Specification of leading and trailing cell features during collective migration in the 
*Drosophila* trachea

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Summary

The role of tip and rear cells in collective migration is still a matter of debate and their differences at the cytoskeletal level are poorly understood. Here, we analysed these issues in the *Drosophila* trachea, an organ that develops from the collective migration of clusters of cells that respond to Branchless (Bnl), a FGF homologue expressed in surrounding tissues. We track individual cells in the migratory cluster and characterize their features and unveil two prototypical types of cytoskeletal organization that account for tip and rear cells respectively. Indeed, once the former are specified, they remain as such throughout migration. Furthermore, we show that FGF signalling in a single tip cell can trigger the migration of the cells in the branch. Finally, we found specific Rac activation at the tip cells and analysed how FGF-independent cell features such as adhesion and motility act on coupling the behaviour of trailing and tip cells. Thus, the combined effect of FGF promoting leading cell behaviour and the modulation of cell properties in a cluster can account for the wide range of migratory events driven by FGF.

Introduction

Collective cell migration is a widespread phenomenon in many biological processes, both in development and in disease conditions. Distinct cell types have been identified in migrating clusters, which have been suggested to display different cell activity, namely leading cells at the tip front and trailing cells at the rear. However, the role of tip and rear cells in collective migration is still a matter of debate (Rorth 2012). Moreover, what makes these cells behave distinctly and their differences at the cytoskeletal level are poorly understood (Friedl and Gilmour 2009; Rorth 2012).

The tracheal system of *Drosophila* is a particularly tractable model for the study of cell migration, and, in particular, of the mechanisms that guide cells to migrate in specific directions (Ghabrial, Luschnig et al. 2003). The Drosophila tracheal system develops from two clusters of cells in the ectoderm, one at each side of the central body segments. These cells invaginate forming the tracheal pits (Ghabrial, Luschnig et al. 2003). The cells of each cluster invaginate and migrate in different and stereotyped directions (Samakovlis, Hacohen et al. 1996) by responding to branchless (*bnl*), a gene encoding an FGF homolog expressed around the developing tracheal
system in cells at each position in which a new branch will form and grow. Activation of the Breathless (Btl) receptor in the tracheal cells by Bnl is thought to stimulate and guide tracheal migration toward these positions (Sutherland, Samakovlis et al. 1996). Tracheal cell division stops just as primary branching begins and that there is no fixed lineage relationship among cells contributing to a given branch or sharing the same position or fate in a branch (Samakovlis, Hacohen et al. 1996). Interestingly, FGF is also required for cell migration in other morphogenetic events such as in the development of the zebra fish lateral line (Aman and Piotrowski 2008; Lecaudey, Cakan-Akdogan et al. 2008), or in gastrulation in both invertebrates and vertebrates (Griffin, Patient et al. 1995; Ciruna and Rossant 2001, see Bae, Trisnadi et al. 2012 for a review). However, in spite of the wide use of the Drosophila tracheal system as a model for FGF-triggered migration it is not known what the role, specification and cytological features of tip and rear cell cells are and which cells require FGF signalling to sustain collective migration. In this work we have investigated these issues by the analysis of a particular branch from the developing tracheal cell cluster.

Results

Morphological features of leading and trailing and cells in the lateral trunk posterior and ganglionic branch (LTp/GB)

Among the tracheal branches from each placode, two grow towards the ventral side of the embryo, one in the anterior and the other in the posterior region of the segment, the lateral trunk anterior (LTa) and the lateral trunk posterior (LTp) respectively (Fig.1A-E). By a combination of migration, intercalation and elongation, the tip cell of the LTp migrates towards the central nervous system (CNS), and the resulting ganglionic branch (GB) connects the CNS to the main tracheal tube (Fig.1E, arrowhead). Another cell from the LTp migrates towards the LTa of the adjacent posterior metamere and makes a fusion branch that connects the two LT branches (Fig.1D,E, red arrow)(Ghabrial, Luschnig et al. 2003). We decided to focus on this branch (LTp/GB) because its complex morphology and pattern of migration make it particularly appropriate for analysing the morphology and behaviour of the tip and trailing cell during tracheal collective migration.

Live recording and individual cell labelling have completely changed our view of how cells migrate collectively (Rorth 2012), and have hence become an essential part
in morphogenesis studies. Individual cell labelling shows tracheal cells upon invagination to be rounded basally and constricted in the apical side, which faces the inner cavity of the tracheal pit (Fig.1F, Fig.2A). Subsequently, these cells, while keeping their constricted apical surface, show an elongated body and become bottle-shaped (Fig.1G, Fig.2B). By early stage 12, the small bud that will give rise to the LTp/GB branch is identifiable (Fig.1H). In particular, a group of 6 or 7 cells are positioned around a common central ring encompassing their apical junctions (Fig.1H,I,M). Subsequently, other cells are allocated into the branch and will connect the LTp/GB to the rest of the tracheal system; however here we limit our analysis to this early and distal group of 6 or 7 cells. At this stage, tip cells in the LTp/GBs can already be distinguished from the remaining cells of the bud, both by their greater elongated shape and higher protrusion activity (Fig.1H,M, Fig.2C). However, protrusion activity is not restricted to tip cells (Fig.2C,E), but as the bud emerges from the placode, protrusion activity declines in all but one or a couple of cells at the leading front, similar to what occurs in late dorsal branches (Ribeiro, Ebner et al. 2002; Caussinus, Colombelli et al. 2008)(Fig.3A, see also Fig.1B-D); trailing cells never completely cease showing protrusion activity, as shown by individual cell labelling revealing that some of their filopodia end up in a front position and could be wrongly attributed to tip cells (Fig.2E). The bodies of tip cells become extremely flat, with a very long and growing lamelipodium-like structure harbouring many filopodia at its edges (Fig.2C,D see also Fig.3A, and Fig1 C-E, J-L), an indication of leading cell morphology (Rorth 2011). This is a specific and early feature of tip cells that is maintained throughout the subsequent extension of the branch (Fig.2D, Fig.3 and movie S1). Indeed, individual cell tracing indicates that, unlike in other systems such as in border cell migration (Bianco, Poukkula et al. 2007; Prasad and Montell 2007), once cells acquire the morphological features of a tip cell, they remain as such during the LTp/GB morphogenesis and do not exchange this morphology/behaviour with the other cells of the branch in all the cases examined (n>30) (Fig. 3A,B, movie S1).

A transient "pyramidal" arrangement reorganizes LTp/GB cells into a longitudinal alignment

Tip and trailing cells in the bud are not all on the same plane and adopt a pyramid-like organisation, in which their apical surfaces cluster in a small circular area (Fig.1M).
This is a transient arrangement, as from stage 12 to 13, LTp/GB cells reorganize into a longitudinal alignment with their apical cell surfaces arranged in a row along a proximo-distal axis (Fig.1H-L,N, Fig.3B, Fig.S1A-D, I-N). In a way, tracheal pyramids are reminiscent of rosettes in *Drosophila* germ band extension, as both appear to be transient organisations suitable to drive cell rearrangements (Blankenship, Backovic et al. 2006). However, unlike rosettes, tracheal pyramidal arrays include unequal cells, namely tip and trailing cells; this is a critical difference because tracheal cell rearrangement is precisely coupled to tip cell behaviour. In the *Drosophila* germ band, rosettes of similar cells act in rearranging cell clusters from being elongated along the embryonic dorsoventral axis to being elongated along the anteroposterior axis, without a significant difference in the type of cell cluster organisation (Zallen and Blankenship 2008). Conversely, LTp/GB pyramids harbouring tip and trailing cells evolve into a new organisation of the cell cluster with increased anisotropy. It has been suggested that rosettes represent functional units of cell behaviour during tissue elongation (Blankenship, Backovic et al. 2006); similarly, we would like to propose that pyramids act as functional units to allocate and organise clusters of cells into the LTp/GBs (Fig.3B).

By stage 12, there is a change in the collective migration path of LTp/GB cell clusters; the two cells at the tip separate and behave as independent leading cells (Fig.3). As they approach two distinct groups of bnl-expressing cells, one cell moves towards a ventral group of bnl-positive cells and becomes the terminal cell, while the other moves posteriorly towards bnl-positive cells near the LTa (Fig.S2H-J) and becomes the fusion cell connecting the LTp with the neighbouring LTa. Associated with the individual changes in the leading cells, the whole LTp/GB cell cluster reshapes following the alternative branching paths (Fig.3A,B).

**FGF signalling in the tip cells is sufficient to confer migratory capacity to the cell cluster**

Can tip cells in the LTp/GB be defined on the basis of cytoskeleton features? Consistent with their morphology, tip cells show strong actin accumulation at their basal membrane through all stages of branch morphogenesis (Fig.4E,F, red arrows). In contrast, in trailing cells, and as cell protrusion declines, high actin accumulation is
progressively restricted to their apical surface (Fig.4E,F white arrowhead and arrow, basal and apical actin respectively). In addition, tip cells retain a constricted apical membrane, while trailing cells increase their apical surface (Fig.S3A, also see Fig. S1). Furthermore, in trailing cells, the apical membrane is parallel to the direction of branch elongation (Fig.4A-D,I white arrows), while that of tip cells remains perpendicular to the direction of branch elongation (Fig.4A-D,I red arrows). Thus, three cytological features distinguish leading from trailing cells: a smaller apical surface, a proximo-distal orientation of their apicobasal axis, and actin accumulation at two prominent sites, one at the small apical junction and another at the basal membrane. Interestingly, all LTp/GB cells acquire these features upon ectopic expression of bnl (see mat & met) (Fig.4G,H), thus indicating that FGF signalling is responsible for the cytoskeletal organisation of leading cells.

Given that FGF signalling can induce the above-mentioned cytological features in any LTp/GB trailing cell, we would like to propose that all the effects of FGF signalling on LTp/GB tracheal morphogenesis could be accounted for by triggering leading cell fate and behaviour. It has been previously shown that among the tracheal cells, those with higher levels of \(btl\) expression adopt the tip position (Ghabrial and Krasnow 2006). However, in that experiment, genetic mosaic animals were generated in which some tracheal cells displayed higher \(btl\) levels than the others but all had some \(btl\) expression. Thus, we aimed to generate embryos with individual cells positive for the FGF receptor while all the other tracheal cells would be completely deficient for the receptor. Remarkably, LTp/GB were observed to migrate and adopt its elongated morphology with just one FGF-receptor-positive cell at the tip position (Fig.5C,D; for examples in other branches see Fig.S2K). These results show that FGF signalling at the tip cells is sufficient to confer migratory capacity to the cluster and that the trailing cells follow the tip cells that act as leading cells. In all cases, FGF-receptor-positive cells accumulated at the tip positions. Interestingly, we found cases in which two FGF-receptor-positive cells occupied the position of the two tip cells, namely the future terminal cell and the future fusion cell, thus enabling the LTp/GB to migrate both towards the ventral midline and towards the neighbouring LTa (Fig.5C,D).

The acquisition of leading cell features requires a shift between Rac activation and inactivation
It was previously suggested that Rac activation is an essential downstream event of tracheal cell motility induced by FGF signalling (Chihara, Kato et al. 2003). To examine whether there is a specific activation of Rac at the tip of the LTp/GB branch we resorted to transgenic *Drosophila* expressing a fluorescence resonance energy transfer (FRET)-based sensor (Kardash, Reichman-Fried et al. 2010) previously used in cultured egg chambers (Wang, He et al. 2010). We worked to detect activity of this sensor in vivo in whole embryos (see mat & met) and upon its expression with a tracheal driver we detected a distinct FRET signal at the tip positions of the LTp/GB, both at the ventral side and at the site of the future fusion with the neighbouring LTa (Fig.5E,F, movieS2).

We then addressed the contribution of Rac activity in tip cells to LTp/GB migration. Since mutant embryos for RhoGTPases do not allow the study of the specific tracheal roles of these proteins and since RNAi technology has a very limited effect at these stages of tracheal development, we turned to the analysis of the dominant negative effect and constitutively activated forms of RhoGTPases (Ridley 2001) under the control of a tracheal driver. First, and as previously described (Chihara, Kato et al. 2003), constitutive Rac activity in all tracheal cells was found to induce the transformation described as an epithelial-to-mesenchymal transition; in particular, we observed the downregulation of junctional (DE-Cadherin (DE-Cadh), a-Catenin (a-Cat) and apical components as Crumbs (Crb) (Fig.6E, Fig.S2G5-7 Fig.S4G-I), associated with this transition (Chihara, Kato et al. 2003). Interestingly, analysis of Rac activity in individual cells by flip out clones (see mat & met) revealed that clones of RacDN in otherwise wild type LTp/GBs are almost never associated with tip cells and correspond to trailing cells (Fig.6K, Fig.S3B), consistent with the above-mentioned suggestion that Rac activation is an essential downstream event of FGF-induced tracheal cell motility. However, and more surprisingly, neither were clones of RacACT in otherwise wild type LTp/GBs associated with tip cells, and the few clones in the branch corresponded to trailing cells (Fig.6L, Fig.S3B). These observations indicate not only a strict requirement of Rac activity for the acquisition of leading cell features but also the need for a temporal shift between Rac activation and inactivation or/and an asymmetric intracellular display of Rac activity.
Individual cell labelling reveals connections between distant cells in the LTp/GB cluster

The experiments reported so far address the specification and features of tip cells; in particular they show that FGF signalling restricted to tip cells is sufficient to provide migratory capacity to whole clusters. We next addressed the trailing cell features in the cluster migration. Once the cell clusters exit the pyramid arrangement and adopt a longitudinal organisation, the LTp/GBs also grow by cell intercalation, as cells evolve from a side-by-side to and end-to-end cell arrangement and exchange intercellular adherens junctions with autocellular ones (Ribeiro, Neumann et al. 2004). Unexpectedly, cells were found to be in contact not only with neighbouring ones, but also with distant ones via long extensions, as appreciated by live imaging (Fig.6A,B). Indeed, in vivo recording of the said cells showed that they had previously been close but had subsequently exchanged neighbours. Connections with distant cells are established by cytoplasmic extensions that are often longer than one cell body (11.8 um ± 1.3) and that accumulate actin (Fig.6B), but not DE-Cad or Crb (data not shown).

Modification of LTp/GB cell properties by Rho dominant negative and constitutively active mutants

In order to analyse how cell features affect cluster migration following the FGF-induced changes in the leading cells, we resorted to the study of the mutant phenotypes for the RhoGTPases Cdc42 and Rho, which are key cytoskeleton regulators (Hall 2005; Jaffe and Hall 2005), as previously done for the RhoGTPase Rac (Chihara, Kato et al. 2003). A unidirectional assignment between a specific cellular process in vivo and a single RhoGTPase is probably an oversimplification as each RhoGTPase may be simultaneously involved in more than one process in the same cell (Pertz 2010). However, the modification of cell properties by RhoGTPases mutant forms, although not a direct effect, has proven very useful as it allows to modify cell features, which can hardly be achieved by modulation of a single of their downstream effectors.

A dominant negative form of Rho (Rho^{DN}) produced breaks and detachment of LTp/GB cells (Fig.6F, asterisks), which then migrated in small groups (in 48 % of branches) or individually (in 23 % of branches) (Fig.S3C,D). In Rho^{DN}, DE-Cadh and
other apical proteins adopted a fragmented distribution (Fig.6F’ and Fig.S2B5-7, Fig.S4J-L), suggesting impaired cell adhesion, which could account for this phenotype. Consistently, upon constitutive activation of Rho, we observed LTp/GB cells held together from the early stages in a pyramidal-like configuration and showed impaired migration (more than 50% of branches not elongating) (Fig.6G, Fig.S3C, Fig.S2C and Fig.S4M-O). Reinforcing the notion of increased cell adhesion as a cause of the Rho<sup>ACT</sup> phenotype, we observed that a decrease in adherens junction components significantly ameliorated the phenotype (methods and Fig.S3E). Conversely, impairment of migration in Rho<sup>ACT</sup> did not appear to depend on decreased cell motility, as cells remained protrusive (Fig.S2C1-4). In particular, tip cells emitted very long projections and adopted a highly elongated shape (Fig.6G, red arrows) and, although they failed to migrate, they still responded to chemoattractant signals, thus indicating that migration and response to chemoattractant are two separable processes. Expression of either Rho<sup>ACT</sup> or Rho<sup>DN</sup> in clones reproduced the main features of the respective phenotypes even when expressed in few cells (Fig.6N,M); thus, for example, we observed that just a few Rho<sup>DN</sup> cells could detach from the branch (Fig.6M). Interestingly, however, Rho<sup>ACT</sup> single cell clones at the tip of normally elongated LTp/GBs did not show the long extensions observed when the whole branches were mutant (compare Fig.6N and 6G). This finding reinforces the interpretation that these extensions are not a cell-autonomous effect of the constitutive activity of Rho but of the incapacity of the cells to migrate although responding to the chemoattractant signals.

**Modification of LTp/GB cell properties by Cdc42 dominant negative and constitutively active mutants**

Upon tracheal expression of a dominant negative form of Cdc42 (Cdc42<sup>DN</sup>), LTp/GB, cells were associated by thin extensions, as if they were overstretched (68% of elongating branches with overstretching) (Fig.6H, arrowheads and Fig.S3C), with discontinuous accumulation of either DE-Cad, a-Cat or Crb, found even in the main cell body (Fig.6H’, Fig.S2D5-7 and Fig.S4P-R). Moreover, in vivo recordings suggested that overstretching causes some breaks in Cdc42<sup>DN</sup>. However, breaks were fewer than in Rho<sup>DN</sup> (small groups of migrating cells detached from 7% of the branches and individual migration of leading cells occurred in 17% of the branches)
(Fig.S3D). The differences in the breaks between Rho\textsuperscript{DN} and Cdc42\textsuperscript{DN} did not appear to depend on variations in the strength of the transformations associated with each construct. Instead, other features of the phenotype suggested that the overstretching, and thus the breaks in Cdc42\textsuperscript{DN}, was due to increased motility. Thus, for example, trailing cells in Cdc42\textsuperscript{DN} mutants showed more protrusions and were more actin-enriched basally than wild-type cells (Fig.S2D1-4), features normally associated with leading cells. Accordingly, we detected many cases in which trailing cells, while still attached to the cluster, appeared to initiate a new migratory path (in 28 \% of branches) (Fig.S3D). Moreover, \textit{in vivo} recording showed some trailing cells taking the lead of the migratory cluster substituting the previously leading cell -a feature never observed in wild-type cells- and eventually one or both cells detaching and migrating individually (movie S3). In addition, and unlike in the wild-type, distal trailing cells also exchanged positions inside the cluster with more proximal trailing cells (movie S3). Consistent with higher motility in Cdc42\textsuperscript{DN}, constitutive activation of Cdc42 (Cdc42\textsuperscript{ACT}) reduced the motility of LTp/GB cells as transient pyramidal organisation did not evolve, or evolved much more slowly, towards branch elongation (Fig.6I, Fig.S3E5-7 and Fig.S4S-U), causing more than 50 \% of branches not to elongate (Fig.S3C). In addition, and reinforcing the interpretation of decreased motility in Cdc42\textsuperscript{ACT}, cells looked smooth and non-protruding (Fig.S2E1-4), in contrast to the appearance of cells in Rho\textsuperscript{ACT} (FigS2C1-4). The corresponding phenotypes were also observed upon expression in clones of each mutated form. Cdc42\textsuperscript{ACT} cells did not move apart, in some cases they did not get even allocated into different branches, and, if at the tip, they were sometimes associated with lack of branch elongation (Fig. 6P). Cdc42\textsuperscript{DN} cells displayed extensions, although with no breaks (Fig. 6O).

Rho and Cdc42 dominant negative and constitutively active mutants modify the migration speed of the LTp/GB

Analysis of kymographs depicting the positions of cells over time allowed us to study how the above-mentioned cell changes impinge on migration of the LTp/GB clusters (example of WT \textit{vs.} Cdc42\textsuperscript{DN} in Fig.7A,B). We found a strong reduction in the migration speed, as measured by the distance reached by the leading cell, in the case of Rho\textsuperscript{CA} (2.5±2.3 nm/s, n=3) and Cdc42\textsuperscript{CA} mutants (2.8±0.64 nm/s, n=8) compared to the wild-type (4.5±0.66 nm/s, n=19). Conversely, speed increased in Rho\textsuperscript{DN} (5.4±1
nm/s, n=36) and Cdc42^{DN} mutants (7.2±1.75 nm/s, n=37), a rise that was higher when mutant conditions were associated with branch breaks (8.5±1.8 nm/s, n=11 for Rho^{DN} and 9.8±2.5 nm/s n=10 for Cdc42^{DN}) than when branches kept their integrity (4±0.67 nm/s n=20 for Rho^{DN} and 5.4±2 nm/s n=21 for Cdc42^{DN}), the latter being consistent with the observations from laser-induced cuts in wild-type branches (Caussinus, Colombelli et al. 2008). Thus, mutant conditions with lower migratory speeds matched compacted LTp/GBs while those with higher migratory speeds matched overstretched and/or broken LTp/GBs.

**Effect of constitutive activity of Rho in a btl mutant background**

Given that we detect an effect of Rho and Cdc42 mutant forms in trailing cells, Rho and Cdc42 appear to modify tracheal cell properties in the LTp/GB cluster in an FGF-independent manner. To confirm this hypothesis we examined the effect of Rho^{ACT} tracheal expression in a mutant background for btl, the gene encoding the FGFR in the trachea. Under these circumstances, tracheal cells were more tightly packed than in just btl mutants. This observation supports the notion that Rho activity increases cell adhesion in a way that is independent of the FGF signalling triggering migration (Fig.S3F).

**Discussion**

**FGF and collective migration**

As pointed out in the Introduction, the FGF signalling pathway is involved in many morphogenetic events requiring collective migration of cell clusters. However, it is not entirely clear whether in these events FGF signalling is directly involved in triggering cell migration, or alternatively if it is required for other processes such as cell determination which only affect cell migration indirectly. Moreover, while FGF might be required it is not clear either whether all the cells or just a subset of those need to directly receive the signal to sustain the migration of the entire cluster. One well-studied case is the role of FGF in the development of the zebra fish lateral line. In that case, FGF appears to be produced by the leading cells which signal to the trailing cells, the cells where FGF signalling is active. Restriction of FGF signalling is
thereafter required for the asymmetric expression of the receptors for the chemokines that guide migration (Aman and Piotrowski 2008).

A very different scenario applies in the case of Drosophila tracheal migration. On the one hand, FGF is expressed in groups of cells outside the migrating cluster (Sutherland, Samakovlis et al. 1996) On the other hand our results in the LTp/GB indicate that FGF signalling is required and sufficient in the leading cells, and not in the trailing cells, for the migration of the whole cell cluster. Therefore, in spite of its widespread involvement, the mechanisms triggered by FGF signalling in collective migration appear to be quite different.

**Rho, Cdc42, adhesion and motility**

The above-described experiments revealed that Rho inactivation produced breaks and detachment in the LTp/GB cluster while its constitutive activation let these cells to hold together impairing migration. Likewise, upon Cdc42 inactivation LTp/GB cells were associated by thin extensions associated in some cases with breaks, while upon its constitutive activation, the LTp/GB transient pyramidal organisation did not evolve, or evolved much more slowly, towards branch elongation. However, the phenotypes from each RhoGTPase mutants don't look alike and our detailed analysis suggests that Rho impinges primarily on cell adhesion while Cdc42 does so on cell motility.

These results are consistent with previous findings that show a role for Rho in regulating adherens junctions stability (Braga, Machesky et al. 1997; Bloor and Kiehart 2002; Magie, Pinto-Santini et al. 2002) and for Cdc42 as the main mediator of filopodia formation (Nobes and Hall 1995; Ridley 2006; Pedersen and Brakebusch 2012). We note, however, that we have found Cdc42 to exert in the LTp/GB an opposite effect to the one identified in other systems, as Cdc42<sup>DN</sup> mutants showed more protrusions and were more actin-enriched basally than wild-type cells and Cdc42<sup>ACT</sup> mutants showed a reduced the motility of LTp/GB.

There is an increasing amount of data pointing to the different effect of RhoGTPases in vitro versus in vivo models and also among various cell types (see Pedersen and Brakebusch 2012). As mentioned before, a unidirectional assignment between a
specific cellular process in vivo and a single RhoGTPase is probably an oversimplification and this was not the aim of our study. Rather we relied on mutant forms of the RhoGTPases to modulate cell features, either individually or collectively, to assess their role in the overall behaviour of the cell cluster. In doing so, our results point to a critical role for a balance between cell adhesion and cell motility for the collective migration of a cell cluster (see below).

**A model for the collective migration of LTP/GB cells**

Our results support the following model for the specification, features and behaviour of leading cells in the migration of the LTp/GB branch (Fig.7C). Upon signalling from the FGF pathway, tip cells reorganise their cytoskeleton features (actin enrichment at the basal membrane, small apical surface and an apicobasal polarity along the proximo-distal axis), thereby enabling them to acquire leading behaviour. Indeed, FGF can induce migratory capacity to the whole cluster by signalling only the tip cells, where a dynamic transition between states of Rac activity is needed to acquire a leading role. How the behaviour of tip cells leads collective migration thereafter depends on the features of the cells in the cluster, which are determined by various different regulators, among these, the RhoGTPases, which act, at least in part, in an FGF-independent manner. Ultimately, the balance between individual cell properties such as cell adhesion, motility and apicobasal polarity will 1) determine the net movement of the overall cell bodies or alternatively changes in cell shape in terms of elongation, 2) control the migratory speed and 3) define whether cells will migrate individually or in clusters and whether clusters will bifurcate in different paths. The combined effect of the changes promoting leading cell behaviour and modulation of cell features is likely to be a widely exploited mechanism in collective migration. In particular, the actual balance between these cell features may dictate the specifics of each migratory process and, consequently, the final shape of the tissues and organs they contribute to generate.

**Materials and Methods**

Drosophila stocks and genetics

Details for all genotypes and transgenes can be found in flybase (http://flybase.bio.indiana.edu) or in the references listed here. Unless otherwise noted, stocks were obtained from the Bloomington Stock Center. The Gal4/UAS
system (Brand and Perrimon 1993) was used to over/miss-express proteins. Unless otherwise noted, transgenes were driven by the pantracheal driver btlGal4 (Shiga 1996) recombined with either srcGFP, catGFP, or GMAGFP. In Fig. S2 and S4 we used it in combination with the btlmoeGFP construct (from M.Affolter). For expression in groups or individual tracheal cells we used (1) the FLP-out technique (Fig. 2A, Fig. 4A-F, Fig. 4C-D, Fig. 6A,B, J-P, Fig. S2K, Fig. S3B) with the hsFlp122; btlRFPMoe, btl>y+>Gal4 stock (Ribeiro, Neumann et al. 2004) combined with the UAS constructs for the diverse RhoGTPase variants and/or UAS-srcGFP and (2) the brainbow system (Fig. 2B,E Fig. 3A, movieS1) with the hsCre; btlGal4, UAS-Brainbow stock (Forster and Luschnig 2012). For the btl rescue experiments and for Rho1 expression in btl mutants we generated the following stocks: a) btlGal4UASsrcGFP; btl^{L19} b) UAS btl; btl^{L19} c) UAS btl, UASrcGFP; btl^{L19} d) hsFlp122; btlRFPMoe, btl>y+>Gal4, btl^{L19} and d) UAS Rho^{ACT}, btl^{L19}. Miss-expression of bnl (UAS-bnl) was induced using either inscGal4 (Fig. 4G) or enGal4srcGFP (Fig. 4H). Flies bearing the Rac construct for FRET are reported by (Wang, He et al. 2010). Genotypes were identified by absence of β-gal expression from “blue” balancers. All crosses and staging were performed at 25°C. For the FLP-out experiments, embryos were heat shocked for 45 min at 37°C and transferred at room temperature for 6 hr. before mounting. Wild-type and mutant LTP/GBs were scored from the central region of embryos (5 metameres 4-8) to ensure the comparison of between branches with similar development. To reduce E-cad and cat levels in genetic interaction experiments we used cat RNAi lines from Bloomington.

The total number (n) of embryos and/or branches is given in text and legends where appropriate. Data are means ± s.e.m.

Immunostaining, whole mount in situ and image acquisition

Embryos were staged as in Campos-Ortega and Hartenstein and stained following standard protocols. A bnl RNA probe was generated from a cDNA corresponding to amino acids 241 to 400. Whole mount in situ followed by immunostaining was done according to standard protocols. The following primary antibodies were used: rabbit anti-b-galactosidase (Cappel) (1:300); goat anti-GFP (1:600) and rabbit anti-RFP (1:300) (Abcam); mouse anti-DE-Cadh (1:100) (DCAD2-DSHB); anti-Crb (1:10) (Cq4-DSHB) mouse anti-Dlg (1:500); rabbit anti-DaPKC (1:500), rabbit anti-SAS (1:250) rat anti-Trh (1:600) and rat anti-Btl (1:10) (N. Martín in our laboratory);
rabbit anti-Dof (1:250) (gift from J.N.Nair and M. Leptin). Embryos were fixed in 4% formaldehyde for 20–30 min and for 10 min for DE-Cadh. Immunostaining was detected with donkey secondary antibodies labelled with Alexa 488, Alexa 555 or Alexa 649 (Invitrogen). Fluorescent images were obtained with confocal microscopy (Leica TCS-SP5-AOBS system, Leica TCS-SPE microscope). Images are maximum projections of confocal Z sections unless otherwise noted. Images were post-processed with Fiji and assembled into figures using both Fiji and the Adobe Photoshop software.

FRET analysis

FRET images of live embryos were acquired with a Leica Sp5 confocal microscope equipped with HyD detectors, providing very low background levels. A 458nm laser was used to excite the sample. CFP and YFP emission signals were collected simultaneously through channel I (470–510 nm) and channel II (525–600 nm) respectively. To capture images, a 63X/1.4 oil immersion objective was used with a zoom of 2 to 3. CFP and YFP images were processed with the Fiji bundle of ImageJ (Wayne Rasband, NIH). Gaussian Blur (sigma=2) filtering was applied to both channels prior to calculations. The YFP image was thresholded and converted to a binary mask with background set to zero and covering only the region of interest. This mask was applied to each channel and the resulting images were used to, in this sequence: i) Normalise intensity in time to correct for photobleaching and/or fluorescence expression increase, ii) Equalize intensities (multiplication factor) to obtain identical average across the whole image. iii) The FRET ratio was obtained by dividing the YFP by the CFP images, thereby leading to a histogram distribution centred on 1 (thanks to the equalization). A specific Look-Up Table (“ICA” in Fiji) was employed to visualize FRET ratios <1 in blue and >1 in yellow, histograms limits were set from 0 to 2, and FRET ratios near 1 appeared black, see Fig. 5E,F and movie S2. The local differences in FRET ratios were thereby visualized within the branch and a specific tip-enhancement was clearly observed in 4 recordings.

Time-lapse experiments, image processing and kymograph analysis

Embryos were mounted as described in Caussinus et al. (Caussinus, Colombelli et al. 2008). Images were collected on a Leica TCS-SP5-AOBS system (Fig.5E,F, Fig.6B, movie S2, S3) or in an Andor Revolution spinning disk confocal (Fig.2E, Fig.3,
Fig.6A, Fig.7, Fig.S2 and movies S1). Sections were recorded every 100 sec (Fig.3A, movie S1), 60 sec (Fig.3B, 5E-F, movieS2), 90 sec (Fig.7A, Fig.S3 and movieS3), and 150 sec (Fig.7B). Z stacks were collected with optical sections at maximum 1μm intervals, and only LTp/GBs metameres from fourth to eighth were analysed. Image processing was made with Fiji (http://fiji.sc/wiki/index.php/Fiji) and custom programming scripts in Fiji. The Z stacks projections were corrected in X and Y dimensions by manual registration using a reference point tracking. Figure 5 and data on the velocity of the tip cell were generated with a custom kymograph macro in Fiji. The number of metameres analysed are indicated for each conditions: WT(19), Rho$^{DN}$ (36), Cdc42$^{DN}$ (37), Rac$^{DN}$ (16), Rho$^{ACT}$ (3), Cdc42$^{ACT}$ (8).

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Figure Legends

Figure 1. A-E: Overview of tracheal development with a focus on LTP/GBs (highlighted in blue). F-L: LTP/GB morphogenesis at budding (F-H), elongation (I-L) and intercalation (K-L). F’-L’: same panels showing DE-Cadherin junctional accumulation; dotted lines outline the contour of LTP/GBs. M1-M2: Imaris 3D reconstitution at two planes of LTP/GB cells labelled with nuclear and junctional marker shows the transient pyramidal-like cell arrangement (in grey, the clipping tool of the Imaris software allowing cutting and following the 3D structure in different planes). N: Schematic representation of cell arrangements during LTP/GB morphogenesis (leading cell in red and trailing cells in green, for simplification only the cell body of some trailing cell is depicted from st 13 to 15).
**Figure 2.** A-B: Individual cell labelling unveils cell shapes in LTp/GB morphogenesis at budding. C: The tip cell (red asterisk) produces a big lamelipodia and filopodia but trailing cells (white asterisks) do also emit filopodia. D: The protrusion from the tip cell changes its orientation at stage 14. E1-E3: Snapshots from a live recording of a LTp/GB with distinct labelling of some cells with the rainbow system to clearly show filopodia from trailing cells (black and white images show the red (E1) and green (E2,E3) channels)

**Figure 3.** A0-A7: Snapshots from an *in vivo* recording of LTp/GB cells randomly expressing the GFP (green) or RFP (red) with the brainbow system (see methods). Two tip cells (in green) move in divergent directions and exchange neighbours. B0-B4: Snapshots from an *in vivo* recording of LTp/GB cells labelled with nuclear (red) and cytoplasmic (green) markers and corresponding cartoons to show individual tracing of the whole cells. (Time in snapshots here and other figures is hours:minutes:seconds:mseconds, unless otherwise stated)

**Figure 4.** A-D: From stages 12 to 15, the apicobasal axis of LTp/GB trailing cells shifts from parallel to perpendicular to the direction of branch elongation (white double arrows) but remains parallel for the tip cells (red double arrows). Individual clones labelled by GFP flip-out clones. E,F: Actin strongly accumulates basally in tip cells (red arrows) while there is a transition from strong basal (white arrowhead) to apical accumulation in trailing cells (white arrow), as detected by expression in group of cells of either actGFP (green) at stage 12 (E) or btl-RFPMod (red) at stage 15 (F). G,H: *bnl* ectopic expression confers LTp/GB trailing cells features of tip cells in terms of apicobasal axis orientation (double arrows) and smaller apical surfaces as unveiled by Crb accumulation (green), and basal actin accumulation (by btl-RFPMod, red in H; red arrows). Ectopic *bnl* expression driven by *insc*-Gal4 (G) and *en*-Gal4 (H). I: Schematic representation of a leading (red) and a trailing (blue) cells according to the features at the different stages indicated.

**Figure 5.** A: Tracheal cells fail to migrate in a *btl* null mutant (*btlLG19*) labelled with src-GFP. B: Rescue of the *btlLG19* phenotype by *btl* tracheal expression (this rescue is not always complete probably due to delayed expression with the GAL4/Uas system).
C,D: Examples of the rescue of the btl<sup>LG19</sup> phenotype by btl expression in individual cells (in red); note that btl positive cells accumulate at tip positions (arrows) and are associated with clusters of migratory cells (compare to A). btl<sup>+</sup> flip-out clones are generated by heat shock (mat&met). E,F: Snapshots from an in vivo recording of a LTp/GB from an embryo bearing a (FRET)-based sensor with a distinct signal at the leading cell positions, both at the ventral side and at the site of the future branch fusion.

Figure 6. A<sub>1</sub>-A<sub>3</sub>: In vivo recordings show long cytoplasmic extensions between close cells that get apart. B: Individual cell labelling in vivo shows these extensions to accumulate actin; same panels to show shape of individual cells (B’) and actin (btl-RFPMoe) accumulation (B”). C-I: Phenotypes upon tracheal expression of constitutively active and dominant negative forms of Rac, Rho, and Cdc42. Most of LTp/GBs do not elongate upon Rho and Cdc42 constitutive activity (red crosses) and Rho and Cdc42 inactivation causes breaks (asterisks); same panels to show DE-Cadh junctional accumulation (C’-I’). J-P: Phenotypes associated with expression of mutant RhoGTPases in individual cells (in green) in otherwise wild-type LTp/GBs (wild-type cells in red); J’-P’: same panels to show DE-Cadh junctional accumulation.

Figure 7. A-B: Snapshots from in vivo recordings of LTp/GBs from a wild-type embryo (A) and from an embryo upon tracheal expression of a dominant negative form of Cdc42 (B); in red, a nuclear marker; in green a-Cat-GFP. The corresponding kymograph of each metamere (indicated by the white arrow) is shown in the right panel, each line indicating the position of each nucleus at different times, thus allowing evaluating their displacement. (C) A model for the specification of leading and trailing cells in LTp/GB morphogenesis.
Individual btl+ cells in btl−/− embryos

btl gal4, UAS-CFP::Rac1::YFP
**A** α-catGfp -nls mcherry WT

**B** α-catGfp -nls mcherry UAS CDC42DN

**C**
- **Rho activity**
- **Cdc42 activity**

Modulation of Cluster cells properties
- Adhesion
- Motility

**FGF signalling**
- **Rac activity**

**Leading cell**
- Large apical surface
- Apico-basal axis perpendicular to the proximo-distal direction of the migration
- Actin accumulation at the apical membrane

**Trailing cell**
- Small apical surface
- Apico-basal axis in the proximo-distal direction of the migration
- Actin accumulation at the basal membrane
**Fig. S1.** Ltp/GB cells at different stages visualised by btlgal4UASsrcGFP (green). In red, and in the corresponding black and white panels localisation of the apical markers Crb (A-D), SAS (I-K), DaPKC (L-M) and the baso-lateral marker Dlg (E-H). Note that images in E-H are single z planes.
Fig. S2. A-G: Details of cell protrusions and cell junctions as visualised by MoeGFp (panels 1-5) and a -catGFP (panels 5-7) respectively in wt (A) and upon tracheal expression of RhoDN(B), RhoACT(C), Cdc42DN(D), Cdc42ACT(E), RacDN(F), RacACT(G) mutant variants. Arrows and arrowheads indicate protrusive activity and the junctional organisation of leading and trailing cells respectively. H-J: Whole mount in situ hybridisation to show bnl RNA accumulation (dark) at early 12 (H), late 12 (I), 13 (J) stages; tracheal cells and nuclei are recognised by tracheal expression of srcGFP and an anti-GFP antibody (green) and by anti-Tracheless antibody (red) respectively. Red and purple arrows indicate the leading cells that will become terminal and the fusion cells respectively; the corresponding coloured arrowheads show the closest bnl-expressing cells. K: Examples of btl rescue in a Dorsal Trunk (DT, K1) and in a Dorsal Branch (DB, K2) by btl expression in individual cells marked with a Btl antibody (red) in otherwise mutant cells visualised with the btlRFPMoe construct (green).
**Fig. S3.** A. Maximal length of apical and basal surfaces in trailing (Tc) and leading cells (Lc). Values were evaluated in 3D using the Imaris software measuring stainings with apical markers and a srcGFP construct; n indicates the number of cells analysed. B. Percentage of clones encompassing tip and non-tip cells in Ltp/GBs. n is the total number of cells belonging to the LTP/GBs in the clones scored for each condition; note the low number of cells in the LTP/GBs for Rac\textsuperscript{ACT} as few clones were found in these branches (Ba,Bb: examples for wt and Rac\textsuperscript{ACT} clone distribution respectively). C: Quantification of the phenotypes upon expression of RhoGTPase mutant variants in terms of number of elongating LTP/GBs per embryo and number of cells per branch; n, number of metameres analysed. D: Percentage of branches with different phenotypes upon expression of RhoGTPase dominant negative variants; numbers of metameres analysed as the ones indicated in C. E. Percentage of different degrees of phenotype severity in terms of elongating LTP/GBs upon expression of Rho\textsuperscript{ACT} or upon expression of Rho\textsuperscript{ACT} and one of two independent cat RNAi lines. RNAi technology has a very limited effect at these stages of tracheal development and accordingly we did not observe any effect upon E-cad RNAi expression, as we have already observed in previous experiments (unpublished data). However, we observed very reproducible phenotypes with two independent cat RNAi lines. In each case, the elongating LTP/GBs per hemi-embryo were counted (in the five central metameres, see methods); branches with greater proximo-distal elongation were scored and listed separately (higher elongation). F. Tracheal placodes in btl mutant embryos (a) and in btl mutants upon tracheal expression of Rho\textsuperscript{ACT} (b); note less elongated placodes and ventral fusion in b, see quantification in % of these phenotypes (c).
Fig. S4. Crb accumulation in the LTp/GB in wild-type and upon the expression of the indicated constructs for mutant rhoGTPases. Crb is in red or black and white in all panels and cell shape is visualised by either btlMoeGFP or btl>>GMAGFP (in green).
**Movie 1.** Two tip cells of the LTp/GB (green) move in divergent directions and exchange neighbours to become the terminal and fusion cells. Cells are labelled by random clones expressing of either GFP or RFP (red) with the brainbow system (see methods). Sections were recorded every 1 min 40 for 4.45 hr.

**Movie 2.** Dynamic FRET pattern observed in the GB/LTp upon expression with btlGal4. (see methods). Sections were recorded every minute for 1 hr.

**Movie 3.** Elongating LTp/GBs from an embryo upon tracheal Cdc42<sup>TM</sup> overexpression; in red and green, nuclear (nls-mcherry) and junctional (a-cat) markers respectively. Dots identify a trailing cell (blue) acquiring the leading position at the expense of the previous one (red) and two proximal trailing cells (green and dark blue) exchanging positions. Sections were recorded every 1 min 30 for 5.45 hr.