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Grainy head controls apical membrane growth and tube elongation in response to Branchless/FGF signalling

Johanna Hemphälä[1,*], Anne Uv[2,*], Rafael Cantera[3], Sarah Bray[4] and Christos Samakovlis[1,†]

[1]Department of Developmental Biology, Wenner-Gren Institute, Stockholm University, S-10691 Stockholm, Sweden
[2]Department of Medical Biochemistry, University of Gothenburg, S-405 30 Gothenburg, Sweden
[3]Department of Zoology, Stockholm University, S-10691 Stockholm, Sweden
[4]Department of Anatomy, University of Cambridge, Cambridge, CB2 3DY, UK

*These authors contributed equally to the work
†Author for correspondence (e-mail: christos@devbio.su.se)

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SUMMARY

Epithelial organogenesis involves concerted movements and growth of distinct subcellular compartments. We show that apical membrane enlargement is critical for lumenal elongation of the *Drosophila* airways, and is independently controlled by the transcription factor Grainy head. Apical membrane overgrowth in *grainy head* mutants generates branches that are too long and tortuous without affecting epithelial integrity, whereas Grainy head overexpression limits lumenal growth. The chemoattractant Branchless/FGF induces tube outgrowth, and we find that it upregulates Grainy head activity post-translationally, thereby controlling apical membrane expansion to attain its key role in branching. We favour a two-step model for FGF in branching: first, induction of cell movement and apical membrane growth, and second, activation of Grainy head to limit lumen elongation, ensuring that branches reach and attain their characteristic lengths.

Key words: Epithelia, Morphogenesis, *Drosophila melanogaster*, Tubulogenesis, Trachea

INTRODUCTION

A characteristic feature of transporting and secretory tubular organs, such as lung, kidney and many glands, is the structural and functional compartmentalisation of their epithelium. Tubulogenesis and branching rely on extensive cell rearrangements and an immense increase of apical lumenal surface, yet in many cases the epithelium remains intact and functional during development (Hogan and Kolodziej, 2002). Thus, the driving forces for cell movement, shape changes and growth must act in the context of prefixed distinct subcellular compartments, and they must be highly co-ordinated with cell adhesion. Although the molecular determinants of epithelial cell architecture are becoming increasingly clear (Tepass et al., 2001), the regulation of the different subcellular compartments during epithelial tissue morphogenesis remains largely unknown. Epithelial cell movement and morphogenesis are commonly induced and guided by secreted factors from the surrounding tissues. How then are these morphogenetic cues integrated to regulate the dynamic cell behaviours that underlie epithelial tube formation and organ growth?

The development of the *Drosophila* trachea, a complex network of epithelial airways that supplies oxygen to the entire animal, provides a well-defined system for the analysis of regulatory mechanisms that control cell migration and branching (Manning and Krasnow, 1993; Samakovlis et al., 1996a). The tracheal system arises from 20 independent sacks of approximately 80 cells that undergo a distinct sequential programme of branch sprouting, directed branch outgrowth and branch fusion. Initially, the actions of at least three independent signals, TGFβ-like (Decapentaplegic; Dpp), Wingless (Wg) and EGF, subdivide the cells in each tracheal placode into branch-specific groups (Affolter and Shilo, 2000). Subsequent branch sprouting and outgrowth occurs without cell division as cells migrate towards localised sources of Branchless (Bnl), an attractant signal of the FGF family (Sutherland et al., 1996). Primary branch growth entails the initial extension of cytoplasmic processes towards the Bnl source, followed by movement of the cell body and a concomitant increase in apical cell surface to promote lumenal extension. The characteristic lengths and diameters of the newly formed branches of the larval trachea are stereotyped and become specified during distinct developmental intervals (Beitel and Krasnow, 2000).

Bnl is the key morphogen co-ordinating branching that acts via the receptor tyrosine kinase Breathless (Btl) (Klambt et al., 1992) and the adaptor protein Dof/Stumps (Vincent et al., 1998; Imam et al., 1999). This pathway leads to phosphorylation and activation of MAPK (Gabay et al., 1997), which in turn may alter the activity of regulatory proteins to control cell behaviour. During primary branching, actin-rich basal extensions are sent by the tracheal cells towards the sources of Bnl, a process that is likely to involve cytoskeletal modulation by the Rho family GTPases (Ribeiro et al., 2002; Wolf et al., 2002). Bnl signalling

is also required for the expression of cell-fate determining genes in specific subsets of tracheal cells in each primary branch. Analysis of these genes has identified key components of the patterning and guidance of the unicellular secondary and terminal branches (Metzger and Krasnow, 1999). However, the role of Bnl in the movement of the cell bodies and the growth of the branch lumen remains unknown.

We have investigated mechanisms that control the elongation of tracheal tubes. We have characterised mutations in three genes that affect branch growth, resulting in abnormally long tubes. Mutations in *fasII* and *Atpα* alter cell adhesion and the basolateral cell domains, causing aberrations in cell shapes, excessive tubular elongation and sporadic lumenal dilations and breaks. In contrast, the transcription factor Grainy head (Grh) is required to specifically control tube elongation. Both loss of function and overexpression of *grh* indicate that it is required to limit lumenal growth and control tubular length. Grh selectively affects the growth of the apical cell membrane, arguing that different genetic programmes regulate distinct sub-cellular domains during branching morphogenesis. Grh is uniformly expressed in the trachea, but its activity is modulated by Bnl/Btl signalling and Grh counteracts the activity of Bnl induced branch growth. Thus, through its regulation of Grh, Bnl regulates epithelial apical membrane growth to accommodate its role in branching morphogenesis.

MATERIALS AND METHODS

Screen and mutants

2460 lethal P-element *Drosophila* strains from the Szeged Stock Center (Deak et al., 1997) were screened as described previously (Samakovlis et al., 1996a). Additional mutants were also chosen for analysis based on previously known phenotypes in other tissues. For *grh* analysis, three strong loss-of-function alleles were used, one EMS allele grh^{B37} (Bray and Kafatos, 1991) and two P-element insertions, grh^{06850} and grh^{s2140}. The P-insertion site of the latter was localised by plasmid rescue and sequencing (Englund et al., 1999) into the first intron of *grh*. Grh protein is not detectable in the mutants. Mutant analysis of *fasII* (*Fas2*) and *Atpα* were carried out using two different alleles of each gene, $fasII^{EB112}$ (Grenningloh et al., 1991), $fasII^{M2225}$ (Cheng et al., 2001), and *Atpα*-P-lacW strains l(3)s067611 and l(3)s083508 (Deak et al., 1997). The P-element insertion site of l(3)s083508 was mapped by plasmid rescue and sequencing to 30 nucleotides upstream of the beginning of the GH23483 EST clone. l(3)s083508 and $fasII^{EB112}$ were chosen for further analysis since no Atpα or FasII protein could be detected in mutant embryos of the corresponding genotypes. The following mutants were used: bnl^{p1} (Sutherland et al., 1996), bnl^{44026} (Deak et al., 1997), a presumptive null allele with the same tracheal phenotype as bnl^{p1}, btl^{LG19} (Klambt et al., 1992), $pnt^{\Delta 88}$ (Scholz et al., 1993) and shg^{IG29} (Tepass et al., 1996).

Genetic interactions were assessed by examining the tracheal phenotypes of the following mutant combinations: grh^{s2140}/shg^{IG29}, $grh^{s2140}shg^{IG29}/grh^{s2140}$, $grh^{s2140}shg^{IG29}/shg^{IG29}$.

grh^{s2140}/grh^{s2140};*Atpα/TM3Z*, grh^{s2140}/*CyOZ*; *Atpα/Atpα*, grh^{s2140}/grh^{s2140}; *Atpα/ Atpα*.

$fasII^{EB112}/Y$; $grh^{s2140}/+$, $fasII^{EB112}/+$; grh^{s2140}/grh^{s2140}, $fasII^{EB112}/+;grh^{s2140}/+$, $fasII^{EB112}/Y$; grh^{s2140}/grh^{s2140}.

$grh^{s2140}/grh^{s2140};bnl^{p1}/+$, grh^{s2140}/grh^{s2140}; *bnl44026/+*

esg-lacZ (*Fusion-1*), and the *trh-lacZ* markers have been described previously (Samakovlis et al., 1996a). *GBE-lacZ* was described by Uv et al. (Uv et al., 1997), two independent transgenic strains where analysed and they were both responsive to ectopic expression of Bnl.

The *UAS-grh* transgenic fly strains were generated by inserting the *grh* cDNA (N′-form) (Uv et al., 1997) into pUAST (*Eco*RI and *Not*I), and injected into *yw* embryos to establish six independent lines. The additional UAS strains used were: *UAS-dpp*, *UAS-tkvQ253D* (Nellen et al., 1996), *UAS-bnl* (Sutherland et al., 1996) and *UAS-btl::tor4021* (a chimeric construct consisting of the extracellular domain of the constitutively active *tor13D* mutant fused to the intracellular domain of *btl* (Vincent et al., 1998), *UAS-Act-GFP* (Verkhusha et al., 1999) and *UAS-EGFPF* (Finley et al., 1998). The *Btl-Gal4* and the *SRF-Gal4* where described previously (Shiga et al., 1996; Jarecki et al., 1999). *SRF-Gal4* expresses *Gal4* in tracheal terminal cells from stage 14. Embryos with one copy of the GAL4 driver and the UAS constructs were collected at room temperature for 6 hours and then aged at 29°C for 10 hours before fixation. In all experiments *CyO, TM3and FM7c* balancer strains carrying *GFP* or *lacZ* transgenes were used as necessary, to unambiguously identify embryos with the desired genotypes.

Immunostaining and TEM

Immunostainings of embryos were performed as described previously (Samakovlis et al., 1996b). Embryos were fixed in 6% paraformaldehyde-saturated heptane and rinsed in ethanol before staining with Alexa Flour-568 and -488 conjugated phalloidin (Molecular Probes). To prevent bleaching the ProLong kit was used (Molecular Probes). The following primary antibodies were used: tracheal lumen-specific mouse IgM antibody mAb2A12 (1:3), mouse monoclonal anti-DSRF (1:1000), rabbit anti-β-gal (1:1500; Cappel), mouse monoclonal anti-Grh (1:5), rabbit anti-Dlg (1:400) (Budnik et al., 1996), rat anti-DE-cad (1:100) (Oda et al., 1994), guinea pig anti-Cor (1:1000), rabbit anti-Nrx IV (1:500), mouse monoclonal anti-Crb (1:20), rabbit anti-β-heavy-spectrin (β_H-spectrin; 1:300), mouse monoclonal anti-FasII (1:10), mouse monoclonal anti-Atpα (1:100) (Lebivitz et al., 1989), rat anti-Trh (1:500) (Wappner et al., 1997), rabbit anti-GFP (1:500; Molecular Probes). Secondary antibodies conjugated to Biotin, Cy2 or Cy3 (Jackson Immunochemicals) or Alexa Fluor-568 and -488 (Molecular Probes) were diluted and used as recommended. Tyramide Signal Amplification (NEN) was used to enhance tracheal Grh and Crb signal detection. Confocal images where obtained with a Leica SP2 confocal microscope and processed in Adobe Photoshop. Embryo preparation and analysis by TEM was as described previously (Englund et al., 1999).

RESULTS

Grh restricts tracheal tube elongation

The transcription factor Grainy head (Grh) is expressed in a number of epithelial structures, including the embryonic epidermis where it has been suggested to be involved in the formation of the cuticular layer that covers the apical surface of epidermal tissues (Ostrowski et al., 2002). Early descriptions of *grh* mutants also revealed a tracheal defect (Bray and Kafatos, 1991), which led us to investigate the expression and phenotype of *grh* in the trachea.

Nuclear Grh is detected in all tracheal cells, appearing first at stage 11, just after they have invaginated from the epidermis, and persisting throughout embryogenesis (Fig. 1L). To investigate its function, an antibody that specifically stains the tracheal lumen (mAb2A12) and several cell fate markers (Samakovlis et al., 1996a) were used to analyse the tracheal phenotype of three strong loss-of-function *grh* alleles (one EMS allele, grh^{B37}; two P-element insertions, grh^{s2140} and grh^{0685}). None of the *grh* mutations affect the patterning, outgrowth and connection of branches or the expression of terminal cell markers (Guillemin et al., 1996) (DSRF; Fig. 1I) and fusion cell

markers (*fusion-3*, not shown). It is only when primary and secondary branching is completed (during stage 16), that *grh* mutant embryos begin to display tubular irregularities. The first signs of a defect are that the dorsal trunk (main airway) appears convoluted and elongated compared to the wild type (Fig. 1B,C; early stage 16). This phenotype subsequently becomes exaggerated, and is also seen in additional branches, including the lateral trunk, transverse connectives and ganglionic branches (Fig. 1D-G). These convoluted branches represent an overgrowth in tracheal tube length, as indicated by an increase of 40% in the tube length of *grh* mutants (based on measurements of dorsal trunk metameres 4-6 from 8 embryos of each genotype at stage 16.4). Despite this substantial increase in tubular lengths, the tubular continuity is not affected in *grh* mutant embryos. Grh is therefore required for the restriction or maintenance of tubular length.

Grh mutants show irregular apical cell shapes

The excessive branch elongation in *grh* mutants could be associated with an increase in cell numbers. We therefore counted the number of cells in hemisegments 4, 5 and 6 of the dorsal trunk (DT) in stage 16 wild-type and *grh* mutant embryos. The tracheal cells normally stop dividing after invagination and each tracheal hemisegment consists of about 80 cells, of which approximately 20 make up the DT (Samakovlis et al., 1996a). Using an antibody against the Trachealess transcription factor, which is expressed in all tracheal cells (Wilk et al., 1996), we found that *grh* mutants contain similar number of cells as the wild type (Fig. 1J,K). Thus, Grh restricts tube length without affecting cell division or the number of cells that become allocated to individual branches.

Tubular growth is accompanied by an immense increase in lumenal surface, and although it has been proposed that the expansion of apical cell surface is an essential cellular process underlying branching morphogenesis, its regulation is poorly understood (Beitel and Krasnow, 2000). To investigate the cellular activities controlled by Grh during tubular morphogenesis, we used antibodies against different membrane-associated proteins to visualise tracheal cell shapes and to monitor the apical basal polarity of the cells. Labelling for DE-cadherin (DE-cad), a protein localised at the apical adherence junctions (AJs) (Oda et al., 1994), shows that the apical circumference of cells in the dorsal trunk of *grh* mutants is highly irregular at stage 16 (compare Fig. 2A and D). In particular, the cells positioned at the outer edge of each curve become excessively elongated (Fig. 2D) compared to the cobblestone-shaped cells that line the lumen of wild-type embryos. The anomalies in cell shapes are first detected at stage 16, and are therefore coincident with the abnormal tube elongation.

The stretched and expanded tracheal cell shapes in *grh* embryos do not appear related to alterations in cell polarity. The transmembrane protein Crumbs (Crb), which confers apical character on the plasma membrane of epithelial cells (Wodarz et al., 1995), is present in the same punctate staining along the lumenal surface of the dorsal trunk in both wild-type and *grh* mutant embryos. No differences in the level of Crb expression between wild-type and *grh* mutant embryos are evident (Fig. 2B,E), neither are there alterations in the minus end of the microtubules [visualized by the expression of a *UAS-NodlacZ* transgene (Clark et al., 1997) (data not shown)]. The subcellular distribution of two lateral membrane-associated proteins, Coracle (Cor) (Fehon et al., 1994) and Neurexin IV (Nrx) (Baumgartner et al., 1996), which are required for the formation and function of the laterally positioned septate junctions (SJs), is also normal in *grh* mutant embryos (Fig. 2C,F,I,L). Double labelling for Nrx and DE-cad further shows that Nrx accumulates just basal to the AJs in both wild-type and *grh* embryos (Fig. 2C,F), suggesting that the increase in apical cell circumference in the mutants is not due to abnormal distribution of apical domain markers into the lateral and basal cell area.

Although the modulation of the apical cytoskeleton plays a key role in epithelial cell shape changes and morphogenesis, we have not detected defects in the apical cytoskeletal structures of tracheal cells in *grh* embryos. Neither the subcellular localization nor the intensity of filamentous actin staining is altered (visualised either by phalloidin or the tracheal expression of actin-GFP; Fig. 2H,K and not shown). In addition, the distribution of two apical cytoskeletal markers Armadillo (β-catenin, not shown) (Peifer and Wieschaus, 1990) and β_H-spectrin (Zarnescu and Thomas, 1999) (Fig. 2G,J) is similar in *grh* and wild-type embryos. Thus, the abnormal tubular extension and irregular cell shapes observed in *grh* mutants are not related to the organisation or maintenance of apical and basal cell polarity and structure, nor to the collapse of the AJs and underlying cytoskeleton.

Grh regulates the growth of apical cell membrane

To investigate further the irregular cell shapes in *grh* mutants we characterized the cellular morphology of *grh* embryos at stage 15 and late stage 16 by transmission electron microscopy. Cross sections of the dorsal trunk typically reveal 2-4 cells that span the lumen circumference. The apical membranes are seen just beneath the secreted cuticle that lines the lumen, and apical junctions appear as electron dense structures near the apical cell surface while septate junctions are visible as ladder-like structures basal to the AJs (Fig. 3E). By analysing several cross sections, we find that the morphology of SJs and the basal part of the cells appear normal in *grh* mutant embryos (not shown). The apical cell domain, however, appears strikingly overgrown and distorted. These defects are first seen in the tracheal dorsal trunk cells of early stage 16 embryos. The apical cell surface continues to enlarge, becoming so expanded that it folds over neighbouring cells, resulting in several layers of cuticle deposition (stage 17; Fig. 3B,G,F). The imbalance in the dimensions of apical membrane thus parallels the occurrence of the convoluted branch phenotype in *grh* mutant embryos. An apical membrane overgrowth is also found in the epidermal cells, and is associated with the production of an enlarged cuticle that lines the apical cell surface (data not shown).

The AJs often appear abnormal in *grh* mutant tracheal cells. They are frequently misplaced, lying parallel rather than perpendicular to the lumen and occasionally appear less electron dense (Fig. 3H). The disruptions of AJs could be a secondary effect, resulting from the excessive apical membrane that forces an increase in the circumference at the apical side. Alternatively, Grh may also directly regulate genes necessary for the maintenance or the function of AJs. Overall the TEM analysis shows that the major defect in *grh* tracheal cells is a continued expansion of the apical membrane, which results in an enlarged and anomalous lumenal surface with cellular protrusions that contrast to the smooth lining of the wild-type lumen. This progressive phenotype first appears at the stage

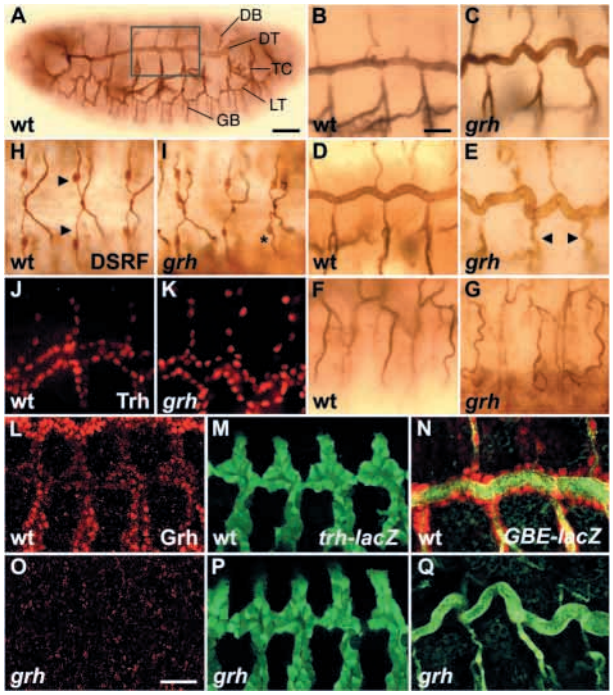

Fig. 1. Grh is expressed in the developing trachea and is required to prevent excessive tube extension. (A) Lateral view of the wild-type embryonic tracheal system at stage 16, visualised by antibodies against the 2A12 (lumenal) antigen. The major tracheal branches, dorsal trunk (DT), dorsal branch (DB), lateral trunk (LT), transverse connective (TC) and ganglionic branches (GB) are indicated. The rectangle outlines the part of the trachea that is shown in the subsequent panels, except for in F-I. (B-G) Tracheal lumens of wild-type (B,D,F) and grhs2140/Df(2R)Pcl7B mutant (C,E,G) embryos at stage 16. At the beginning of stage 16, grh mutants (C) have more elongated DTs than wild-type embryos (B). The DT growth continues as stage 16 proceeds, and at the end of this stage (D,E), the excessive tubular extension is evident in additional branches, including the TC (arrowheads in E), LT (not shown) and GB (F,G; ventral lateral view). (H-I) Dorsal view of wild-type (H) and grhs2140/Df(2R)Pcl7B mutant (I) embryos stained for 2A12 and DSRF, showing three pairs of unicellular terminal branches (arrowheads) emanating from the dorsal branches. Terminal branching and DSRF expression is normal in grh mutant embryos, but at the end of embryogenesis, the terminal branches become convoluted and often make loops in grh mutants (I, asterisk). (J,K) Wild type (J) and grh mutants (K) labelled with antisera against the nuclear protein Trh, which is expressed in all tracheal cells. The number of DT and DB cells is not greater in grh mutants than wild type. (L,M) Wild-type and (O,P) grhB37/grhB37 mutant embryos carrying the cytoplasmic trh-lacZ marker were labelled for Grh (red; L,O) and b-Gal (green; M,P). Grh is expressed in all tracheal cells in wild-type embryos (L; stage 14), and is absent in grh mutants (O; stage 14). (N,Q) Wild-type (N) and grhB37 embryos (Q) carrying the Grh activity reporter, GBE-lacZ, were double labelled with mAb2A12 (green) and anti-b-Gal (red). GBE-lacZ is expressed in the wild-type trachea (N; red nuclear staining), but is absent in grh mutants. Scale bar in A: 25 mm; B-K, 10 mm; L-Q, 20 mm. In this and all subsequent figures anterior is left and dorsal up.

when tube extension normally ceases, suggesting that Grh activity restricts branch elongation by limiting apical membrane growth. Given the proposed key role of apical cell surface expansion in branch morphogenesis (Beitel and

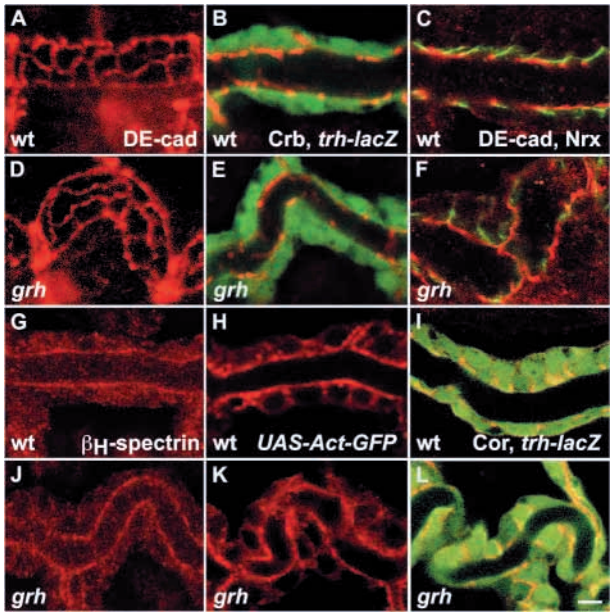

Fig. 2. Irregular cell shapes, but normal apical-basal epithelial cell organisation in *grh* mutants. (A,D) Confocal projections of a DT segment of wild-type (A) and *grh* mutants (D) labelled with antibodies against DE-cad to visualise the apical cell circumference. The tracheal cell shapes in *grh* mutants are irregular and elongated compared to the wild type. (B,C,E-L) Confocal longitudinal sections of one segment of the DT, to visualise the subcellular localisation of apical, lateral and cytoskeletal markers in wild-type and *grh* mutant embryos. Embryos carrying the cytoplasmic *trh-lacZ* marker (B,E) were double labelled with antibodies against β-gal (green) and Crumbs (red). The levels and subcellular localisation of Crumbs at the apical cell surface is the same in wild-type (B) and *grh* mutants (E). Double labelling of wild-type (C) and *grh* (F) embryos with anti-DE-cad (red) and anti-Nrx IV (green) shows that cadherin is localised more apically than Neurexin both in *grh* mutants and in wild-type embryos. Labelling for β-heavy spectrin (G,J) shows concentrated localisation at the apical surface of the tracheal cells, both in wild-type (G) and *grh* mutants (K). Wild-type (H) and *grh* mutants (J) expressing *UAS-Act-GFP* in all tracheal cells were labelled with GFP. The apical localisation of actin-GFP is not affected in *grh* mutants. Wild-type (I) and *grh* mutants (L) carrying the *trh-lacZ* reporter were double labelled for β-gal (green) and Coracle (red). The lateral membrane localisation of Coracle is not affected by *grh*. Scale bar: 5 μm.

Krasnow, 2000), the specific effect of Grh on this compartment argues that it has a pivotal role in controlling the process.

Basolateral proteins modulate tube shape and integrity independently of *grh*

In search for genes that are functionally related to Grh, we identified and characterised two mutations that give rise to convoluted tubes, similar to those of *grh* mutants (Fig. 4A,D). One mutation inactivates the *fasciclinII* gene (*fasII*^{EB112}), encoding a homophilic cell adhesion protein (Grenningloh et al., 1991), and the other disrupts the gene *Atpα*, encoding the sodium/potassium-transporting ATPase alpha subunit (ATPα). In addition to the extended and curvy dorsal trunk phenotype, detection of DE-cad reveals that both *fasII* and *Atpα* mutants display an enlarged apical circumference (Fig. 4C and F). Unlike

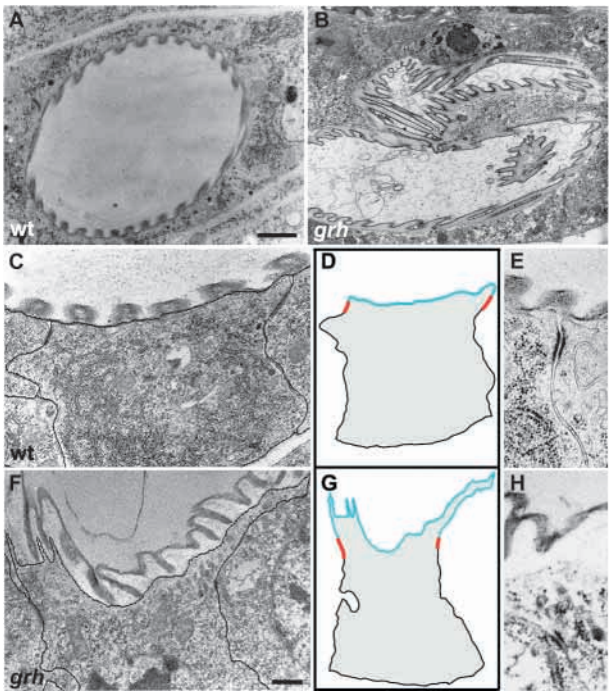

Fig. 3. Expanded apical membrane in *grh* mutants. (A,B) Electron micrographs of the DT (cross section) in wild-type (A) and *grh* mutants (B) at early stage 17. The lumen is disorganised in *grh* mutants, with tongues of cell masses extending from the apical surface to occupy part of the lumen. *grh* mutant embryos can secrete lumenal and cuticular components, but the characteristic taenidial structure is in places disrupted. (C,F) DT cross section at higher magnification showing a single cell of wild-type (C) and a *grh* mutant (F) with the membranes outlined in black. (D,G) Drawings of single cells based on EM cross sections in C and F. The cell membrane is shown in black, the apical junctions are marked with red, and the apical membrane is blue. In *grh* mutants, the amount of cell membrane apical to the AJs is significantly greater than in the wild type and excessive membrane folds over neighbouring cells. (E,H) High magnification images focused on a single AJ. In the wild-type cell (E), the AJs are positioned near the apical surface perpendicular to the lumenal surface. In the *grh* mutant (H), AJs are generally positioned further away from the lumen and are occasionally smaller. Scale bars in A,B, 1 μm; C,F, 0.5 μm.

grh mutant embryos, however, TEM cross sections of the dorsal trunk in *Atpα* mutants show that the apical cell domain is indistinguishable from that of wild type (Fig. 5I). In addition *fasII* and *Atpα* mutants also display local tubular dilations (Fig. 4A,D) and lumenal breaks (Fig. 4B,E). ATPα localises to the lateral cell surface of all tracheal cells throughout development (Fig. 5B), and the *Atpα* mutation appears to affect the integrity of the lateral septate junctions since the characteristic septa between cells are sparse (not shown) and the septate junction protein Nrx appears more diffuse in the dorsal trunk of *Atpα* mutants (Fig. 5F,H). As FasII protein is also localised to the lateral surface of all cells in the developing trachea, (Fig. 5A), the defects in tube and cell shapes could arise similarly through the destabilisation of adhesion complexes between the tracheal cells. Thus, FasII and ATPα affect cell shape and tracheal tube length, as well as tubular diameter and epithelial integrity, most likely through their action on the lateral cell surface.

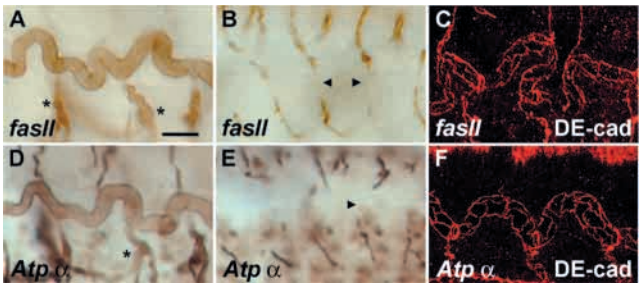

Fig. 4. Tracheal lumen morphology in *fasII* and *ATPα* mutants . (A-F) Lumens (A,B,D,E) and dorsal trunk cell shapes (C,F) visualised with anti-DE-cad of stage 16 *fasII*^{eb112} (A-C) and *Atpα* mutants (D-F). (A,C,D,F) Dorsal views; (B,E) ventral views showing the ganglionic branches. (A,D) Embryos mutant for *fasII* and *Atpα* develop long and convoluted DTs reminiscent of those of *grh* mutants. Tubular dilations in the transverse connectives are indicated by asterisks. (B,E) The lumen of several branches is discontinuous (arrowheads at the GBs). In *fasII*, (C) and *Atpα* (F) mutants the apical cell surface appears elongated and expanded. Scale bar: 20 μm.

Despite the difference in cellular phenotypes of *grh*, *fasII* and *Atpα* mutants, the similarity of their dorsal trunk phenotypes prompted us to investigate a functional relationship between these genes. We first examined the expression of FasII or ATPα in *grh* mutants and in embryos overexpressing Grh in the trachea (see below). Both proteins are expressed at normal levels and are localised correctly in these mutants (Fig. 5C,D, and not shown). In addition, *fasII grh* and *Atpα grh* double mutants display an additive tracheal phenotype with increasingly fragmented tubes, suggesting an additive effect of weakened epithelial cohesion and the physical strains exerted by excessive apical growth. These data therefore indicate that FasII and ATPα are not functionally related to Grh. Instead, they imply that tubular dimensions depend on the control of distinct subcellular domains; the growth of the apical cell surface, exemplified by Grh and the integrity and cohesion of epithelial structure mediated by the lateral membrane proteins FasII and ATPα.

Grh overexpression prevents lumenal growth

The apical membrane overgrowth and excessive tubular elongation phenotypes suggest a key role for *grh* in the regulation of branch extension. We therefore characterised the effects of its overexpression by directing *UAS-grh* expression in all tracheal cells after invagination (late stage 11) using the *Btl-Gal4* driver strain. Detection of lumenal and cellular tracheal markers at stage 16 reveals a pattern characteristic of the wild type at stage 13 (Fig. 6A,E,F). This indicates that the cells have migrated towards their targets, but they have not formed full-length primary branches or a normal lumen. The visceral and dorsal branches only form rudimentary buds and the dorsal and lateral trunk branches in each hemisegment remain unconnected (Fig. 6G). Although the specialized fusion cells that normally form unicellular anastomoses and mediate branch fusion are in close contact and express the correct differentiation markers (*esg-lacZ;* Fig. 6D) (Samakovlis et al., 1996b), they fail to form the interconnecting sprouts. In addition, no secondary branch lumen is formed, but the expression of the terminal cell marker DSRF is not affected (Fig. 6B,C). Thus, Grh appears to inhibit branch extension without changing tracheal cell fates.

To assess whether the lumen and branch growth defects are due to failure in cellular extensions towards target tissue, we analysed embryos co-expressing *UAS-grh* and the membrane marker eGFP (Finley et al., 1998) in all tracheal cells. Grh overexpression does not affect the basolateral projections of the tracheal cells, since these extend towards their normal orientations (Fig. 6F). However, the cells appear unable to develop a lumen since their apical side, which surrounds the cavity of the presumptive dorsal trunk and the short stumps extending from it, seems limiting (Fig. 6F,F′). Thus, ectopic Grh appears to restrict tracheal branch extension by directly targeting cellular activities that underlie lumenal growth.

We also assayed the effect of Grh overexpression in single terminal cells, using the *Term-Gal4* driver. In wild-type embryos, the terminal cells form unicellular branches by extending long cytoplasmic processes that subsequently become penetrated by a lumen (Fig. 6B,H). Ectopic Grh does not affect the initial extension of cytoplasmic processes, revealed by a *trh-lacZ* marker, but prevents the formation and elongation of the intracellular lumen (Fig. 6H,I), demonstrating that Grh can also regulate the cellular processes involved in intracellular branch formation.

Control of Grh activity by Bnl/FGF signalling

The tracheal phenotypes produced by alterations in Grh levels imply that Grh activity must be carefully controlled during branching morphogenesis to ensure branch extension at the right stage and to the right extent. Consequently, tracheal Grh activity is likely to be modulated during branching morphogenesis. To assay the in vivo activity of Grh, we used strains carrying a transgene with four high-affinity Grh response elements (*GBE-lacZ*) (Uv et al., 1997). *GBE-lacZ* expression is detected in all tissues where Grh is expressed, is absent in *grh* mutants, and becomes activated upon ectopic Grh expression (data not shown). It is thus representative of Grh transcriptional activity in vivo. During tracheal development *GBE-lacZ* is expressed in all tracheal cells after invagination, and requires Grh for its expression (Fig. 1N,Q). However, *GBE-lacZ* expression is not uniform, it becomes temporarily enhanced in the fusion and terminal cells during branching (stage 14; Fig. 7A and not shown). As Grh itself appears to be uniform in all tracheal cells (Fig. 1L, Fig. 7B), the enhanced expression of *GBE-lacZ* indicates that the activity of Grh is regulated post-translationally during branching.

One possible mechanism for regulation of Grh activity is through Bnl signalling, which is instrumental in the formation and extension of all tracheal branches. Initially, we established that apical cell surface growth is an intrinsic component of Bnl-induced tube extension, by combining alleles of *grh* and *bnl*. This revealed that a subset of the branch outgrowth defects seen in embryos that carry only one copy of the *bnl* gene are partially rescued by a reduction in *grh* function (grh^{s2140}/grh^{s2140}; $bnl^{P1}/+$). Thus, in embryos heterozygous for *bnl* 40% of the ganglionic branches (n=380) fail to reach the CNS, whereas the simultaneous removal of *grh* restores this phenotype so that 78% (n=380) of the branches now enter the CNS. These data therefore show that Grh-mediated modulation of the apical cell surface has an active inhibitory role on Bnl-induced branch extension.

In order to analyse whether tracheal Grh activity could be targeted by Bnl/Btl signal transduction, we analysed *GBE-lacZ* expression in embryos with altered levels of Bnl and Btl activity. When Bnl is ectopically expressed in all tracheal cells, *GBE-lacZ* expression becomes significantly upregulated (compare Fig. 7A and C), although the levels of Grh protein are not altered (Fig. 7B,D). This suggests that Bnl controls Grh activity post-translationally, and surprisingly, upregulates the expression of this artificial Grh target. Nevertheless, the effects of Btl appear specific since with more limited Bnl expression using the *Term-Gal4* driver, *GBE-lacZ* expression becomes enhanced specifically in the cells that respond to Bnl by ectopically expressing the terminal marker DSRF (compare Fig. 7F and H). Similar enhancement of *GBE-lacZ* expression is evident upon tracheal expression of an activated form of the Btl receptor itself (*UASBtl-Tor*) (Vincent et al., 1998) (data not shown). In all instances the augmented *GBE-lacZ* expression is dependent on Grh, as embryos that express ectopic Bnl or the activated form of Btl, but lack Grh activity, do not express *GBE-lacZ* (not shown). Furthermore, ectopic activation of Dpp, another signalling pathway that promotes the growth of dorsal and ganglionic branches during tracheal development (Ribeiro et al., 2002), has no effect on *GBE-lacZ* (data not shown), indicating that the effects on GBE-lacZ are specific for Bnl/Btl.

We next tested whether Bnl signalling is a prerequisite for the transcriptional activity of Grh, by analysing the levels of *GBE-lacZ* expression in mutants for *bnl*, *btl* or *pointed* (*pnt*) (Klambt, 1993). Tracheal *GBE-lacZ* expression is both reduced and uniform in *bnl* and *btl* mutant embryos (Fig. 7K,L and not shown), but is unchanged in *pnt* embryos (Fig. 7I, J) that lack the activity of a downstream transcriptional effector of the ETS family (Samakovlis et al., 1996a). Since Grh is a substrate for activated MAPK (ERK2) in vitro (Liaw et al., 1995), its activity could be modulated directly during branching by Bnl-induced phosphorylation. This would account for the fact that *GBE-lacZ* expression is affected by mutations in *bnl* and *btl*, but not by mutations in the nuclear effector *pnt*.

The apparent upregulation of Grh activity by Bnl signalling and the fact that Grh and Bnl exert opposing effects on branch extension suggests that there are two possible models of Grh activity. The first assumes a two-step process, where upregulation of Grh activity represents a second function of Bnl to prevent excessive tube extension. Alternatively, the Bnl signalling augments some aspects of Grh function (e.g. activation of *GBE-lacZ*) but inhibits others (e.g. the restriction of apical membrane growth) allowing for branch extension. These two models are discussed below.

DISCUSSION

Grh specifically controls apical membrane growth and tube elongation

Fundamental to tubular organ function are the sizes and shapes of the constituent branches. Each branch is shaped into a tube of precise diameter and length to accommodate specific transport demands. Errors in the control of tube dimensions have vital consequences during organ development and homeostasis during adult life, for example, autosomal dominant polycystic kidney disease (ADPKD), a common human genetic disorder, results in tubular overgrowth and cysts leading to renal failure. Several studies in mammalian and invertebrate systems have highlighted the importance of cell proliferation, cell polarity and epithelial cell cohesion in

tubulogenesis. In this work we show that the transcriptional regulation of apical membrane size is a key determinant of branch length and tubulogenesis.

During tracheal development, Grh is required to restrict apical membrane growth, thereby preventing excess elongation of tracheal tubes. Loss of zygotic Grh protein produces branches that are too long, whereas ectopic Grh has the converse effect. Tracheal branching is initiated in cells receiving the FGF signal, which respond by extending basal projections towards the FGF source, and subsequently move the cell body. Concurrently, a lumen is generated in the extending branch, facilitated by the enlargement of the apical cell membranes to accommodate the necessary expansion in lumenal surface. In embryos that lack *grh*, branch extension is initiated and proceeds as in wild-type embryos. However, when individual tubes have reached their approximate length and their extension is supposed to halt, the apical membrane growth continues in *grh* mutants embryos resulting in an enormous apical cell surface that folds over neighbouring cells and forms tortuous tubes. Overexpression of Grh, in contrast, does not affect the basal cytoplasmic extensions towards the Bnl source or cell motility, but specifically prevents lumen extension. Thus, Grh activity is necessary and sufficient to terminate apical membrane growth and tubular extension. Grh may exert its function as an activator of genes that promote homeostasis of apical cell membrane, or a repressor of genes that enhance apical membrane growth.

Several lines of evidence argue that Grh has a specific and restricted function in apical membrane growth control. Firstly, epithelial polarity is not altered in *grh* mutants. TEM analysis of *grh* mutants and embryos overexpressing Grh showed that cuticular and lumenal components are made and secreted and the expression and subcelluar localisation of Crb, DE-cad, Nrx, Cor and Disc large (not shown) is normal in *grh* mutant trachea. In addition, no genetic interaction was detected in embryos carrying different combinations of *grh* and *shg* (encoding DE-cad) mutant alleles (data not shown). Secondly, no cytoskeletal defects were detected in *grh* mutant embryos. The filamentous apical actin cytoskeleton, and the expression of Cadherin, Armadillo and β_H-spectrin are unchanged by overexpression or inactivation of *grh*. Likewise, no anomalies were evident in the minus-end microtubules visualised by the apical distribution of *Nod-lacZ* in the trachea of *grh* and wild-type embryos. Finally, changes in the basolateral cell domain influence tracheal tube size and integrity independently of *grh*. FasII and ATPα localize to the lateral cell membrane, and disruption of either gene function causes distinct irregularities in tubular diameter, length and continuity. No regulatory or functional relationship between Grh and these two lateral proteins were detected in spite the fact that all three proteins influence tubular length.

There are specific differences between the tracheal phenotypes caused by the expansion of apical membrane, seen in *grh*, and the ones caused by disruption of lateral domain functions, seen in *fasII* and *Atpα* mutants. The increase in apical cell surface in *grh* embryos has no apparent effect on the diameter of the tracheal tubes, whereas mutations in *fasII* and *Atpα* result in a longer lumen with local dilations. The additional tubular breaks observed in the latter suggest that they function in the lateral cell compartment to maintain epithelial cohesion and structure. The programmed changes in tube diameter that take place in the different tracheal branches during development occur by expansion of the inner lumenal diameter, whereas the outer diameter remains the same (Beitel and Krasnow, 2000), requiring a decrease in the distance from the apical to basal surface and major remodelling of the lateral cell compartment. Therefore, the size and shape of epithelial tubular structures appears to depend on the modulation of distinct subcellular domains during morphogenesis. In addition to the regulation of apical membrane growth by *grh*, there may be separate regulatory programmes modulating the dynamics of the lateral cell surface. Together these two aspects of regulation co-ordinately determine tubular dimensions.

Most of the well-studied examples, in which morphogenesis involves changes in the apical membrane of polarised epithelia, are mediated through the function of the apical membrane determinant Crb, an EGF-repeat-containing transmembrane protein. In the *Drosophila* embryo overexpression of the Crb intracellular domain causes cytoskeletal re-organisation and apical membrane expansion. Crb also functions during photoreceptor morphogenesis, where its intracellular domain is required to maintain the integrity of zonula adherens during rhabdomere elongation (Izaddoost et al., 2002), and its extracellular domain has an additional and distinct function in the extension of the stalk, by stabilizing the membrane-associated β_H-spectrin cytoskeleton to facilitate apical membrane growth (Pellikka et al., 2002). As neither the expression nor the localisation of Crb and β_H-spectrin is detectably affected in the tracheal cells where the apical membrane is altered by loss of or overexpression of grh, an alternative mechanism for apical membrane growth must be involved in mediating the *grh* function on branching morphogenesis. One possibility is via an effect on membrane trafficking, which is highly regulated in polarised epithelial cells (Mostov et al., 2000; Lipschutz and Mostov, 2002). Apical membrane growth during branching may be achieved by directly modulating the relative rates of exocytosis or endocytosis and membrane metabolism.

Regulation of Grh activity by Bnl

Grh levels appear uniform in all tracheal cells throughout development. However, Grh functions in the regulation of branch extension and apical membrane growth, allowing branch extension during a certain time frame and to different extents in the various branches. Grh activity must therefore be modulated post-translationally as branch growth proceeds. Such a regulation of Grh activity could be exerted through extracellular signals or by branch-specific co-factors, modulating its ability to regulate gene expression in a branch and time specific manner.

Using a *lacZ* reporter gene for Grh activity that reflects the in vivo ability of Grh to activate transcription (*GBE-lacZ*), we find that Grh activity is controlled by FGF signalling during tracheal development. Ectopic expression of Bnl or the activated form of its receptor Btl up-regulates the expression of *GBE-lacZ*, whereas *GBE-lacZ* expression is reduced in mutants for *bnl* or *btl*. Thus, Bnl signalling converts Grh to a more potent activator of its *GBE-lacZ* target. Since Grh becomes phosphorylated by MAPK in vitro (Liaw et al., 1995), and MAPK is a downstream effector of Btl signal transduction, the alteration in Grh activity may be brought about by MAPK-mediated phosphorylation of the Grh protein.

Currently, we see two ways of explaining the biological consequence of the regulation of Grh. In the first model, the

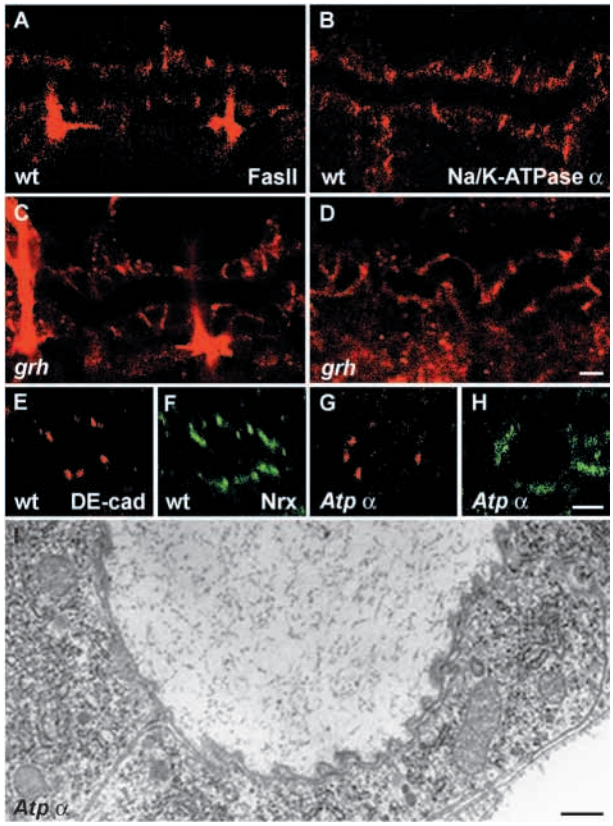

Fig. 5. FasII and ATPα are localised to the lateral cell surface and do not affect apical membrane growth. (A,B) Confocal longitudinal sections of the DT of wild-type embryos labelled with antibodies against FasII (A) and ATPα (B) show that both proteins localise to the lateral cell surface. (C,D) The expression and localization of FasII and ATPα are not affected in *grh* mutants. (E,H) Confocal cross sections of the DT of wild-type (E,F) and *Atpα* mutant (G,H) embryos labelled with antibodies against DE-cad (red; E,G) and Nrx (green; F,H). The lateral localization of Nrx is diffuse in the *Atpα* mutants. (I**)** Electron micrograph revealing a partial cross section of the DT in an *Atpα* mutant embryo at late stage 16. No excessive growth of the apical membrane can be seen in the *Atpα* mutants, and the AJs appear correctly localised near the lumenal surface. Scale bar: A-D, 10 μm; E-H, 5 μm; I, 0.5 μm

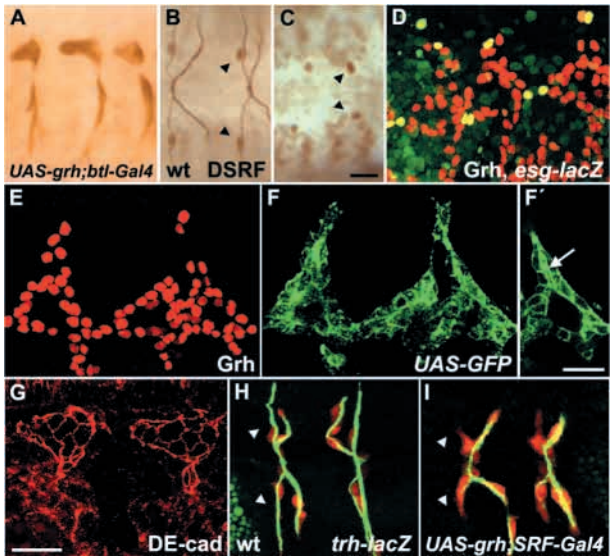

Fig. 6. Ectopic tracheal expression of Grh inhibits branch extension. (A) mAb2A12 (lumenal) labelling of a *btl-GAL4*/*UAS-grh* embryo at stage 16 reveals that primary branch extension is impeded. No secondary branches are detectable and the individual tracheal segments fail to connect (compare to wild type in Fig. 1A). (B,C) Dorsal view of stage 16 wild-type (B) and *btl-Gal4*/*UAS-grh* (C) embryos labelled for DSRF and 2A12, showing that terminal branch identity is not altered by ectopic Grh expression (arrowheads). (D) A stage 16 *btl-GAL4*/*UAS-grh* embryo carrying the fusion cell marker *esg-lacZ* double labelled with antibodies against Grh (red) and β-gal (green) showing that fusion cell differentiation (yellow; double labelled cells) is not affected. (E,F) Confocal optical section (E,F′) and projections (F) of *btl-GAL4*/*UAS-grh*;*UAS-eGFP* embryo labelled for Grh (E; red) and GFP (F,F′; green). The positions of the cell nuclei of the dorsal trunk and dorsal branches in E indicate that they have migrated away from the tracheal sac. The cells extend elaborate basolateral projections (F), but their apical surface does not elongate to form a branch (arrow in F′ marks the tip of the short stump that forms instead of the dorsal branch). (G) Confocal projections of stage 16 *btl-Gal4*/*UAS-grh* embryo labelled with anti-DE-cad, showing apical cell circumferences and outline of the apical (lumenal) surface in two dorsal trunk metameres. The lumenal cavities fail to elongate and fuse despite the fact that the DT cells migrate to become juxtaposed (see D,E,F). (H,I) Stage 16 wild-type (H) and *term-GAL4*/*UAS-grh* (I) embryos carrying the cytoplasmic marker *trh-lacZ*, labelled with antibodies against β-gal (red) and 2A12 (green). Ectopic Grh expressed in single terminal cells prevents lumen growth into the cytoplasmic extensions. Terminal branches (TB) are indicated by arrowheads. Scale bars: 10 μm.

regulation of Grh by Bnl increases its activity, and thereby delimits lumen growth. This invokes a hierarchical two step function for Bnl in which it first promotes branching and tube elongation and it then activates Grh to halt excess apical surface growth and establish a functional lumen. In this model active restriction of morphogenetic processes is required to achieve stereotyped tube dimensions and is an intrinsic part of the program that induces branching morphogenesis. In the second model, regulation by Bnl has differential consequences on Grh, activating some functions (like the one necessary for *GBE-lacZ* expression) and inactivating others, necessary for inhibiting apical membrane growth. In this model, high levels of Btl signalling would temporarily inactivate Grh, in order to allow for apical membrane expansion during the process of branch extension. Both models are consistent with the genetic interactions, which indicate an antagonistic relationship between *grh* and *bnl*, and add the control of apical membrane growth to the repertoire of cellular activities regulated by FGF signalling during morphogenesis.

Of the two models we currently favour the former, where Btl coordinates branching through a sequence of activities, since this model is consistent with the activation of the *GBE-lacZ* reporter. It can also be well integrated with the apical overgrowth phenotype of *grh* mutants, which becomes apparent first in the branches that have reached their final length and only after the completion of branch elongation at stage 16. If Grh were acting to restrict membrane growth continuously, the *grh* mutant phenotype would be expected to appear at earlier stages. A two step model could also explain the inhibiting effect on tube elongation that is seen upon

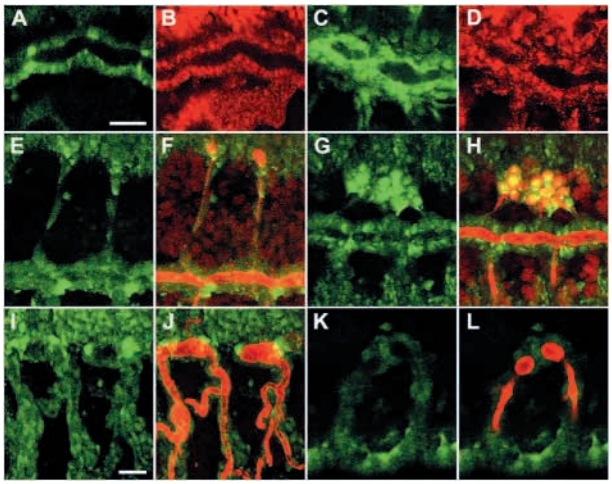

Fig. 7. Bnl activity modulates *GBE-lacZ* expression. (A-D) Ectopic Bnl expression in the trachea causes up-regulation of *GBE-lacZ* expression. Lateral view of a portion of the DT of wild-type (A,B) and *btl-GAL4/UAS-bnl* (C,D) stage 16 embryos, carrying *GBE-lacZ* and double labelled for β-gal (green; A,C) and Grh (red; B,D). *GBE-lacZ* expression is enhanced in most tracheal cells upon ectopic Bnl signalling (compare A and C), but Grh levels remain the same (compare B and D). (E-H) Ectopic expression of Bnl in single terminal cells results in increased levels of *GBE-lacZ* expression in this and neighbouring cells. Dorsal lateral views showing part of the DT and two dorsal branches of wild-type (E,F) and *Term-GAL4/UAS-bnl* (G,H) embryos carrying *GBE-lacZ*, and labelled for β-gal (green, E,G), and lumenal antigen 2A12 and DSRF (both in red; overlaid with green in F and H). Ectopic Bnl signalling in terminal cells results in the expression of the terminal marker DSRF in additional cells (H) and in the same cells the level of *GBE-lacZ* expression is increased (G,H). (I-L) Bnl is required for *GBE-lacZ* expression. Lateral view of two tracheal segments in embryos mutant for *pnt* (I,J) and *bnl* (K,L) that carry *GBE-lacZ*. Double labelling for 2A12 (red) and β-gal (green) shows that tracheal *GBE-lacZ* expression is reduced in *bnl* mutant embryos (K,L) as compared to the epidermal expression, or to the tracheal expression in *pnt* mutants (I,J), which represents wild-type levels. Scale bars: 10 μm.

expression of activated forms of Btl receptors in all tracheal cells of wild-type embryos (Lee et al., 1996).

As restriction of apical membrane growth depends on Grh-mediated alterations in transcriptional activity, the induction of apical membrane expansion upon branch elongation may also rely on changes in gene expression. The nuclear factor Ribbon (Rib) is required for branch elongation (Bradley and Andrew, 2001), and may act as an activator of apical membrane growth. In *rib* mutants, the extension of basal cytoplasmic processes towards the Bnl source appears normal, but the movement of the cell body fails and the apical membrane does not expand, causing a tracheal phenotype that is reminiscent of that seen with ectopic Grh expression (Shim et al., 2001). It is thus conceivable that a balance between Rib and Grh activity determines the extent of apical membrane growth and is coordinated by Bnl through direct modulation of Grh, and perhaps also of the Rib protein. Such a regulation of apical cell surface size by signals deriving from the target tissue could co-ordinate branch elongation, and would provide an elegant allometric control of organ size depending on the signal strength, size and respiratory demand of the target tissue.

Non-tracheal Grh expression and function of mammalian homologues

Apart from its tracheal expression, Grh is found in the embryonic epidermis and all primary epithelial tissues. The epidermal expression of *grh* is also essential as *grh* mutant embryos show a ‘blimp’ phenotype, where the embryonic cuticle stretches to a much greater extent than the wild-type cuticle upon removal of the vitelline membrane (Ostrowski et al., 2002). We find that the epidermal cells in *grh* embryos also show an abnormal apical membrane expansion (data not shown). This is associated with the production of an enlarged cuticle that lines the apical cell surface. Grh may therefore have a common biological function in the epithelial tissues where it is expressed, being required to regulate apical cell membrane growth. Grh protein is continuously expressed in epithelial tissues during larval life (Uv et al., 1997), a period of extensive organ growth to accommodate the dramatic increase in animal size. Thus, Grh is likely to be required not only for organogenesis, but also for the continuous modulations in organ size and shape that occurs throughout the animals life. The temporal and spatial control of Grh activity must however be accomplished through distinct mechanisms in different tissues, as Bnl signalling does not operate in the epidermis.

Grh belongs to a small family of transcription factors that is found only in higher eukaryotes. The specific, but basic function of Grh in the regulation of epithelial apical cell membrane growth raises intriguing questions as to its functional conservation in higher organisms. Two mammalian Grh homologues, MGR and BOM have been recently identified (Wilanowski et al., 2002). Like Grh, MGR and BOM form dimers and MGR interacts specifically with Grh DNA binding sites in vitro. Intriguingly, these mammalian homologues display similar expression patterns to that of Grh. During mouse development MGR is expressed predominantly in the epidermis, and BOM is expressed in the epidermis as well as in several internal tubular organs including the kidney and lung. Thus the biological function of Grh may be conserved in its murine homologues. Given the functional conservation of FGF signalling in tracheal and lung morphogenesis, it will be of great interest to test whether the mammalian homologues of Grh participate in the growth of the lung and to investigate their functional relationship with FGF signalling.

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