Cell Sci* 109*:* 941-952.

PANTALONI D., BOUJEMAA R., DIDRY D., GOUNON P. and CARLIER M.F. (2000). The Arp2/3 complex branches filament barbed ends: functional antagonism with capping proteins. *Nat Cell Biol 2:* 385-391.

PANTALONI D., LE CLAINCHE C. and CARLIER M.F. (2001). Mechanism of actin-based motility. *Science* 292*:* 1502-1506.

PERRIMON N., NOLL E., McCALL K. and BRAND A. (1991). Generating lineage-specific markers to study *Drosophila* development. *Dev Genet* 12: 238-252.

PETIT V. and THIERY J.P. (2000). Focal adhesions: structure and dynamics. *Biol Cell* 92*:* 477-494.

REICHMAN-FRIED M., DICKSON B., HAFEN E. and SHILO B.Z. (1994). Elucidation of the role of breathless, a *Drosophila* FGF receptor homolog, in tracheal cell migration. *Genes Dev 8:* 428-439.

RIDLEY A.J. and HALL A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70*:* 389-399.

ROHATGI R., MA L., MIKI H., LOPEZ M., KIRCHHAUSEN T., TAKENAWA T. and KIRSCHNER M.W. (1999). The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* 97*:* 221-231.

SAMAKOVLIS C., MANNING G., STENEBERG P., HACOHEN N., CANTERA R. and KRASNOW M.A. (1996). Genetic control of epithelial tube fusion during *Drosophila* tracheal development. *Development* 122*:* 3531-3536.

SANDERS L.C., MATSUMURA F., BOKOCH G.M. and DE LANEROLLE P. (1999). Inhibition of myosin light chain kinase by p21-activated kinase. *Science* 283*:* 2083-2085.

SCHLESSINGER J., PLOTNIKOV A.N., IBRAHIMI O.A., ELISEENKOVA A.V., YEH B.K., YAYON A., LINHARDT R.J. and MOHAMMADI M. (2000). Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol Cell* 6*:* 743-750.

SCHOENWAELDER S.M. and BURRIDGE K. (1999). Bidirectional signaling between the cytoskeleton and integrins. *Curr Opin Cell Biol* 11: 274-286.

SELLS M.A., KNAUS U.G., BAGRODIA S., AMBROSE D.M., BOKOCH G.M. and CHERNOFF J. (1997). Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. *Curr Biol* 7*:* 202-210.

SHAW L.M., RABINOVITZ I., WANG H.H., TOKER A. and MERCURIO A.M. (1997). Activation of phosphoinositide 3-OH kinase by the alpha6beta4 integrin promotes carcinoma invasion. *Cell* 91*:* 949-960.

SHEETZ M.P., FELSENFELD D., GALBRAITH C.G. and CHOQUET D. (1999). Cell migration as a five-step cycle. *Biochem Soc Symp* 65*:* 233-243.

SHILO B.Z., GABAY L., GLAZER L., REICHMAN-FRIED M., WAPPNER P., WILK R. and ZELZER E. (1997). Branching morphogenesis in the *Drosophila* tracheal system. *Cold Spring Harb Symp Quant Biol* 62*:* 241-247.

STRUTT D.I., WEBER U. and MLODZIK M. (1997). The role of RhoA in tissue polarity and Frizzled signalling. *Nature* 387*:* 292-295.

SUTHERLAND D., SAMAKOVLIS C. and KRASNOW M.A. (1996). branchless encodes a *Drosophila* FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell* 87: 1091-1101.

TOKER A. (1998). The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate. *Curr Opin Cell Biol* 10: 254-261.

VAN AELST L. and D'SOUZA-SCHOREY C. (1997). Rho GTPases and signaling networks. *Genes Dev* 11: 2295-2322.

VINCENT S., RUBERTE E., GRIEDER N.C., CHEN C.K., HAERRY T., SCHUH R. and AFFOLTER M. (1997). DPP controls tracheal cell migration along the dorsoventral body axis of the *Drosophila* embryo. *Development* 124*:* 2741-2750.

VINCENT S., WILSON R., COELHO C., AFFOLTER M. and LEPTIN M. (1998). The *Drosophila* protein Dof is specifically required for FGF signaling. *Mol Cell* 2: 515-525.

WAPPNER P., GABAY L. and SHILO B.Z. (1997). Interactions between the EGF receptor and DPP pathways establish distinct cell fates in the tracheal placodes. *Development* 124*:* 4707-4716.

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

WOLF C. and SCHUH R. (2000). Single mesodermal cells guide outgrowth of ectodermal tubular structures in *Drosophila*. *Genes Dev* 14*:* 2140-2145.

YAMADA K.M. and GEIGER B. (1997). Molecular interactions in cell adhesion complexes. *Curr Opin Cell Biol* 9: 76-85.

ZELZER E. and SHILO B.Z. (2000). Interaction between the bHLH-PAS protein Trachealess and the POU-domain protein Drifter, specifies tracheal cell fates. *Mech Dev* 91*:* 163-173.

ZHAO Z.S., MANSER E., CHEN X.Q., CHONG C., LEUNG T. and LIM L. (1998). A conserved negative regulatory region in alphaPAK: inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Rac1. *Mol Cell Biol* 18: 2153-2163.

ZIGMOND S.H. (2000). How WASP regulates actin polymerization. *J Cell Biol* 150: 117-120.