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Distinct Roles for Two Receptor Tyrosine Kinases in Epithelial Branching Morphogenesis in *Drosophila*

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Summary

Branching morphogenesis is a widespread mechanism used to increase the surface area of epithelial organs. Many signaling systems steer development of branched organs, but it is still unclear which cellular processes are regulated by the different pathways. We have used the development of the air sacs of the dorsal thorax of *Drosophila* to study cellular events and their regulation via cell-cell signaling. We find that two receptor tyrosine kinases play important but distinct roles in air sac outgrowth. Fgf signaling directs cell migration at the tip of the structure, while Egf signaling is instrumental for cell division and cell survival in the growing epithelial structure. Interestingly, we find that Fgf signaling requires Ras, the Mapk pathway, and Pointed to direct migration, suggesting that both cytoskeletal and nuclear events are downstream of receptor activation. Ras and the Mapk pathway are also needed for Egf-regulated cell division/survival, but Pointed is dispensable.

Introduction

The elaboration of highly ramified tubular epithelia contributes to the formation of numerous organs. The lung, the kidney, the mammary gland, as well as a number of smaller epithelial glands make use of distinct mechanisms to drive tubulogenesis from bud-like outgrowth. Different cellular functions have been linked to the tube-forming processes as driving forces, including cell division, cell migration, cell rearrangement, cell shape changes, cell death, and cell repolarization (Affolter et al., 2003; Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003). In all cases investigated, growth factors control these processes, but how cellular behavior is coordinated in time and space to establish these beautiful branched tissues remains somewhat a mystery.

The *Drosophila* tracheal system serves as a paradigm by which to investigate the genetic and molecular basis underlying epithelial branching morphogenesis (reviewed in Affolter et al., 2003; Ghabrial et al., 2003; Uv et al., 2003). In a series of stereotyped events, architecturally distinct tubes are generated from an epithelial invagination via controlled cell migration, cell rearrangement, cell shape changes, and branch fusion events. The stereotyped branching process is controlled by a growth factor of the Fgf family encoded by the *branchless/fgf* (*bnl/fgf*) locus, which acts both as a chemoattractant and a patterning factor (Sutherland et al., 1996). Bnl/Fgf is secreted from distinct, nontracheal cells in positions around the tracheal bud, and it is toward these positions that tracheal cells ultimately migrate. The branching process occurs after cell division in the embryonic tracheal invagination has ceased; thus, branch formation and elongation does not involve the addition of cells within the branching structure. Therefore, studies on the development of the tracheal system during fly embryogenesis are characterized both by their strength to investigate cell shape changes and cell rearrangements linked to epithelial branch elongation and by their weakness to directly compare mechanisms of tubulogenesis to organs that involve considerable growth during development.

In late larval development, the tracheal system is remodeled extensively to give rise to new structures that will ultimately serve the adult organism. The remodeling events have been characterized at the morphological level, both in *Drosophila melanogaster* and in other insects (Manning and Krasnow, 1993; Whitten, 1980), but little is known about the genes and the molecules that orchestrate the remodeling process. In a pioneer study, Sato and Kornberg (2002) have shown that the formation of a particularly intriguing structure, the air sac of the dorsal thorax, develops during the period of the third larval instar under the control of the same molecule, Bnl/Fgf, that steers the branching process in the embryo. During the third larval instar, air sac precursor cells bud from a particular tracheal branch in response to Bnl/Fgf, and they proliferate and migrate to the adepithelial layer of the wing imaginal disc. In this system, Fgf signaling is proposed to act as a mitogen, a chemoattractant, and an instructive determinant, reprogramming the cells in the tracheal branch to become air sac tracheoblasts.

Since Fgf signaling is the driving force for tracheal branching morphogenesis in the embryonic and larval tracheal system in *Drosophila* and has also been linked to branching morphogenesis in the vertebrate lung (Affolter et al., 2003; Hogan and Kolodziej, 2002), both molecular and cellular studies have been undertaken to better understand how this signaling pathway controls the morphogenesis process. Live imaging studies have shown that Bnl/Fgf signaling induces dramatic cytoskeletal rearrangements manifested in filopodia formation (Ribeiro et al., 2002; Sato and Kornberg, 2002), and thus presumably induces the motility of tracheal cells. Bnl/Fgf signals via a transmembrane receptor tyrosine kinase encoded by the *breathless/fgfr* (*btl/fgfr*) locus (Klambt et al., 1992). Previous studies have identified an adaptor protein called Downstream-of-Fgfr (Dof) (Vincent et al., 1998), which associates directly with Btl/Fgfr in a constitutive manner and serves as a receptor substrate (Petit et al., 2004; Wilson et al., 2004). Upon signal-induced phosphorylation, Dof recruits additional proteins, such as the phosphatase Corkscrew (Csw), the homolog of mammalian Shp2, to the activated ligand/receptor complex (Petit et al., 2004). Although receptor tyrosine kinase (RTK) signaling is known to be mediated to a large extent by the Ras/Map kinase

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pathway, it has been difficult to link this particular signaling module to cellular events underlying tracheal branching, partly due to the important maternal contribution of the proteins functioning in the Ras/Map kinase pathway in the egg. In addition, the maternal contribution of numerous other important components participating in a process that underlies cytoskeletal reorganization is to be expected since the cytoskeleton already has very important functions before zygotic transcription starts. Therefore, studies at later developmental stages should more readily allow for the use of genetic approaches (such as the analysis of mutant clones of cells generated by mitotic recombination) to investigate the requirement of such factors. Obviously, the developing air sac represents an ideal situation by which to further study the role of Fgf signaling in the control of epithelial organogenesis in general and in epithelial tubulogenesis in particular.

Results

Air Sac Outgrowth Is Equivalent to the Directed Enlargement of an Epithelial Sheet

The air sac of the dorsal thorax grows from a bud that arises during the third larval instar from a wing disc-associated tracheal branch (Figure 1A). To illustrate the development of the air sac, we used a GFP trap line expressing membrane bound GFP rather ubiquitously; tracheal cells were counterstained with an mRFP1-moesin construct under the direct control of the trachea-specific *breathless* (*btl*) enhancer (Ribeiro et al., 2004). From the early (Figure 1B) to late (Figure 1D) third instar stage, a bud-like structure grows out of the transverse connective and spreads on the wing imaginal disc epithelium; this outgrowth corresponds to the primordium of the air sac of the dorsal thorax.

In a previous study, it has been proposed that the air sac of the dorsal thorax forms de novo from a small group of wing imaginal disc cells, and that the resulting sac subsequently generates a tracheal lumen by an unknown process (Sato and Kornberg, 2002). Since, in the early *Drosophila* embryo, the lumen arises from an epithelial invagination via cell migration, we were wondering whether the cells in the growing air sac were epithelial in nature with a clear apical/basal polarity. For this purpose, we expressed a Dα-Catenin-GFP (Dα-Cat-GFP) fusion construct in the developing air sac and analyzed the distribution of GFP from early to late third instar larvae (Figures 1E–1G). Dα-Cat-GFP labels the adherens junctions (AJs) of epithelial cells (Oda and Tsukita, 1999). Clearly, the growth of the air sac was accompanied from the early stages onward by an outbulging of an AJ network, suggesting that most or all of the cells in the growing bud were epithelial in nature, and that a luminal space was generated at the apical side of the epithelial tracheal cells during outgrowth. To confirm this interpretation, we have made use of the recent identification of a protein, Piopio (Pio), which is apically secreted into the tracheal lumen in the embryo (Jazwinska et al., 2003). Indeed, the prospective luminal space in the outgrowing air sac was filled with Pio protein (Figures 1H–1J: Pio only; Figures 1H′–1J′: Pio and Dα-Cat-GFP), demonstrating that the air sacs

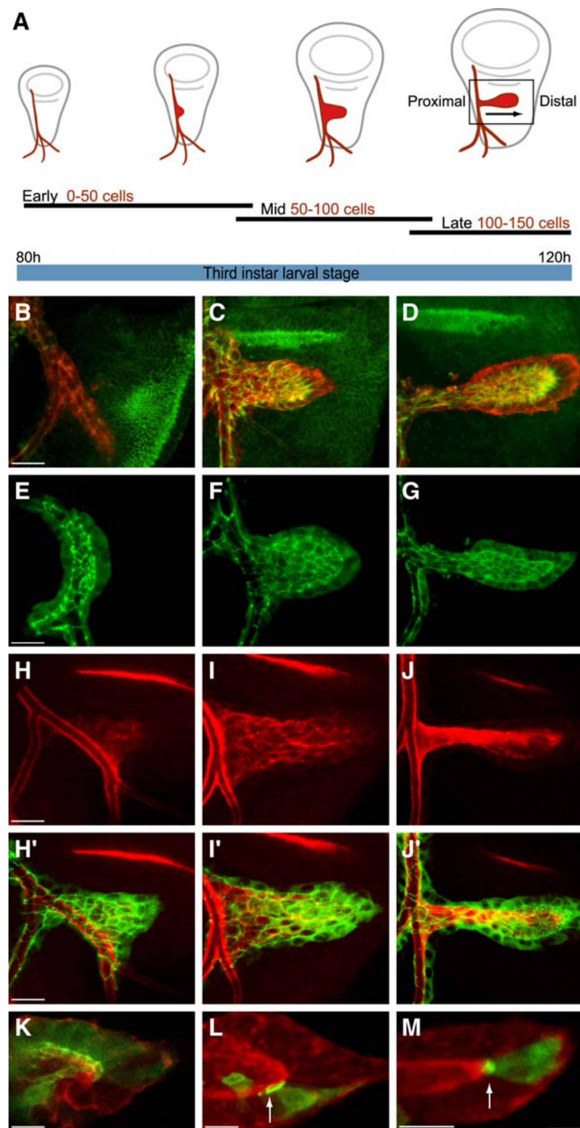


Figure 1. Development and Cellular Organization of the Air Sac of the Dorsal Thorax

(A) Schematic illustration of air sac development; adapted from (Sato and Kornberg, 2002).

(B–D) (B) Early, (C) mid, and (D) late third larval instar air sacs outlined with the *btlenhancer-mRFP1moesin* construct. Wing disc cells are visualized with a ubiquitously expressed GFP protein trap line.

(E–G) (E) Early, (F) mid, and (G) late third larval instar air sacs outlined with *btlGal4-UAS-Dα-cat-GFP* (*btlGal4* is a trachea-specific driver).

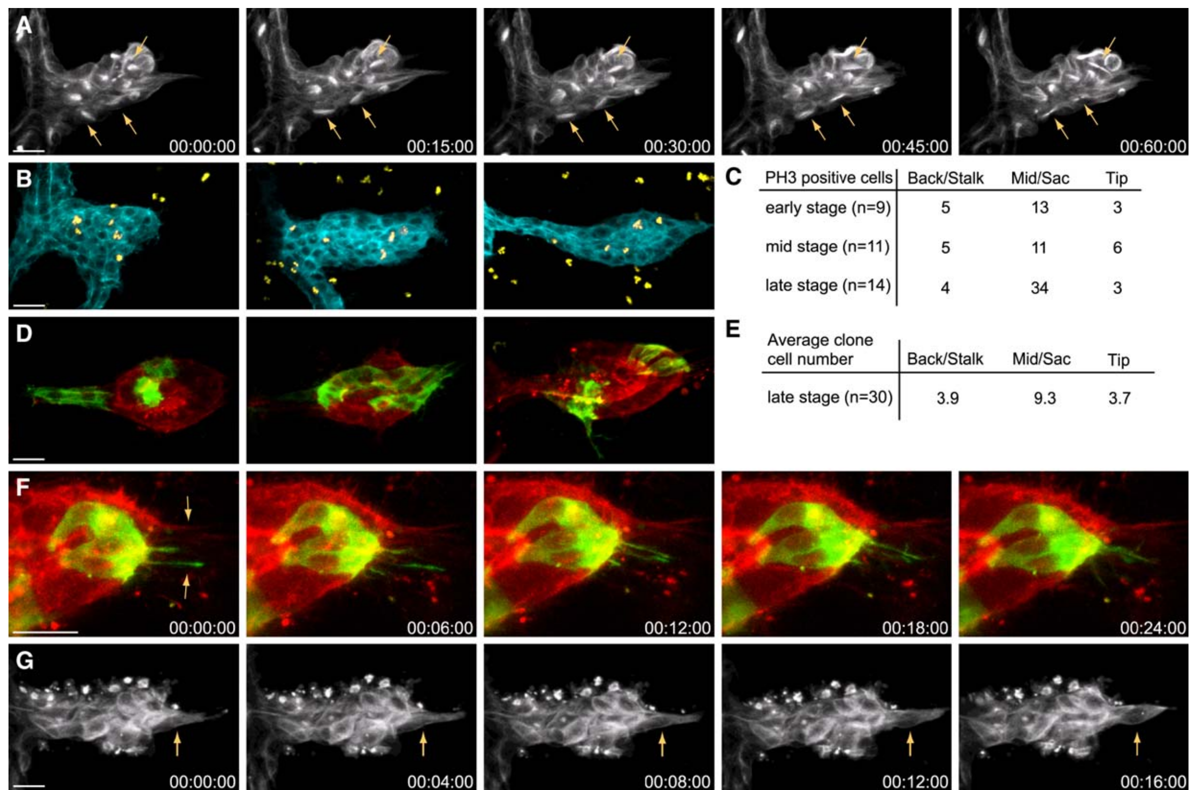
(H–J) Lumen formation visualized with anti-Pio. Pio is apically secreted into the luminal space. (H′–J′) Overlay with *btlGal4-UAS-Dα-cat-GFP.*

(K–M) Three independent flip-out clones, labeled with Dα-cat-GFP (green) and mRFP1-moesin (red). Dα-cat-GFP predominantly localizes to the subapical adherens junctions of the cell (see also white arrows in [L] and [M]).

The scale bars are 20 μm, except for (K) and (L) (10 μm) and (M) (8 μm).

consist of a sac-like epithelial sheet, generating a luminal space as they grow.

To test whether all cells maintained an apical-basal polarity during air sac budding, we labeled single tracheal cells by using a recently developed assay system that allows for the visualization (and manipulation) of individual tracheal cells in vivo (Ribeiro et al., 2004). When



PH3 positive cells	Back/Stalk	Mid/Sac	Tip
early stage (n=9)	5	13	3
mid stage (n=11)	5	11	6
late stage (n=14)	4	34	3

Average clone cell number	Back/Stalk	Mid/Sac	Tip
late stage (n=30)	3.9	9.3	3.7

Figure 2. The Thoracic Air Sac Develops via Cell Division and Cell Migration

(A) Pictures taken from a time-lapse analysis of air sac development; all cells express GFP-*tau*. Mitotic spindles can be observed at various locations in the air sac (yellow arrows).

(B) Proliferating cells visualized with anti-phospho Histone H3 antibody (yellow); air sac cells express GFP-actin.

(C) Quantification of anti-phospho Histone H3 staining. “Back/stalk” refers to the proximal part of the air sac; “Tip” refers to the distal part of the air sac.

(D) Flip-out clones in the “stalk-,” “mid-,” and “tip-” regions of the thoracic air sac. Clones are labeled with GFP-moesin (green); all air sac cells express mRFP1-moesin.

(E) Quantification of flip-out clones.

(F) Pictures from a time-lapsed flip-out clone labeled with GFP-moesin (green), highlighting the dynamics of filopodia at the tip (arrow). All air sac cells express mRFP1-moesin (*btlenhancer-mRFP1moesin*).

(G) Time-lapsed air sac expressing GFP-Tau. Note the changing position of the migrating cell at the tip (yellow arrow).

The scale bars are 20 μm, except in (F) (8 μm).

we used this scenario in the presence of a *UAS-Dα-Catenin-GFP* chromosome, we found that, in virtually all cases, such individually labeled air sac cells contacted the lumen and formed AJs with neighboring cells, even when these cells were located at the tip of the outgrowing air sac (Figures 1K–1M, arrows). The same conclusion was reached when we analyzed the expression of GFP-moesin in single air sac cells; cells at the tip made clear contact with the lumen (Figure 2F). Therefore, we conclude that the air sac is sculpted from an epithelial cell layer, which expands and at the same time generates an apical luminal space filled with secreted proteins.

Directional Air Sac Outgrowth Is Brought About by Unlocalized Cell Division and Directed Migration

The surface expansion of the air sac could be brought about by cell migration, cell shape changes, cell additions (cell proliferation), or a combination of these processes. Tracheal cells in the air sac have been shown to divide (Sato and Kornberg, 2002), and we wanted to investigate whether cell division was restricted to certain areas during development. Therefore, we analyzed the regional dynamics of spindle formation in air sacs expressing a GFP-Tau protein, by using live imaging (Figure 2A and Movie S1; see the Supplemental Data available with this article online), or, alternatively, we stained developing air sacs with antibodies against phosphorylated Histone H3 (pH3), a marker often used to identify dividing cells (Shibata et al., 1990). In cultivated air sacs (see Experimental Procedures for culture conditions and live imaging procedures), spindles formed at all positions (arrows in Figure 2A), and we did not find large differences in the frequencies of spindle formation between proximal or distal regions. To quantify cell division rates in defined regions more precisely, we stained fixed preparations by using the pH3 antibody and counted mitotic cells (Figures 2B and 2C). The growing air sac was subdivided into three regions, the proximal stalk, the centrally enlarged sac, and the distal tip. Although there was an approximately 2-fold increase in H3-staining nuclei in the central region (which also has more cells), cell divisions occurred in the proximal and the distal regions at similar rates, both in

early and in late third instar stages (Figure 2C), arguing that directional outgrowth is not controlled by local cell proliferation at the tip. We also measured the size of clones induced just prior to air sac outgrowth in early third instar larvae that were analyzed in the late third instar. We found that clones in the center of the air sac were approximately twice as large as clones at the tip or in the stalk (Figures 2D and 2E).

It has previously been shown that Bnl/Fgf acts as a chemoattractant for air sac cells and is expressed in a small group of cells in the columnar epithelium of the wing imaginal disc, just in front of the growing air sac (Sato and Kornberg, 2002). Using two-color live imaging on cultivated air sac preparations, we found that several cells at the tip of the air sac formed extensive filopodial and lamelipodial extensions, suggesting that several cells at the tip respond to Fgf signaling (Figure 2F and Movie S2). In addition, time-lapse movies revealed that cells indeed moved in the direction of the extensions, demonstrating that cells change their respective position with regard to the underlying wing disc epithelium over time (arrow in Figure 2G; see also Movie S3).

Fgfr and Egfr Signaling Are Essential for Proper Air Sac Outgrowth

Throughout development, a limited number of signaling pathways are used repetitively to control growth, patterning, and function of organ systems. Since only Fgf signaling has thus far been linked to air sac formation during the larval stages, we wanted to find out whether other pathways were also involved. The occurrence of cell divisions during air sac formation in larval stages allows for the application of somatic genetic approaches by which to study gene function. Therefore, we generated marked loss-of-function clones of key components acting in different signaling pathways by using the MARCM technique (Lee and Luo, 1999), and we analyzed the capacity of mutant cells to contribute to air sac development and to populate different areas of the developing air sac (see Experimental Procedures).

Using clones of labeled wild-type cells, we first determined how wild-type cells behave during air sac formation. FRT-driven recombination was induced in the early embryo, before tracheal cells divide twice during the invagination process. The distribution of marked patches of cells was then analyzed in the large dorsal air sacs of late third instar larvae (Figures S1D–S1G; see the Supplemental Data available with this article online). We classified the wild-type clones with regard to two criteria. First, we determined whether a marked clone reached the tip of the growing air sac, and, second, we analyzed the size of the clones (large, medium, and small; see Experimental Procedures for specific criteria). Approximately 70% of the wild-type clones reached the tip of the air sac; 50% of these clones were rather large, 30% were of medium size, and 20% were small. Although larger clones had a higher tendency to contribute to the tip of the growing air sac (or to any other region of the sac), small clones were also capable of reaching the tip (50% of the small clones reached the tip of the extending air sac; see Figures S1D, S1E, and S1H).

We then induced marked clones of cells unable to respond to a number of signaling molecules to test for an involvement of the latter in air sac development. In order to do so, we generated homozygous mutant clones lacking either Thick veins (necessary for Dpp/BMP signaling; Figures 3C and 3D), Smoothened (necessary for Hh signaling; Figures 3E and 3F), Frizzled1/2 (necessary for Wnt signaling; Figures 3G and 3H), Pvr (necessary for VEGF ligand-dependent signaling; Figures 3I and 3J), Egfr (necessary for Egf signaling; Figures 3K and 3L), or Btl/Fgfr (necessary for Bnl/Fgf signaling; Figures 3M and 3N). Only mutations in *egfr* and *btl/fgfr* dramatically changed the behavior of clones with regard to their size and their capacity to populate the tip of the air sac. Clones mutant for *egfr* were generally much smaller and contained fewer cells than wild-type clones (Figure 3L, compare with Figure 3B). A few clones still did populate the tip of the growing air sac (Figure 3K; also see below), but the frequency of *egfr* clones found at the tip ($\sim$20%) was $\sim$2.5-fold lower when compared to the frequency with which small wild-type clones were identified at the tip ($\sim$50%; see Figure S1). Quite in contrast to *egfr* mutant clones, cells lacking Btl/Fgfr grew to large sizes, similar to wild-type clones. However, such clones were never found to contribute to the tip of the air sac (Figure 3N).

Since it has been shown that air sac cells directionally migrate toward sources of Bnl/Fgf both in the embryo and the larvae (Sato and Kornberg, 2002; Sutherland et al., 1996); that tracheal cells form filopodia and lamelipodia, structures characteristic of migrating cells, toward Bnl/Fgf sources (Ribeiro et al., 2002; Sato and Kornberg, 2002); and that cells lacking the Btl/Fgfr receptor do not die in the air sac (data not shown; also see below), we interpret the failure of cells lacking Btl/Fgfr to populate the tip of the growing air sac as a failure of cells to actively migrate toward Bnl/Fgf (see also Discussion); neighboring wild-type cells take up the position of the leading, migrating cells, leaving the mutant cells behind. Among the signaling pathways we analyzed, we found that only the Bnl/Fgfr signaling pathway was strictly required for cells to be at the tip. Although clones lacking Btl/Fgfr did not populate the tip of the air sac, clones did grow to large sizes, indicating that Fgf signaling is not essential for cells to divide during dorsal air sac growth; indeed, *btl/Fgfr* mutant cells can be labeled with the pH3 antibody (see below and Figures 5J and 5J′). Quite in contrast, we find that Egfr is essential for the formation of large-size clones; Egfr signaling might thus be required for cell division and/or survival during air sac outgrowth.

Dissection of the Fgf Signaling Pathway in Air Sac Formation

In the *Drosophila* embryo, Fgf signaling is mediated by Downstream-of-Fgfr (Dof), a cytoplasmic protein linking the activated Btl/Fgfr to downstream signaling mediators such as the phosphatase Csw (Petit et al., 2004; Vincent et al., 1998). When we analyzed the behavior of cells mutant for *dof*, we found that although such clones grew to large sizes, they never reached the tip of the growing air sac (Figure 4C), suggesting that the mutant cells were impaired in their migratory behavior, thus mimicking the phenotype of *btl/fgfr* mutant cells. Therefore, we used this experimental approach to analyze the requirement of the Ras/Map kinase pathway in Fgf-regulated cell migration. We argued that the induction of clones lacking

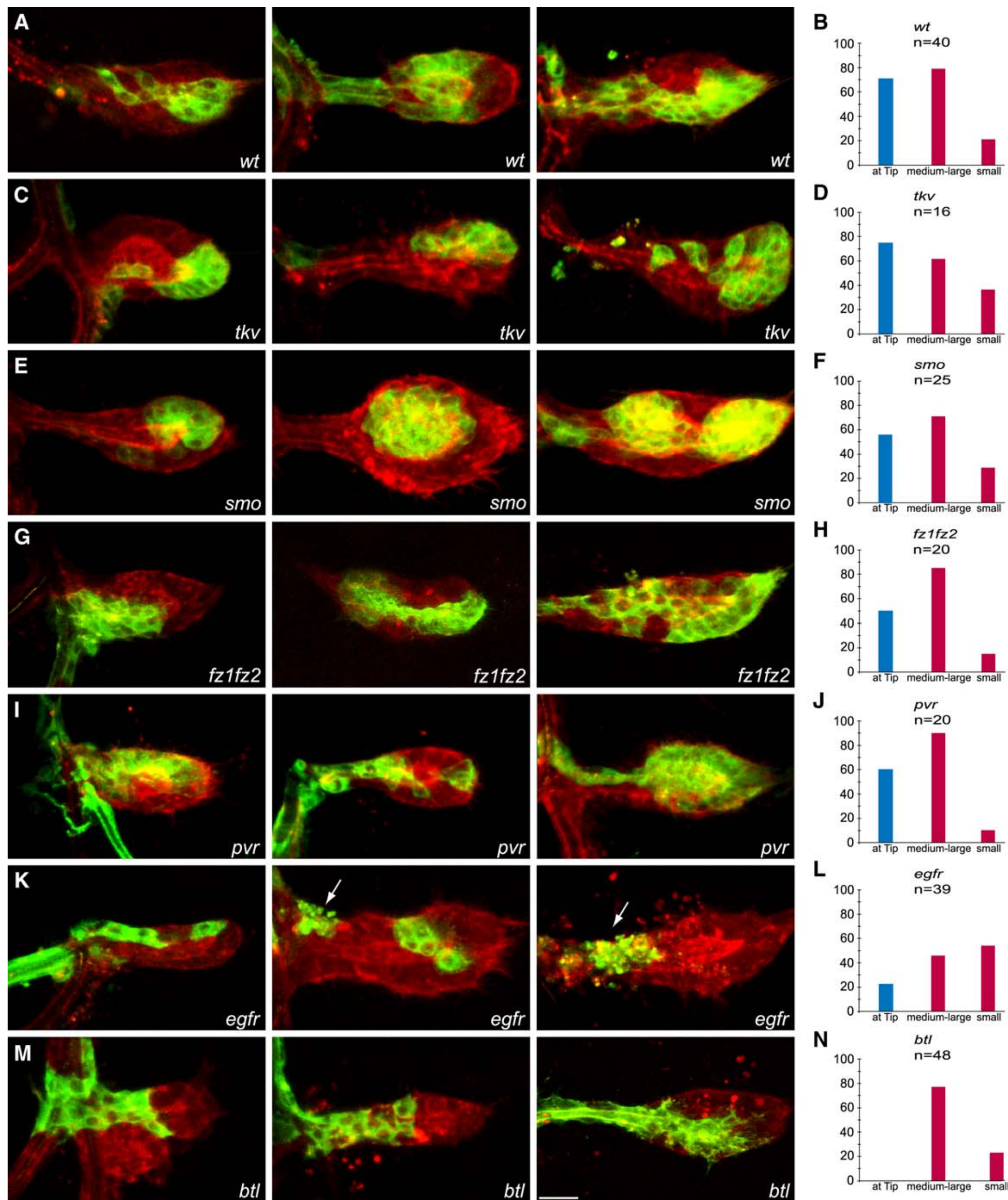


Figure 3. Two RTK Signaling Pathways Are Essential for Air Sac Development

(A) Three representative wild-type clones labeled with CD8:GFP. The clones are labeled with GFP (green); the entire air sac is labeled with mRFP1-moesin (*btl* enhancer directly fused to mRFP1-moesin).

(B) Statistics of wild-type clones. In this and all subsequent panels showing a graphic display of the results, the blue bar indicates the percentage of clones found to extend to the tip. Red bars represent size distribution. Units in percentage. n = number of air sac clones analyzed.

(C) tkv^{Q12} mutant clones (amorphic allele).

(D–N) (D, F, H, J, L, N) Statistics are as described above. (E) smo^{3} mutant clones (amorphic allele). (G) $fz^{p21}fz2^{C1}$ mutant clones. (I) pvr^{5363} mutant clones (amorph or strong hypomorph allele). (K) $egfr^{K35}$ mutant clones (amorphic allele). Some clones appear fragmented (white arrow). (M) $btl^{H82\Delta3}$ mutant clones (strong hypomorph or amorphic allele. Identical results were obtained with the amorphic allele btl^{LG18}).

The scale bar is 20 μm.

the small GTPase Ras (or other components with a strong maternal contribution) in the early embryo would allow the mutant cells to use the maternal protein for migration, division, and survival until the early third instar larval stage, when we assayed whether such clones were able to migrate or divide and thus contribute to air sac development during the third larval stage. Indeed, *ras* mutant clones were recovered in the air sac, but, in all cases, such clones were small and never reached the tip (Figure 4G), mimicking the phenotype

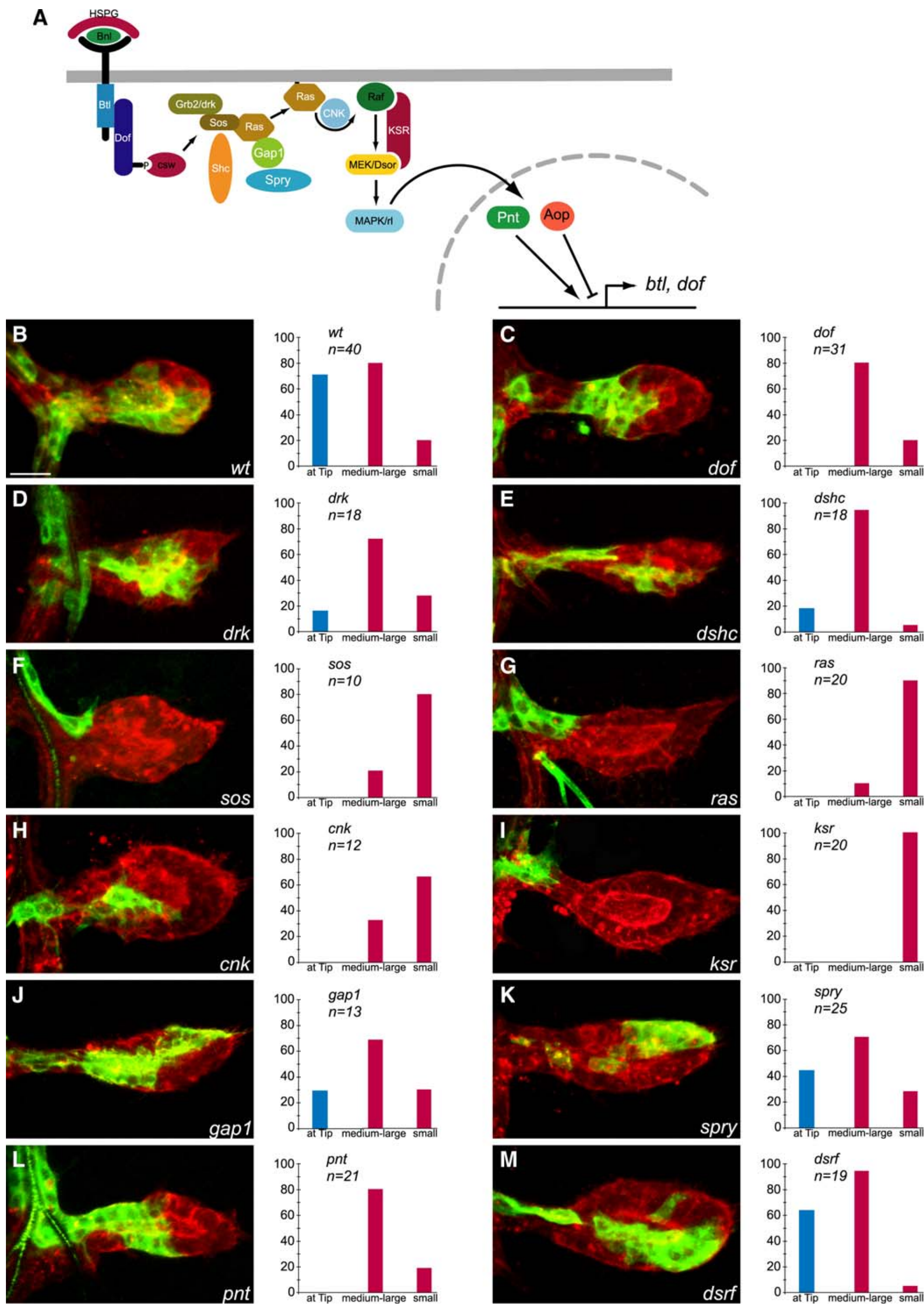


Figure 4. Analysis of the Fgf Signaling Pathway in Air Sac Development

(A) Components of the Fgf signaling pathway (see text for details). Components tested in this study are indicated in white fonts.
(B) Wild-type clone.
(C) dof^{P1740} mutant clone (amorph or strong hypomorph).
(D) $drk^{\Delta P24}$ mutant clone (amorphic allele).
(E) $dshc^{BG}$ mutant clone (amorphic allele).
(F and G) (F) sos^{X122} mutant clone. Identical results were obtained with sos^{SF15}. (G) ras^{X7B} mutant clone (amorphic allele). (F and G) Note that neither large *ras* nor *sos* clones were recovered. A few clones were classified as medium sized.
(H) $cnk^{l(2)16314}$ mutant clone.
(I) $ksr^{S\text{-}638}$ mutant clone.
(J) $gap1^{B1}$ mutant clone (amorphic allele).
(K) $spry^{D5}$ mutant clone.

of both *btl/Fgfr* mutant cells (in not reaching the tip) and *egfr* mutant clones (in being of small size). Virtually identical results were obtained for Son-of-Sevenless (Sos) (Figure 4F), which is required for Ras activation, and with Connector enhancer of Ksr (Cnk) (Figure 4H) and Kinase suppressor of Ras (Ksr) (Figure 4I), two modulators of the Map kinase pathway. Clones lacking the adaptor proteins Drk and Shc also showed a reduced frequency at the air sac tip (only ∼17% reached the tip as compared to 70% in the case of wild-type clones, Figures 4D and 4E); this finding is consistent with previous reports demonstrating a certain redundancy in the functional requirement for these adaptor proteins in RTK signaling (Hou et al., 1995; Luschnig et al., 2000). Two negative regulators of the Fgf signaling pathway, Gap1 and Sprouty, were not essential for migration since cells mutant in either of these components were frequently found at the tip, although with a 2- to 3-fold reduced frequency when compared to marked wild-type cells (Figures 4J and 4K).

Thus far, our results demonstrate that Ras signaling is absolutely essential for cell migration at the tip of the air sac, and that it is also required for cell division and/or cell survival throughout the air sac during the outgrowth phase. The results obtained with Cnk and Ksr strongly suggest that Ras signals via the Map kinase pathway to regulate migration and division. We wanted to confirm this interpretation by using mutations in generic components of the Mapk pathway such as Raf, Mek/Dsor, or Mapk/rl. Unfortunately, the latter is between the centromer of the second chromosome and the FRT we used for the analysis, making its study impossible. Both *raf* and *mek/Dsor* are on the X chromosome, and, so far, we failed to induce clones at a significant frequency by using FRT-containing X chromosomes. However, our interpretation was indirectly confirmed when we analyzed Pointed, a nuclear mediator of Map kinase signaling (Klambt, 1993).

To investigate whether Ras signaling in tracheal cell migration requires transcriptional changes, we determined whether Pointed (Pnt), an Ets domain transcription factor mediating Ras/Map kinase signaling in many cells in *Drosophila* (Brunner et al., 1994; O'Neill et al., 1994), was required for cells to populate the migrating tip of the air sac. We found that cells lacking Pnt never migrated to the tip, although they grew to large sizes (Figure 4L). Apparently, Pnt is required to mediate Fgf signaling in cell migration in the air sac, but it is not essential to mediate Egfr signaling in cell division and cell survival. In contrast to *pnt*, we find that *blistered/DSrf*, a second gene important to mediate Bnl/Fgf patterning function in the embryonic trachea (Guillemin et al., 1996) and required for the migration of border cells (Somogyi and Rorth, 2004), was neither required for cells to populate the tip nor for cell division (Figure 4M).

Egfr Signaling Is Essential for Cell Division and Cell Survival during Air Sac Outgrowth

Our initial analysis of the requirement of several signaling pathways revealed that two RTKs, Btl/Fgfr and Egfr, as well as the Ras/Mapk pathway, were essential for dorsal air sac formation. We wanted to better understand how Ras can be used in the same tissue at the same time for different cellular processes (Halfar et al., 2001).

As shown above, *egfr* mutant cells can contribute to the tip of the growing air sac, although the clones are relatively small. In the stalk of the air sac, cells lacking Egfr often appeared fragmented, a sign of cell death (arrows in Figures 3K and 5B). Indeed, when we stained *egfr* mutant cells with anti-Drice, a marker for apoptotic cells, we found a strong accumulation of this protein (Figures 5C, 5C′, 5C″, and 5C‴). When we expressed p35, a viral anti-apoptotic protein (Bump et al., 1995), in *egfr* mutant cell clones, these clones grew to larger sizes and were able to populate the air sac tip at a significantly higher frequency than in the absence of p35 (Figure 5D; compare to Figure 5B). These experiments establish that Egfr is dispensable for migration, and that migration is exclusively triggered by one of the two RTKs, Btl/Fgfr. The experiments shown in Figure 3M and Figure 5J also demonstrate that, during the growth phase, Btl/Fgfr signaling is dispensable for cell division; clones can grow to large sizes, although they fail to populate the tip. This same result was obtained with two other components, which are exclusively used by the Fgfr signaling pathway in the air sac (and not the Egfr pathway), namely, Dof and Pointed (Figures 4C and 4L). Thus, migration and cell division are controlled by two different RTKs, but both RTKs signal via the activation of Ras and the Map kinase pathway to regulate these different cellular outcomes.

How does Ras control cell migration in the tip and cell division in the remaining air sac? To start to address these questions, we first tested whether high levels of constitutive active Ras were compatible with directional cell migration and expressed RasV12 in wild-type tissue in small cell clones (see Experimental Procedures). Interestingly, such clones expanded considerably and grew to large sizes in the center of the air sac or in the stalk, resulting in bulgy outgrowths; however, clones expressing RasV12 never contributed to the tip of the air sac (Figure 5E). This finding suggests that unrestricted levels of Ras in a cell perturbed its capacity to read out the migratory cues (presumably the Bnl/Fgf ligand); wild-type cells were apparently much better in taking up the leading position. In line with this interpretation, we found that expression of an activated version of Btl (Torso-Btl/Fgfr) also resulted in bulky outgrowths. In addition, cells expressing the chimeric Btl receptor never populated the tip (Figure 5F). Quite in contrast, activated Egfr (Egfr fused to a lambda dimerization site) was not able to perturb air sac guidance, but it also triggered higher division rates in clones, generating bulgy outgrowths (Figure 5G).

To test whether single cells expressing activated receptor constructs changed their behavior with regard to cytoskeletal dynamics, we induced the expression of either the activated version of Fgfr or Egfr in early third instar stages and analyzed the behavior of such cells with live imaging of cultured discs. Cells in the stalk of the air sac expressing activated Fgfr showed extremely

(L) *pnt*$^{\Delta 88}$ mutant clone. Identical results were obtained with *pnt*$^{\Delta 33}$ and *pnt*$^{\Delta P78}$.
(M) *bs*^{14} mutant clone.
In all panels, blue bars represent the percentage of clones at the tip. Red bars indicate clone size distribution. Clones are labeled with (D, E, H, and M) CD8:GFP or (B, C, F, G, and I–L) GFP-actin. The scale bar is 20 μm. n = number of air sac clones analyzed.

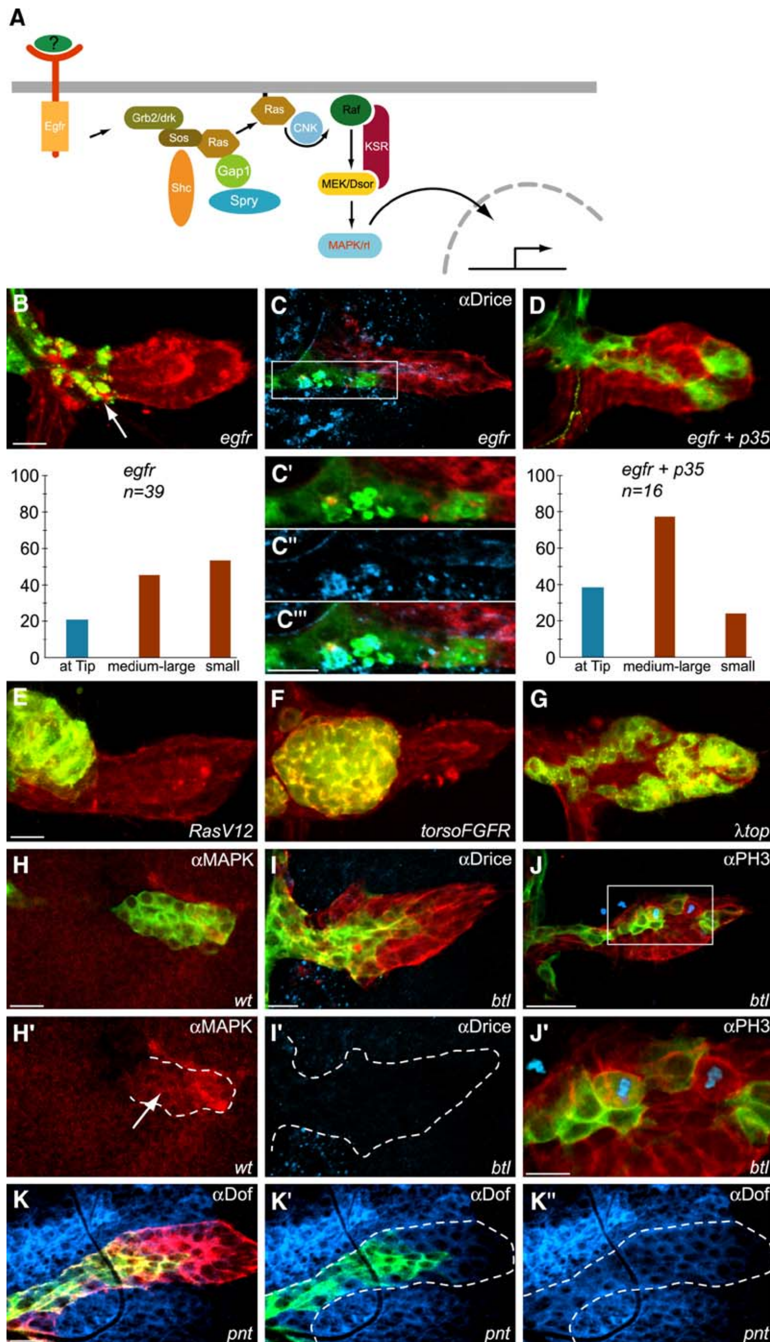


Figure 5. Distinct Requirement for Two RTKs in Air Sac Development

(A) Egfr signaling pathway components (see also Figure 4 and text for details).

(B) *egfr*^{K35} (amorphic allele) mutant clones; statistics are as in previous figures. White arrows point to clones showing signs of cell death.

(C–C‴) (C) *egfr*^{K35} mutant clones probed with anti-Drice antibody to detect apoptotic cells. (C′–C‴) Higlights of boxed area in (C) displaying the (C′) egfr mutant clone, (C″) anti-Drice staining only, and (C‴) overlay.

(D) *egfr*^{K35} mutant clones expressing UAS-p35 to prevent apoptosis. Note the shift in clones found at the tip (blue bar) as well as the shift in clone size (red bars) compared to (B).

(E–G) (E) MARCM gain-of-function clone expressing an activated version of (E) Ras (*UAS-Ras*^{V12}), an activated version of (F) Btl/Fgfr (*UAS-Torso*^{4021}*-Btl/Fgfr*), and an (G) activated version of Egfr (*UAS-λTop*).

(H and H′) (H) Section of an air sac expressing *btlGal4-UAS-actinGFP* (green) and stained with anti-dpERK antibody (red). (H′) dpERK staining only. The air sac is outlined with the dotted line. Note that dpERK staining is strongest in tip cell nuclei and is also visible in the cytoplasm behind the leading tip (arrow).

(I and I′) *btl*^{LG18} mutant clone probed with anti-Drice (blue).

(J and J′) (J) *btl*^{LG18} mutant clone probed with anti-pH3 (blue). (J′) Highlight of boxed region in (J).

(K–K″) Section of a *pnt*$^{\Delta 88}$ mutant clone (green) stained with anti-Dof antibody (blue). (K) Overlay picture. (K′) *pnt*$^{\Delta 88}$ clone (green) and Dof protein (blue). (K″) Dof protein shown only. Note that Dof is expressed in air sac cells, even in the absence of *pnt* (dotted line outlining air sac), as well as in adepithelial cells of wing imaginal disc.

The scale bar is 20 μm, except in (C) 15 μm, (J) 30 μm, and (J′) 10 μm. n = number of air sac clones analyzed.

dynamic cytoskeletal activity and formed large lamelipodia extending away from the air sac (Movie S4), similar to cells at the tip. Quite in contrast, cells expressing activated Egfr did not show increased lamelipodia formation, and their basal side remained relatively inactive (Movie S5).

Since the expression of constitutive active versions of the two different RTKs during air sac growth had different effects, we wanted to investigate whether the endogenous receptors activated the Ras/Mapk pathway to different levels in wild-type air sacs. In order to monitor the strength of Mapk signaling, we used an antibody recognizing the double-phosphorylated form of Erk, dpErk. Indeed, we detected high levels of dpErk in the nucleus of tip cells (Figures 5H and 5H′); lower dpErk levels were found in the cells in the center of the air sac, and dpErk was mostly cytoplasmic (Figure 5H′, arrow).

From all of the above-mentioned data, we conclude that air sac development makes use of two distinct RTKs to control directed organ extension via cell migration (Fgfr) and organ growth via cell division (Egfr). The possible implications of these findings will be considered in the Discussion.

Discussion

Branching morphogenesis is a process often used in the formation of tubular epithelial structures, such as the

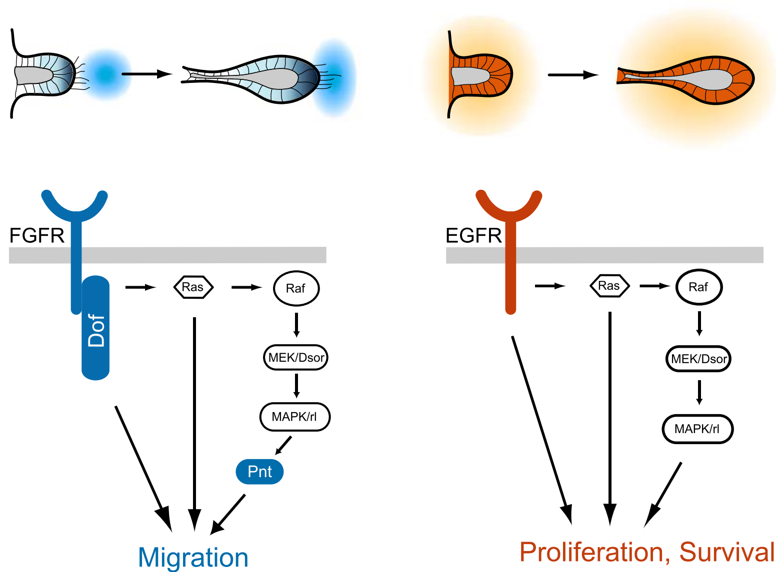


Figure 6. Distinct Roles for Two RTKs in Epithelial Branching Morphogenesis

Coordinated outgrowth of the air sac of the dorsal thorax requires signaling from two RTKs. Btl/Fgfr is absolutely required at the tip of the growing air sac (dark blue), whereas Egfr signaling is required in all areas of the air sac (red color). Btl/Fgfr signaling triggers cell migration at the tip of the growing structure and coordinates the direction of outgrowth of the air sac with the underlying wing imaginal disc from which the Bnl/Fgf ligand (light blue) is secreted. Egfr signaling is required for cells to divide properly and/or for cells to survive (putative EGF expression indicated with light orange). Both RTKs share core components of the Ras/MAPK pathway (indicated with black rim and font in the schematic pathways below). The essential components downstream of the two RTKs are different, triggering distinct cell behaviors within the same cellular field.

lung, the kidney, a number of glandular organs in mammals, as well as the tracheal system in *Drosophila*. For a number of these structures, signaling molecules steering the branching process, such as Fgf-like molecules (lung and tracheal system [Ghabrial et al., 2003; Warburton et al., 2000]) and GDNF (kidney [Karihaloo et al., 2005]) have been isolated. It is a major aim of present investigations to decipher the downstream events of signaling pathways acting during the branching process, as well as to identify important cellular events triggered as part of the signal response. Here, we have analyzed branching morphogenesis in the *Drosophila* larva by using the generation and analysis of mitotic clones, which allows for a detailed dissection of different signaling pathways and largely circumvents the problem of maternal contribution.

Formation of the Thoracic Air Sacs Does Not Require a Mesenchymal-to-Epithelial Transition

The development of the air sacs of the dorsal thorax during the third larval instar was first described in a seminal study by Sato and Kornberg (2002). Although these authors described how the air sac primordium grows out during the third larval instar, they did not describe how the lumen of this tracheal extension is made and whether the cells forming the air sac during the growth phase were of mesenchymal or epithelial nature. We have carefully analyzed air sac outgrowth from early to late stages, by using a number of different markers labeling either membranes or AJs of individual air sac cells, or the apical luminal compartment. We find that the thoracic air sac is modeled out of the existing tracheal epithelium, and that a luminal space is generated by the migration of a few cells away from the cuticle of the existing tracheal branch; the luminal space is then expanded by increasing the cell number in the sac-like epithelial structure via cell division (Figure 6). We find that, during this process, all cells remain within the epithelium and only round up when they divide. Even those cells that send out filopodia and lamelipodia and migrate in the direction of Bnl/Fgf remain embedded within the epithelium, contact the lumen, and form AJs with their neighbors. Thus, the directed outgrowth of the thoracic air sac during larval development is very similar to the budding of tracheal branches in the early embryo, in that epithelial cells form extensions from the basal side, ultimately resulting in cell movement toward the Fgf ligand. In contrast, during tubule formation of MDCK cells in culture, cells initially depolarize and migrate to form chain-like structures before they repolarize and form the luminal cavity; tubulogenesis is thus accompanied with partial epithelial-to-mesenchymal as well as mesenchymal-to-epithelial transitions (O'Brien et al., 2002; Zegers et al., 2003). The tube-forming process has been subdivided into different stages such as cyst, extension, chain, cord, and tubule. In the case of the MDCK model system, growth factors have been proposed to trigger branching by inducing a dedifferentiation that allows the monolayer to be remodeled via cell extension and chain formation. Similar to the MDCK system, we find that growth factor signaling induces the formation of cellular extensions, the first sign of outgrowth. Also, in both systems, cell division is an integral part of the process, but it occurs randomly throughout the structure and not locally at the point of outgrowth (Yu et al., 2003; this study). However, two different RTKs are used in the air sac to control extension (migration) and cell division, and chain and chord stages are not observed. It thus appears that both similarities and differences exist between these different cellular systems.

Cell Division/Survival Is Controlled by Egfr Signaling

It has already been reported that cells divide during air sac formation. We have semiquantified the cell division rates and found that the elongating structure does not grow preferentially at the tip. Our genetic analysis demonstrated that the Egfr is essential for cells to divide and survive efficiently in the air sac. Egfr signals via Ras and the Mapk pathway, but it does not require the Pnt transcription factor to regulate cell division. We do not know yet which ligand activates Egfr, and whether expression of this ligand is induced at early stages of

development by Fgf signaling. As shown before (Sato and Kornberg, 2002), the complete lack of Fgfr signaling results in the absence of air sacs; Fgf signaling might thus be used at the onset of the budding process to initiate or trigger cell division, but it is clearly dispensable in later stages. Since cells in the tracheal branch, which gives rise to the air sac primordium, also divide in the absence of Fgf signaling (see, for example, Figure 2A, top left), it is possible that the role of Fgf signaling consists in generating an outgrowth via directed cell movement, triggering cell division indirectly.

Directed Cell Migration Is Controlled by Fgf Signaling

Interestingly, a recent study addressing the role of GDNF/Ret signaling in kidney branching morphogenesis in vivo has shown that *ret* mutant cells (which are unable to respond to GDNF) can contribute to the primary outgrowth of the ureteric bud, but are excluded from the ampulla that forms at its tip. Apparently, a Ret-dependent proliferation of tip cells under the influence of GDNF controls branch outgrowth (Shakya et al., 2005). We find that in the developing air sac, cells lacking Fgfr are also excluded from the tip. However, we provide evidence that Fgf signaling is translated into directed migration in the leading structure and not into a local increase in cell proliferation. The isolation and cultivation of wing imaginal discs allows for using 4D imaging to document cell behavior during air sac growth. We find that numerous tip cells extend long filopodia and lamelipodia, similar to the findings reported earlier (Sato and Kornberg, 2002). We also documented that tip cells not only produce extensions, but that they indeed change their respective position with time, and move forward over the substrate in the direction of the filopodia/lamelipodia (Figures 2F and 2G and Movies S2 and S3). Thus, tips cells are clearly motile and migrate in the direction of Bnl/Fgf. We have induced marked cell clones incapable of responding to different families of ligands and analyzed these clones with regard to their capacity to populate the air sac tip. Among the receptors analyzed, only Btl/Fgfr was strictly required for cells to populate the leading tip of the air sac. Considering the observation that cells in the tip actively migrate, that Btl/Fgfr signaling is required for tracheal cell migration in the embryo (Sutherland et al., 1996), that tracheal cells migrate to ectopic sources of Bnl/Fgf in the embryo (Sutherland et al., 1996) and the larva (Sato and Kornberg, 2002), and that cells form numerous filopodia and lamelipodia upon constitutive activation of the Fgf signaling pathway (Ribeiro et al., 2004; Movie S4), we conclude that Fgf steers cell migration in the tip of the air sac and leads to its directional outgrowth on the surface of the wing imaginal disc. The demonstration that the MARCM system can be used to analyze gene function with regard to cell migration in the developing air sac prompted us to investigate the role of Ras and the Mapk pathway in Fgf-directed cell movement.

Ras and Pointed Are Required for Fgf-Controlled Cell Migration

Using the MARCM system, we find that Ras activation is essential for cells to migrate at the tip of the air sac. The requirement for Cnk and Ksr strongly suggests that one important branch downstream of Ras in the control of cell migration is the Mapk pathway. This interpretation is supported by the somewhat surprising finding that the transcription factor Pnt is also strictly required for cell migration. In the *Drosophila* embryo, genes regulated by Fgf signaling at the transcriptional level and essential for migration have not been identified so far; although both *pnt* itself and *blistered/DSrf* are targets of Fgf signaling with important functions in tracheal morphogenesis, they are not required for migration (Guillemin et al., 1996; Ribeiro et al., 2002). One possible target of Fgf signaling in the dorsal air sac cells might be the *btl/fgfr* gene itself (Ohshiro et al., 2002). We have tried to rescue the *pointed* defects in air sac development by supplementing a *btl* transgene under the control of UAS sequences. We find that even when Btl/Fgfr is provided by the transgenes, *pnt* mutant clones do not reach the tip. A second gene that might have been a transcriptional target of Pointed is *dof*; however, we find that Dof protein is still present in *pnt* mutant clones (Figure 5K).

Coordinated Organ Growth Controlled by Two RTKs

Our results demonstrate that the outgrowth of the dorsal air sac along the underlying wing imaginal disc is controlled by two RTKs, Btl/Fgfr and Egfr. Fgf signaling is required for directional outgrowth via cell migration, and Egf signaling is required for organ size increase sustaining cell division/cell survival. Both signals use the Ras/Mapk pathway to elicit their cellular responses. To what extent these two pathways regulate different downstream targets is not known at present. However, our study shows that Pointed is only required downstream of Fgf signaling in the control of directed cell migration, and not downstream of Egf signaling in the control of cell division/survival. Since the activation of the Map kinase pathway is much stronger in the cells at the tip as compared to the cells in the central portion or in the stalk of the air sac (according to the levels of dpErk; see Figure 5H), we think that the local availability of the Bnl/Fgf ligand results in a local signaling peak. Egf signaling in more central and proximal cells does not result in a strong activation of the Map kinase pathway, yet this activation is apparently sufficient to control cell division and survival. The independent regulation of cell migration and cell division by two different RTKs might be even more important in later stages of dorsal air sac development, when the growing tip is yet farther away from the main body of the air sac. It will be interesting to find whether other growing branched tissues use similar mechanisms to uncouple directional expansion and size increase.

Experimental Procedures

Drosophila Strains and Genetics

Targeted gene expression was achieved with the Gal4/UAS system (Brand and Perrimon, 1993). A *btlGal4* strain, which drives tracheal Gal4 expression throughout embryonic, larval, and pupal stages, was used in order to drive different GFP transgenes in the trachea (Ribeiro et al., 2002 and references therein). Air sac tracheoblasts were visualized by using *UAS-GFP-actin*, *UAS-Dα-cat-GFP*, *UAS-tau-GFP*, *UAS-GFP-moesin* (Ribeiro et al., 2002 and references therein), and *UAS-CD8-GFP* (Lee and Luo, 1999).

Recombinant lines were generated via standard genetic methods. The protein trap line *316-1 snake* (insertion site currently not known)

was a kind gift of Alain Debec (http://biodev.obs-vlfr.fr/gavdos/protrap.htm). For dual-color imaging, the *btlenhancer-mRFP1moe* line was used (Ribeiro et al., 2004).

For a list of all alleles used, see the Supplemental Data. The following UAS lines were used: *UAS-p35* (1., 2., 3. chromosome) (Bloomington stock center), *UAS-Ras^{V12}* (2. chromosome) (Fortini et al., 1992), *UAS-torso^{4021}-btl* (1. chromosome), *UAS-λTop* (1. chromosome) (Queenan et al., 1997), and UAS-btl (2. chromosome).

Mosaic Analysis

Flip-Out Clones

Embryos of the genotype *70FLP/70FLP; btlenhancer>y$^+$>Gal4, UAS-Dα-cat-GFP/CyO; btlenhancer-mRFP1moe/TM6C* or *70FLP/70FLP; btlenhancer>y$^+$>Gal4/CyO; btlenhancer-mRFP1moe/UAS-GFPmoe* were kept at 25ºC until they reached early third instar stages. After a 5–8 min heat shock at 34ºC, the larvae were transferred back to 25ºC and dissected about 5–6 hr later.

MARCM Clones

The following MARCM strains were constructed by using standard genetic procedures:

For 2L: *70FLP/70FLP; tubgal80, FRT40A/CyO; btlenhancer-mRFP1moe, UAS-CD8-GFP/TM6C*.

For 2R: *70FLP70/70FLP; FRTG13, tubgal80/CyO; btlenhancer-mRFP1moe, UAS-CD8-GFP/TM6C or 70FLP/70FLP; FRT42D, tubgal80/CyO; btlenhancer-mRFP1moe, UAS-CD8-GFP/TM6C*.

For 3L: *70FLP/70FLP; btlenhancer-mRFP1moe, btlGal4-UAS-GFP-actin/CyO; tubgal80, FRT2A/TM6C*.

For 3R: *70FLP70/70FLP; btlenhancer-mRFP1moe, btlGal4-UAS-GFP-actin/CyO; FRT82B, tubgal80/TM6C*.

All *tubGal80*-containing FRT chromosomes (Lee and Luo, 1999), except for *FRT42D tubGal80* (Goldstein et al., 2005), were obtained from the Bloomington stock center.

Alleles of interest were crossed to the appropriate MARCM strain. 4- to 6-hr-old embryos were heat shocked in a water bath for 1 hr at 38ºC, and afterwards they were incubated at 25ºC until they developed into third instar larvae. Clone-bearing larvae were dissected in 1× PBS.

Clone size was always measured in relation to the total size of the air sac. Small clones consist of only very few cells, medium clones populate between 20%–50% of the entire air sac, whereas large clones take up 50%–100% of the air sac.

Due to difficulties in unambiguously distinguishing between medium- and large-sized clones, these two classes were merged into one class in Figures 3–6.

For MARCM misexpression experiments in wild-type backgrounds, we crossed *FRT40A* or *FRT2A* isogenized chromosomes to *UAS-torso^{4021}-btl*, *UAS-Ras^{V12}*, and *UAS-λTop.* Male offspring bearing the nonmutagenized FRT chromosome as well as the UAS line of interest were crossed to the appropriate MARCM strain.

For misexpression experiments in *pnt$^{\Delta 88}$ FRT82B* or *egfr^{K35} FRT42D* mutant backgrounds, *UAS-btl*, or *UAS-p35* in the latter mutant, was crossed to the mutant allele of interest, and male offspring bearing the UAS chromosome as well as the allele of interest were crossed to the appropriate MARCM strain.

Imaging

Freshly dissected discs were put on a slide containing a drop of S2 Schneider cell media (Schneider's insect medium [Invitrogen] supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin) surrounded by a ring of Voltalef immersion oil. In order to avoid tissue damage upon placing the coverslip, two small coverslips were placed on each side of the Voltalef-S2 media ring acting as a support.

Images or time-lapse recordings were taken on a Leica TCS SP2 confocal system with the Leica Confocal Software. Pictures and movies were processed with Imaris 4.0.4/4.1.1 (Bitplane) software.

Immunostainings

The following primary antibodies were used: anti-rabbit Pio (1:100) (Jazwinska et al., 2003), anti-PH3 (1:100) (Shibata et al., 1990), anti-dpMAPK (1:1000, Sigma). As secondary antibodies, we used anti-mouse Cy5 (1:100, Jackson Immunoresearch), anti-rabbit Cy5 (1:100, Jackson Immunoresearch), anti-mouse Cy3 (1:300, Jackson Immunoresearch), anti-rabbit Alexa 568 (1:300, Molecular probes, Invitrogen). Discs were fixed and immunostained as described previously; for dpERK stainings, imaginal discs were fixed for 30 min in 8% formaldehyde (Sato and Kornberg, 2002).

Supplemental Data

Supplemental Data including the analysis of wild-type clones, a table of all the alleles used in this study, and five movie sequences are available at http://www.developmentalcell.com/cgi/content/full/9/6/831/DC1/.

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