Development 134, 3665-3676 (2007) doi:10.1242/dev.007328

Tramtrack regulates different morphogenetic events during *Drosophila* tracheal development

Sofia J. Araújo[1,2], Carolina Cela[1] and Marta Llimargas[1,*]

Tramtrack (Ttk) is a widely expressed transcription factor, the function of which has been analysed in different adult and embryonic tissues in *Drosophila*. So far, the described roles of Ttk have been mainly related to cell fate specification, cell proliferation and cell cycle regulation. Using the tracheal system of *Drosophila* as a morphogenetic model, we have undertaken a detailed analysis of Ttk function. Ttk is autonomously and non-autonomously required during embryonic tracheal formation. Remarkably, besides a role in the specification of different tracheal cell identities, we have found that Ttk is directly involved and required for different cellular responses and morphogenetic events. In particular, Ttk appears to be a new positive regulator of tracheal cell intercalation. Analysis of this process in *ttk* mutants has unveiled cell shape changes as a key requirement for intercalation and has identified Ttk as a novel regulator of its progression. Moreover, we define Ttk as the first identified regulator of intracellular lumen formation and show that it is autonomously involved in the control of tracheal tube size by regulating septate junction activity and cuticle formation. In summary, the involvement of Ttk in different steps of tube morphogenesis identifies it as a key player in tracheal development.

KEY WORDS: *ttk*, Tramtrack, Tracheal system, *Drosophila*, Morphogenesis, Organogenesis, Tubulogenesis

INTRODUCTION

One of the main challenges of developmental biology is to understand how three-dimensional structures are formed during embryonic development. Originally flat epithelia undergo organogenesis and morphogenesis to give rise to complex tissues and organs. Branched tubular organs, such as kidneys, lungs or mammary glands, perform vital functions in all metazoans. Morphogenesis of these organs, so-called tubulogenesis, uses a plethora of different cellular processes (Affolter et al., 2003; Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003). Full identification of the genetic circuits and molecular mechanisms used to control these cellular responses will represent a step forward in our understanding of organogenesis.

The *Drosophila* tracheal system represents one of the best models for organogenesis and tubulogenesis. We use this system to approach how morphogenesis is controlled both genetically and at the cellular level. Tracheal patterning occurs via different cellular processes, including cell migration and intercalation, branch fusion and formation of luminal structures. Throughout the tracheal tree, these processes involve the acquisition of different cell fates and the ability of cells to respond to both intracellular and extracellular cues (Manning and Krasnow, 1993). Over the last decade, several studies have identified genes required for the orchestration and coordination of these aspects. Transcription factors have been reported to play key roles (reviewed in Ghabrial et al., 2003). For instance, Trachealess (Trh) and Ventral veinless (Vvl) are involved in orchestrating early events by inducing many early tracheal-specific genes. At later stages, subpopulations of cells giving rise to different branches with distinct properties express specific transcription regulators, such as Knirps (Kni) and Spalt (Sal). Other transcription factors, such as Grainy head (Grh) are broadly expressed later to fulfil other specific requirements, such as the control of apical membrane growth.

The transcription factor Tramtrack (Ttk) was first identified as a zinc-finger protein involved in the regulation of the pair-rule gene *fushi tarazu* (*ftz*) (Harrison and Travers, 1990). *ttk* encodes two isoforms, Ttk69 and Ttk88, which share N-terminal sequences containing a BTB/POZ domain, but differ in their C-terminal region, in which their DNA-binding zinc-fingers reside. For this reason, it is assumed that they have different DNA-binding specificities and functions (Read and Manley, 1992).

Ttk has been most extensively characterised in the developing embryonic nervous system, in which it acts as a repressor (Badenhorst, 2001; Giesen et al., 1997; Guo et al., 1995), and during photoreceptor differentiation, during which it additionally plays a positive role (Lai and Li, 1999). During early embryogenesis, Ttk regulates the pattern of several pair-rule genes (Brown et al., 1991; Brown and Wu, 1993; Read and Manley, 1992). In addition, a role for Ttk in cell cycle regulation has also been proposed (Audibert et al., 2005; Badenhorst, 2001; Baonza et al., 2002). To date, most of the described requirements for Ttk rely on its ability to regulate cell fate specification. Conversely, very little is known about other roles of Ttk in morphogenesis regulation downstream of cell fate determination (French et al., 2003).

In this study, we have analysed tracheal developmental dynamics with an emphasis on the functional orchestration of diverse morphogenetic steps. In addition to previously defined roles, we report here that Ttk controls various cellular responses downstream of cell fate specification. We find that Ttk is autonomously involved in a pathway leading to cell rearrangements and intercalation, most probably via the regulation of cell shape and the remodelling of adherens junctions (AJs). Remarkably, Ttk also controls tube size autonomously, regulating septate junction (SJ) activity and cuticle formation. Moreover, we define Ttk as the first identified regulator of intracellular lumen formation, and as a factor required autonomously and non-autonomously to specify different tracheal

[1]Institut de Biologia Molecular de Barcelona (IBMB-CSIC) and [2]Institut de Recerca Biomedica de Barcelona (IRB), Parc Cientific de Barcelona, Josep Samitier 1-5, 08028 Barcelona, Spain.

*Author for correspondence (e-mail: mlcbmc@cid.csic.es)

Accepted 2 August 2007

cell identities. The non-autonomous requirement, mediated by *branchless* (*bnl*) modulation, is also involved in the establishment of primary branching. In summary, we propose that Ttk plays a key role in the regulation of multiple steps during tracheal development.

MATERIALS AND METHODS

Drosophila strains

The following stocks are described in FlyBase (http://flybase.bio.indiana.edu): *ttk*^{1}, *ttk*$^{D2\text{-}50}$, *ttk*^{rM730}, *ttk*^{1e11}, *UAS-ttk69*, *UAS-ttk88*, *vvl*^{GA3}, *trh*^{10512}, *Df(3L)ri-XT1*, *esg*$^{B7\text{-}2\text{-}22}$, *UAS-H*, *UAS-N*^{act}, *UAS-btl*^{act}, *btl-moeGFP*, *UAS-srcGFP*, *UAS-tauGFP*. *btlGFP* was obtained from M. Affolter (University of Basel, Switzerland). To recognise the chromosomes carrying the desired mutations, we used second or third blue or GFP-marked balancers.

The wild-type strain used was *yw*. *Drosophila* stocks and crosses were kept on standard conditions at 25°C. Overexpression experiments were conducted at 29°C.

Molecular analysis

The GS element in line *346* was mapped by inverse PCR techniques following standard protocols (BDGP, http://www.fruitfly.org/about/methods/index.html).

Immunostaining, in situ hybridisation and permeabilisation assays

Embryos were staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985) and stained following standard protocols. Immunostainings were performed on embryos fixed in 4% formaldehyde for 20-30 minutes, except for DCAD2 (encoded by *shotgun* – FlyBase) stainings, which were fixed for 10 minutes. The following antibodies were used: anti-Ttk69 (C. Murawsky and A. Travers, MRC-LMB, Cambridge, UK), anti-Dys (S. Crews, University of North Carolina, USA), anti-Sal (R. Schuh, Max Plank Institut, Göttingen, Germany), anti-Cora (R. G. Fehon, University of Alberta, Canada), anti-FasIII (7G10, Developmental Studies Hybridoma Bank, DSHB), anti-Verm (S. Luschnig, University of Bayreuth, Germany), anti-Lac (M. Strigini, IMBB, Crete, Greece), mAb2A12 (DSHB), anti-DSRF (2-161, Cold Spring Harbor Laboratory, CSHL), anti-Kni (developed by J. Reivitz and provided by M. Ruiz-Gomez, CBM, Madrid, Spain), anti-DE-cad (DCAD2, DSHB), anti-Trh (made by N. Martín in J. Casanova's laboratory, IRB, IBMB-CSIC, Barcelona, Spain), anti-GFP (Molecular Probes and Roche), anti-βGal (Cappel and Promega) and anti-Pio (from M. Affolter). Biotinilated or Cy3-, Cy2- and Cy5-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1/300. For HRP histochemistry the signal was amplified with the Vectastain-ABC kit. For fluorescent staining, the signal was amplified using TSA (NEN Life Sciences) when required. Chitin was visualised with Fluostain (Sigma) at 1 μg/ml or CBP (NEB) at 1:500. Permeabilisation assays were performed by injecting rhodamine-labelled dextran (M_r 10,000; Molecular Probes) into the body cavity of embryos (Lamb et al., 1998). In situ hybridization was performed according to standard protocols, with ribo-*pyd* probe (gift from M. Neumann, University of Basel, Switzerland). ribo-*bnl* and ribo-*mmy* were generated using the whole cDNA as template and using the Megascript kit (Ambion). Photographs were taken using Nomarski optics or fluorescence in a Nikon Eclipse 80i microscope. Confocal images were obtained with a Leica TCS-SPE or TCS-SP2 system.

Unless otherwise stated, in all panels labelled 'GFP' the embryos carried *btlGal4* driving GFP-fusion proteins (*btl>xGFP* in Figures). *btlGal4* also drove the expression of other indicated *UAS* constructs. We used mAb2A12 or CBP to visualise the lumen.

Luminal vesicle quantification

We quantified the number of 2A12-positive vesicles in the same two-dimensional areas of confocal projections in both fusion and terminal cells in wild-type and *ttk* embryos. We counted 2A12 vesicles within these areas and subtracted the background measured outside fusion and terminal cells in each case. We used the AnalySIS software v.3.2 (Soft Imaging System GmbH) to quantify the number of vesicles.

Electron microscopy

Wild-type and *ttk* mutants at stage 16-17 were selected under a stereomicroscope, and cryo-fixed and analysed according to Araujo et al. (Araujo et al., 2005).

Time-lapse experiments

Embryos carrying *btlGal4UAS-srcGFP;ttk*$^{D2\text{-}50}$ or *btlGal4UAS-srcGFP* were collected at 25°C and dechorionated for 2 minutes with sodium hypochlorite diluted 1/100. They were glued to a coverslip and mounted in 10S Voltaleff oil with the hanging drop method to improve optics and to avoid desiccation in an oxygen-permeable chamber. Images were collected from stage 14 embryos at 21°C on a Leica TCS-SP2-AOBS or TCS-SP5-AOBS system, Leica DM IRE2 microscope and LCS software. The 488 nm emission line of an Argon laser was used for excitation and sections were recorded every 4 or 5 minutes over a 3- to 6-hour period. Laser intensity was kept at a minimum to minimise phototoxicity. TIFF projection images were processed into 3D and 4D LCS software, and the movie was assembled using ImageJ (NIH Image).

Quantification of the intercalation defects

ttk$^{D2\text{-}50}$ mutants and *ttk*$^{D2\text{-}50}$ mutants expressing *ttk69* in the tracheal tissue (obtained from the cross *btlGal4/btlGal4; ttk*$^{D2\text{-}50}$*/TM3lacZ* × *ttk*$^{D2\text{-}50}$*UASttk69/TM3lacZ* and selected by the absence of blue balancers) were immunostained with DCAD2. To determine the intercalation state, we carefully analysed the presence of intercellular versus autocellular AJs in each dorsal branch (DB) and lateral trunk (LT) of stage 15 or 16 embryos under the microscope. Each branch was classified into one of the four categories we describe in Fig. S1 in the supplementary material.

RESULTS

Ttk is expressed and required during tracheal development

To identify new genes involved in tracheal development, we used the Gene Search (GS) system (Toba et al., 1999) to generate an original collection of lines whose tracheal phenotypes were analysed by crossing with *breathless-Gal4* (*btlGal4*) (C.C. and M.L., unpublished). GS line *346* was selected because of its impairment of tracheal branch fusion (Fig. 1A). This insertion was mapped to 100D, close to the *ttk* gene. When an independent *UASttk69* line was crossed to *btlGal4*, the *GS346* tracheal phenotype was reproduced (data not shown), indicating that the tracheal phenotype was indeed due to *ttk* over- or mis-expression.

ttk is maternally supplied, subsequently declines and zygotic expression reappears during germ band extension (Harrison and Travers, 1990; Read and Manley, 1992). From stage 11 until the end of embryogenesis, clear expression of *ttk* is observed in all tracheal cells (Fig. 1B,C). We found that *ttk* tracheal expression does not depend on genes known to induce tracheal fate, such as *vvl*, *trh* or *kni*, on their own (data not shown), suggesting that it might depend on a combination of these inducers or directly on the same anteroposterior (A-P) and dorsoventral (D-V) embryonic cues regulating tracheal inducers (de Celis et al., 1995; Wilk et al., 1996).

Strong tracheal pattern defects were detected in amorphic mutants (*ttk*$^{D2\text{-}50}$) whereas milder defects were observed in hypomorphic mutants (*ttk*^{rM730}) (Fig. 1E and data not shown). The *ttk* locus encodes *ttk88* and *ttk69* (Read and Manley, 1992). Mutants for *ttk88* (*ttk*^{1}) (Xiong and Montell, 1993) are viable and do not show a tracheal phenotype. In addition, overexpression of *ttk88* did not result in tracheal defects (data not shown). Conversely, mutants for *ttk69* (*ttk*^{1e11}) (Lai and Li, 1999) displayed a clear tracheal phenotype (Fig. 1F) and, as already indicated, overexpression of *ttk69* affected tracheal development (Fig. 1A). These results suggest a specific role for *ttk69* during tracheal development. In this work, we used either *ttk*$^{D2\text{-}50}$ or *ttk*^{1e11} to analyse *ttk* tracheal requirements.

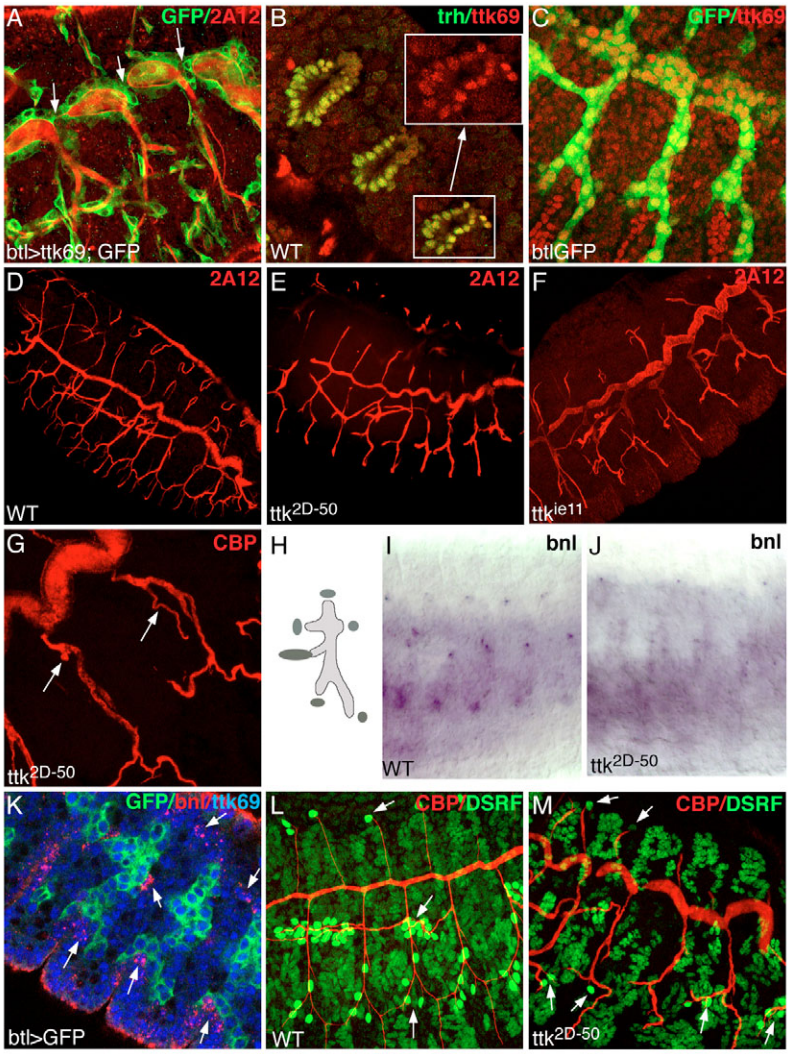

Fig. 1. Ttk tracheal expression and requirements. (**A**) Late-stage *ttk* mutant embryo showing an absence of branch fusion in the dorsal trunk (DT) (arrows). (**B**,**C**) Ttk69 protein accumulation in wild-type embryos at stage 11 (B) and 14 (C). Ttk (red) is expressed in all tracheal cells (green). (**D-F**) Embryos at late embryogenesis. Notice the rudimentary aspect of the tracheae in *ttk* mutants (E,F) as compared with wild type (WT; D). (**G**) Details of two tracheal metameres, showing the absence or reduction (arrows) of visceral branches (VBs) in a *ttk* mutant. (**H**) Schematic representation of wild-type *bnl* expression (dark grey) at stage 13. (**I**,**J**) Expression of *bnl* at stage 13. Notice the reduction in size and number of *bnl* spots in the *ttk* mutant (J). (**K**) A stage 12 embryo showing co-expression of *bnl* mRNA and Ttk69 protein (arrows). (**L**,**M**) Details of stage 15 embryos labelled to highlight DSRF-positive terminal cells (arrows). DSRF is expressed in several presumptive terminal cells in *ttk* mutants (arrows in L). All panels, except H, I and J, show projections of confocal sections of laterally viewed embryos. In this and all figures, dorsal is up and anterior to the left.

Ttk is non-autonomously required to establish proper tracheal identities

Early steps of tracheal development, such as tracheal induction and invagination, proceeded normally in *ttk* mutants. The first tracheal defects were visible from stage 13, when visceral branches (VBs) were often missing or reduced (Fig. 1G) and, if present, contained fewer cells. Cell counts indicated that the rest of the primary branches contained grossly the normal number of cells, except for the transverse connective (TC), which contained more cells (19.2 cells, n=10, in the TC of the fifth tracheal metamere of *ttk* mutants as compared with 8-10 cells in wild type) (Samakovlis et al., 1996a), suggesting that the TCs incorporate the presumptive VB cells.

The Bnl/Breathless (Btl) pathway plays a key role in the establishment of primary branching (reviewed in Ghabrial et al., 2003). The receptor Btl, expressed in all tracheal cells, is activated by its ligand, Bnl, which is dynamically expressed outside the tracheal tissue in positions towards which the primary branches will grow (Sutherland et al., 1996). We found that, from stage 12-13, *bnl* expression was slightly reduced and disappeared earlier from the dorsal and from most ventral spots, and was almost lost from the visceral mesoderm (Fig. 1J), which is presumably responsible for VB formation (Sutherland et al., 1996). Consistent with a positive regulation of *bnl* expression by *ttk*, we found that *ttk* is co-expressed in *bnl*-expressing cells (Fig. 1K).

Besides its role in primary branching, the Bnl/Btl pathway is also required for terminal cell specification via the regulation of *DSRF* (also known as *blistered* – FlyBase) (Sutherland et al., 1996). We investigated whether terminal cells were specified in *ttk* mutants and found that cells expressing DSRF protein were generally present in some branches [i.e. lateral trunk anterior (LTa)], but only occasionally present in others, such as in dorsal branches (DBs), VBs and lateral trunk posterior-ganglionic branches (LTp-GBs) (Fig. 1M). This pattern of DSRF is consistent with the defective pattern of *bnl* expression observed in *ttk* mutants: in those spots in which *bnl* is lost or reduced, DSRF is rarely expressed.

Altogether these results indicate that *ttk* regulates the allocation of cells to particular primary branches and the specification of the terminal cells by modulating *bnl* expression outside the tracheal tissue.

Specification of fusion fate requires Notch-mediated regulation of Ttk levels

In wild-type embryos, tracheal cells that mediate branch fusion express specific markers, such as *escargot* (*esg*) and *dysfusion* (*dys*) (Jiang and Crews, 2003; Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996) (Fig. 2A and data not shown). Protein expression of these markers was not detected in *btlGal4-UASttk*

embryos (Fig. 2B and data not shown), revealing that tracheal cells failed to acquire the fusion identity. This results in an absence of branch fusions (Fig. 1A).

The above results suggested that high levels of *ttk* have to be avoided to correctly specify fusion cells. Consistent with this hypothesis, we found subtle differences in the levels of Ttk69 protein, which appeared to be lower in the presumptive fusion cells and higher in the fusion-adjacent cells (Fig. 2D-F′). These differences were functional, because overexpressing *ttk* specifically in fusion cells prevented branch fusion (Fig. 2C). The results suggested that a precise modulation of Ttk levels is necessary for proper fusion identity specification.

The fusion phenotype of *ttk* overexpression resembles that of constitutive activation of the Notch (N) pathway, which also blocks fusion. Indeed, *N* is active in fusion-adjacent cells, restricting the fusion fate specification in that area (Ikeya and Hayashi, 1999; Llimargas, 1999; Steneberg et al., 1999). This pattern of *N* activity correlates with the protein pattern of Ttk69. Furthermore, Ttk acts as an effector of N signalling in several developmental contexts (Guo et al., 1996; Jordan et al., 2006; Okabe et al., 2001). Interestingly, we found low Ttk69 protein levels in the extra fusion cells present in *N* loss-of-function conditions [achieved by overexpressing Hairless (H) (Llimargas, 1999), Fig. 2G]. Conversely, we did not find cells expressing low levels of Ttk69 protein in constitutively activated N conditions (Fig. 2H). These results suggest that *ttk* acts as a downstream effector of *N* during fusion cell type specification, although N might have other targets to fulfil this function (see below and Discussion).

Ttk is involved in the pathway leading to tracheal cell rearrangements

By late embryogenesis, the tracheal pattern of *ttk* mutants resembles that of stage 13 or 14 embryos, as if branches did not extend properly (Fig. 1E,F). During wild-type development, branches extend by directed cell migration and cell rearrangements. Cell intercalation, a particular type of cell rearrangement, occurs in most primary multicellular branches except the dorsal trunk (DT) (Fig. 3A-B′). Intercalation has been divided into four steps (Ribeiro et al., 2004): (1) pairs of cells connected by intercellular AJs arrange side-by-side; (2) one of the two cells reaches around the lumen with its distal end while the other does it with its proximal end, thereby forming autocellular AJs at the points at which the AJs of each single cell meet and seal; (3) the nascent autocellular AJs elongate and zip up as the two cells arrange in an end-to-end position; (4) the zipping-up process is stopped, leaving the two cells connected by a small ring-like intercellular AJ.

We analysed cell intercalation in *ttk* mutants by monitoring DE-cadherin (DE-cad) protein accumulation, an marker of AJs (Fig. 3C-D′). Tracheal cells in branches in which intercalation usually occurs did not rearrange and remained positioned in side-by-side pairs by late embryogenesis (Fig. 3D). Autocellular AJs (visualised as lines after using AJ markers, Fig. 3B′) only occasionally formed or zipped up. Indeed, we found several DBs with no signs of autocellular AJs (Fig. 3D′, arrowhead; see Fig. S1 in the supplementary material) and others with short stretches of autocellular AJs followed by long stretches of intercellular ones (visualised as a mesh-like structure, Fig. 3D′, arrows; see Fig. S1 in the supplementary material). Similar results were observed in the lateral trunk (LT) (Fig. 3E-F′; see Fig.

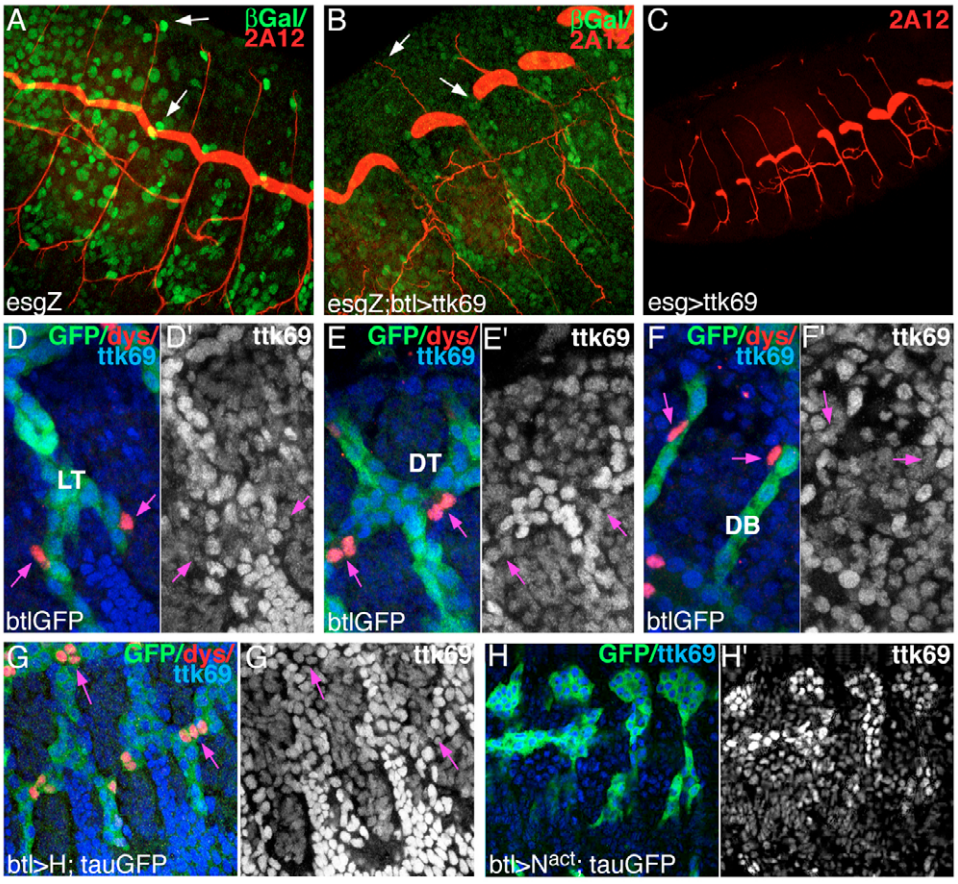

Fig. 2. Regulated levels of Ttk specify fusion cell identity. (**A**,**B**) Stage 15 embryos labelled to highlight Esg-positive fusion cells (arrows in A). Tracheal overexpression of *ttk* (B) blocks fusion fate acquisition (arrows) and branch fusions. (**C**) Late-stage embryo overexpressing *ttk* specifically in fusion cells, which also leads to a lack of branch fusion. (**D-F′**) Details of one or two tracheal metameres focused at the lateral trunk (LT; D,D′), dorsal trunk (DT; E,E′) or dorsal branches (DBs; F,F′). Dys-positive fusion cells (arrows) show lower levels of Ttk69 (in greyscale in panels D′,E′ and F′) when compared with the neighbouring tracheal cells. (**G**,**G′**) Two tracheal metameres focused at the LT. All extra fusion cells (arrows) in N loss-of-function conditions show lower levels of Ttk69 (in greyscale in G′). (**H**,**H′**) Four tracheal metameres focused at the DT. No cells show low levels of Ttk69 (in greyscale in H′) when N is constitutively active. All panels show projections of confocal sections of laterally viewed embryos.

S1 in the supplementary material). These results indicate that, in *ttk* mutants, the step involving reaching around the lumen is generally prevented and the zipping up, when it occurs, is incomplete.

To check whether intercalation defects were caused by cell fate misspecification, we analysed the pattern of primary branching markers in *ttk* mutants. The transcription factor Salm (Spalt-major) is normally expressed in the DT (Kuhnlein and Schuh, 1996), in which it prevents intercalation (Ribeiro et al., 2004). Conversely, the transcription factors Kni and Knrl are expressed in all other primary branches except the DT (Chen et al., 1998), and they repress Salm, thereby promoting intercalation (Ribeiro et al., 2004). Salm and Kni proteins were correctly accumulated in *ttk* mutants (Fig. 3H,I), indicating that the allocation of cells to different primary branches was correct. Additionally, we found that *piopio* (*pio*), required for the intercalation process (Jazwinska et al., 2003), was normally expressed and that Pio protein accumulated normally (Fig. 3K) in *ttk* mutants.

We next asked how *ttk* was required for intercalation. We found that adding *ttk* to tracheal cells in *ttk* mutants rescued intercalation (Fig. 3G, see Fig. S1 in the supplementary material). This indicated an autonomous requirement for *ttk* during intercalation and ruled out the possibility that impaired intercalation was due to defects in other tissues (for instance, due to impaired dorsal closure).

Ttk regulates cell shape changes and modulates AJs during intercalation

How does Ttk affect intercalation? To approach this question, we performed time-lapse experiments in embryos carrying *btlGal4 UAS-srcGFP*, in which the outline of tracheal cells is highlighted (Fig. 3E-F′, Fig. 4 and see Movies 1-4 in the supplementary material). In an otherwise wild-type background, paired cells of intercalating branches showed a short period of rapid relative movement followed by a directional sliding. These movements were accompanied by a conspicuous change in shape, which transformed originally paired-

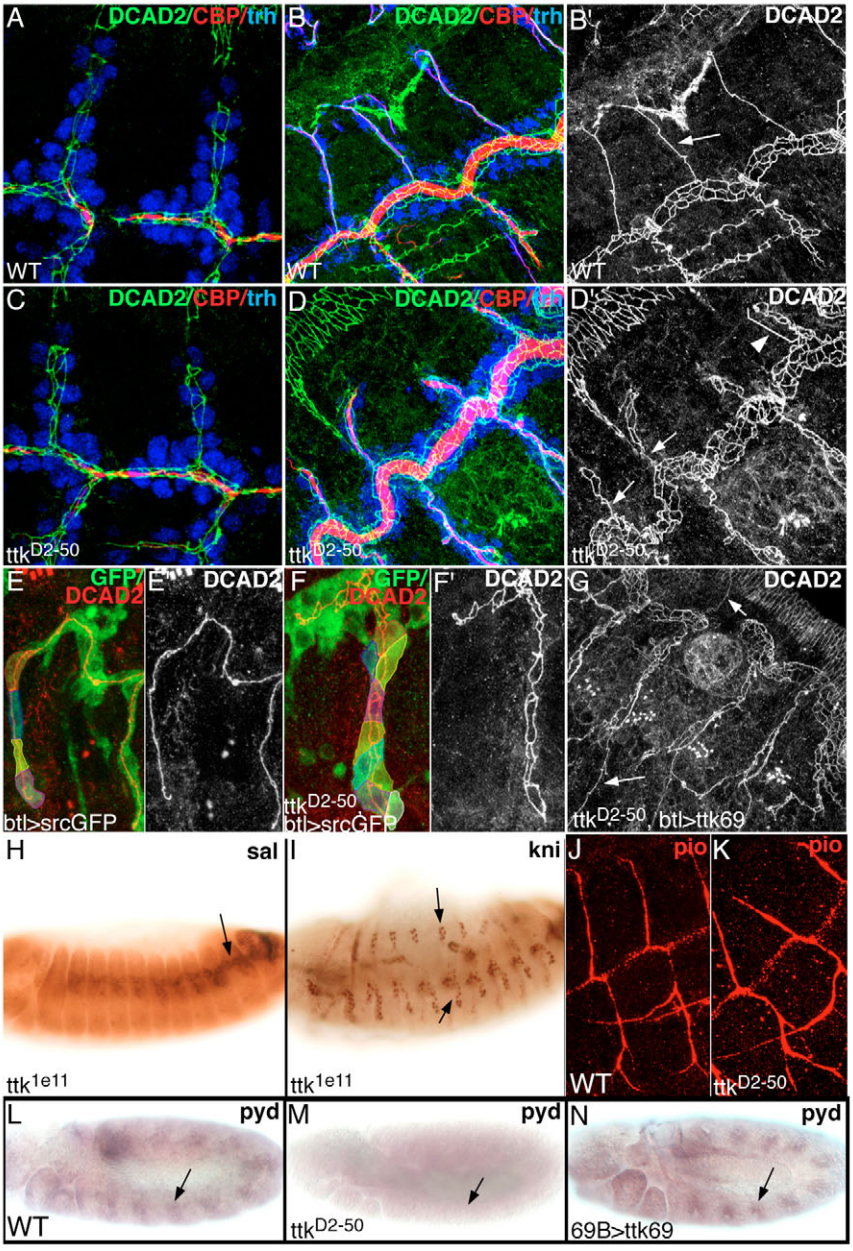

Fig. 3. Ttk is required during tracheal cell intercalation. (**A-D′**) Projections of confocal sections showing details of stage 13 (A,C) and 16 (B,D) embryos focused at the dorsal trunk (DT) and dorsal branches (DBs). No apparent differences are observed at the early stages (compare A with C). Accumulation of DE-cad (labelled with DCAD2 and shown in greyscale in B′ and D′) shows the presence of autocellular adherens junctions (AJs; arrow in B′) in wild type (WT). (D′) *ttk* embryos exhibit mainly intercellular AJs (arrowhead) and only occasional short stretches of autocellular ones (arrows). (**E-F′**) Projections of confocal sections showing lateral trunk (LT) details of stage 16 embryos. Notice the presence of autocellular AJs in wild type (E′), and the elongated shape of the intercalated cells (E, cells have been coloured to facilitate observation of cell rearrangements and shape). Conversely, *ttk* mutants show mainly intercellular AJs (F′) and cuboidal cells positioned side-by-side (F). (**G**) Projection of confocal sections showing a late-stage *ttk* mutant. The formation of autocellular AJs (arrows) is restored by tracheal expression of *ttk*. (**H**,**I**) Lateral views of *ttk* embryos at stage 13-14 showing normal accumulation of Sal and Kni proteins (arrows). (**J**,**K**) Projections of confocal sections showing details of stage 16 wild-type and *ttk* mutant embryos labelled to show Pio accumulation. (**L-N**) Expression pattern of *pyd* at the placode stage (arrows). Notice the absence of expression in *ttk* mutants (I) and increased levels in several structures (arrows in J) when *ttk* is generally overexpressed.

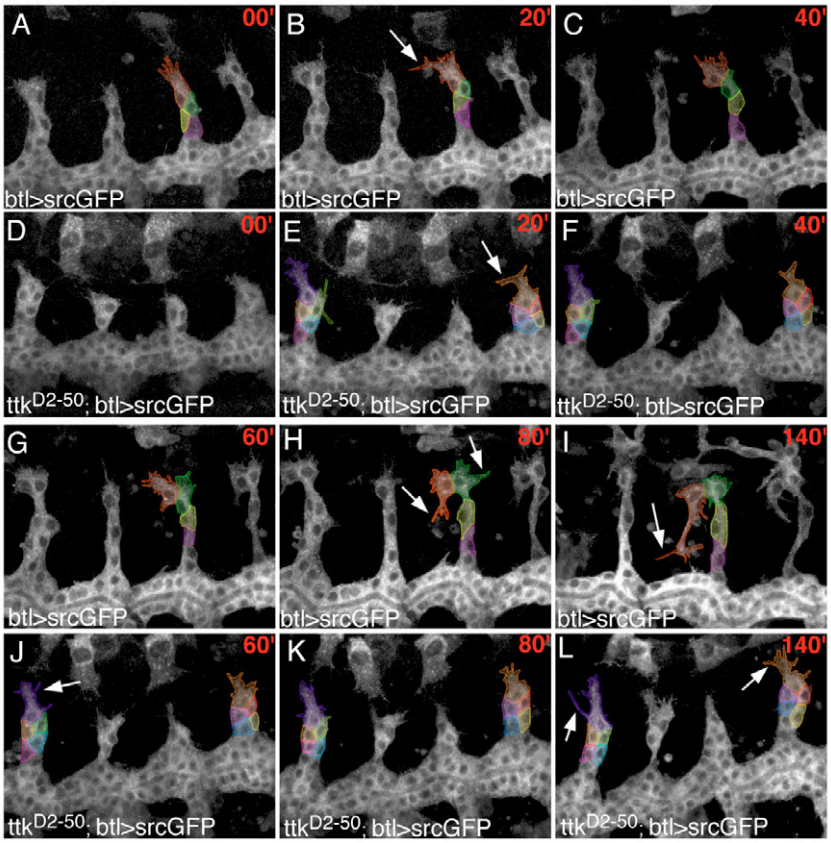

Fig. 4. Ttk is required for tracheal cell rearrangements and cell shape changes. (**A-L**) Projections of confocal sections from a time-lapse experiment, showing the development of dorsal branches (DBs). Time is indicated in the top right corner of each panel. The cells of selected DBs have been coloured to facilitate observation of cell rearrangements. In *ttk* mutants (D-F,J-L), paired cells remain paired and cuboidal, and do not intercalate. Notice the formation of filopodia (arrows).

cuboidal cells into a single row of elongated ones. Strikingly, *ttk* mutant cells remained cuboidal throughout development, and only occasionally could weak signs of cell elongation be detected. These cuboidal-paired cells still showed the relative movement (for longer periods than in wild type), but this was not usually followed by a shift to a directional displacement. *ttk* mutant cells appeared unable to undertake cell shape changes, which we suggest (see Discussion) prevents the paired cells to slide one over the other and intercalate.

Additionally, time-lapse experiments suggested that the external force proposed to drive branch extension and intercalation, Bnl (Ribeiro et al., 2004), was working in *ttk* mutants. In wild type, numerous thin filopodia extended at the tips of primary branches in response to Bnl (Fig. 4, see Movies 1 and 2 in the supplementary material) (Ribeiro et al., 2002). Although in *ttk* mutants *bnl* expression was affected, we detected filopodia at the tips of the branches, presumably in response to Bnl (Fig. 4, see Movies 3 and 4 in the supplementary material). This result suggests that the *bnl* mRNA remaining in *ttk* mutants should be sufficient to drive intercalation. Supporting this hypothesis, we found that *ttk* tracheal expression rescues the intercalation defects of *ttk* mutants (Fig. 3G), ruling out the possibility that impaired intercalation was due to the non-autonomous requirement of *ttk* for *bnl* expression.

A correlation between the modulation of AJs and intercalation during tracheal development has been recently established in a report on the role of Polychaetoid (Pyd). *pyd* encodes a MAGUK protein that localises to AJs, and loss of *pyd* prevents intercalation (Jung et al., 2006). We found that *pyd* is a target of Ttk; *pyd* expression was lost in *ttk* embryos (Fig. 3M) and enhanced by *ttk* overexpression (Fig. 3N). Our results indicate that *ttk* autonomously regulates intercalation not by regulating cell fate but by allowing cell shape changes and by modulating AJs via Pyd.

Ttk is required for the proper fusion of branches

Besides its role in fusion fate specification (see above), we also found that *ttk* mutants show impaired (in DBs and the LT) or delayed (in the DT) fusion events. In particular, we observed that 30% of *ttk* embryos showed a complete absence of LT fusion, 40% showed one single anastomosis out of the total nine per hemisegment, and the remaining mutants never showed more than three anastomosis. It is unlikely that this is caused by defects in fusion fate specification, because fusion markers, such as *esg* and *dys* (Fig. 5A and data not shown), are expressed in a normal pattern but at slightly lower levels than in wild type, although we cannot rule out this possibility. At the cellular level, the fusion process has been well-characterised (Lee et al., 2003; Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996). It begins when two fusion cells extend cytoplasmic processes and make contact. Then they form a new DE-cad contact at the interface (Fig. 5C), promoting the formation of an actin-containing track that guides the invaginating apical surfaces of the fusion cells. Finally, the two apical surfaces meet and fuse, giving rise to two doughnut-shaped fusion cells containing an intracellular junctionless lumen. It has been proposed that this intracellular lumen forms by assembly and coalescence of luminal vesicles that appear at the tip of the growing lumen (Uv et al., 2003). We observed that the first steps of branch fusion appear normal in *ttk* mutants: the presumptive fusion cells extended filopodia and established contact, and they formed a new DE-cad contact at the interface (Fig. 5D). However, *ttk* mutant cells seemed defective in generating an intracellular lumen that penetrates the fusion cell (Fig. 5B). In agreement with this, we detected fewer luminal vesicles in *ttk* fusion cells as compared with wild type (0-5 vesicles in *ttk* mutants, n=10, versus 10-20 in wild type, n=10; see Materials and methods) (Fig. 5E′,F′, arrowheads). Altogether, our

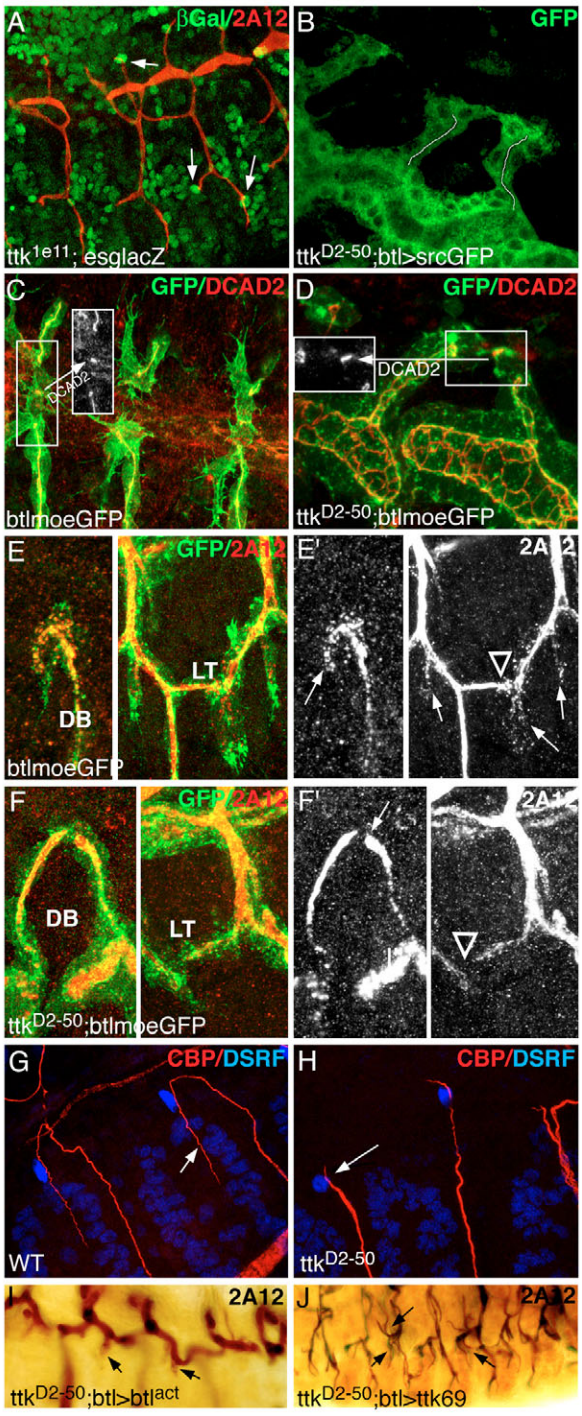

Fig. 5. Ttk is involved in the formation of terminal and fusion branches. (**A**) Stage 15 embryo labelled to highlight Esg-positive cells (arrows). (**B**) Two adjacent dorsal branches (DBs), the tip cells (probably fusion cells) of which make contact. The lumen (highlighted in white) does not penetrate the contacting cells. (**C**,**D**) Contralateral (C) or adjacent (D) DBs, the tip cells of which make contact. An E-cad-rich structure (in greyscale in insets) forms at the contact point (arrows). (**E-F′**) Details of DBs and lateral trunks (LTs). The lumen marker 2A12 (in greyscale in E′,F′) reveals the presence of abundant luminal vesicles at the tips of branches in the presumptive fusion (arrowhead) and terminal (arrows) cells in wild type (E′). *ttk* mutants (F,F′) show a decreased density of luminal dots. (**G**,**H**) DBs showing DSRF-positive cells and terminal branches (arrow in G). Notice the absence of terminal branches (arrow in H) in spite of DSRF expression in *ttk* mutants (H). (**I**,**J**) Fragments of LTs of *ttk* mutants at stage 16. Terminal branch formation is rescued when *ttk* is expressed in tracheal cells (arrows in J), but not when the Btl pathway is constitutively activated (arrows in I). All panels except I and J show projections of confocal sections of laterally viewed embryos.

observations suggest that *ttk* regulates a step downstream of fusion fate specification: contact of fusion cells and formation of a new DE-cad contact.

Ttk is required for terminal branch formation

Although several cells expressed terminal markers in *ttk* mutants (Fig. 1M), terminal branches which normally arise from terminal cells did not extend in these embryos (Fig. 5G,H). This phenotype is not due to reduced DSRF accumulation caused by lower *bnl* expression, because although DSRF expression was restored in *ttk* mutants expressing *bnl* (data not shown) or a constitutively active form of Btl in tracheal cells, terminal branches remained short and rudimentary (Fig. 5I). This indicates that, apart from its non-autonomous role, *ttk* must also play an autonomous role in terminal tube formation. Accordingly, we found that restoring *ttk* tracheal expression in *ttk* mutants was able to rescue terminal branch formation, mainly in the LT, in which DSRF is normally expressed (Fig. 5J).

At the cellular level, terminal branches are intracellular junctionless tubes formed inside the terminal cells. Terminal cells form an F-actin-rich structure and extend cytoplasmic protrusions that are invaded by an intracellular lumen, which presumably grows by the fusion of intracellular vesicles (Oshima et al., 2006; Uv et al., 2003). Time-lapse experiments of *btlGal4 UAS-srcGFP* embryos revealed the formation of cytoplasmic protrusions in *ttk* mutants, although these filopodia were never stabilised and no internal lumen was detected (Fig. 4, and see Movies 3 and 4 in the supplementary material). In addition, we detected a lower density of luminal vesicles in presumptive terminal cells in *ttk* mutants as compared with wild type (0-3 vesicles in *ttk* mutants, n=10, versus 20-30 in wild type, n=10; see Materials and methods) (Fig. 5E′,F′, arrowheads), suggesting a defect in intracellular lumen formation.

Ttk plays a key role in the control of tube size

By late embryogenesis, the tracheal tubes of *ttk* mutants appeared thicker and more convoluted than those in wild type. On average, the diameter of the largest part of the DT (between abdominal segments 6 and 8) is 25% wider in *ttk* mutants than in wild type (n=12) (Fig. 6A,B). This phenotype is reminiscent of other mutants affecting tube size. To date, two different systems have been reported to regulate tube size: the septate junctions (SJs) and a transient chitin filament [(Swanson and Beitel, 2006; Wu and Beitel, 2004) and references within].

We investigated whether the intraluminal chitin matrix was properly organised in *ttk* mutants. Using a fluorescent chitin binding protein (CBP) or Fluostain, which label chitin fibrils, we detected clear differences between *ttk* and wild-type embryos (Fig. 6C,D and data not shown). Instead of an organised cylindrical filament composed of parallel chitin polymers, *ttk* mutants showed an amorphous, decreased and disorganised labelling, with a scratched perpendicular pattern. These results point to a defect in the assembly of the chitin filament, consequently affecting tube size.

Several genes participate in a pathway devoted to chitin synthesis in the trachea. *mummy* (*mmy*) encodes a UDP-N-acetylglucosamine pyrophosphorylase enzyme required for the synthesis of the building blocks of chitin (Araujo et al., 2005; Devine et al., 2005; Tonning et al., 2006). We found that *mmy* is a target of Ttk, because its expression was increased in *ttk* embryos at stages in which chitin is being synthesised and lowers when *ttk* is overexpressed (Fig. 6E-G). The chitin filament has to be properly assembled and modified to become functional. *serpentine* (*serp*) and *vermiform* (*verm*, also known as *LCBP1* – FlyBase) encode two ChLD (Chitin and LDL-receptor binding motifs) proteins required to assemble the intraluminal chitin filament and restrict tube elongation (Luschnig et al., 2006; Wang et al., 2006). We detected a decrease in levels of these proteins in the lumen of *ttk* mutants (Fig. 6K). These results indicate a defect of filament synthesis and maturation.

It has been suggested that the proper secretion of Verm and Serp depends on SJ activity (Wang et al., 2006). Thus, we next analysed SJ complexes in detail. At confocal resolution, we could only detect very subtle differences between *ttk* and wild-type embryos using different markers. In particular, with antibodies recognising Fasciclin III (FasIII, Fas3) or Lachesin (Lac), a slight decrease and/or diffusion was detected in the trachea, salivary glands and hindgut of *ttk* mutants, whereas we found no detectable differences in these tissues with respect to *Coracle* (Cora) accumulation (Fig. 6L,M and data not shown). In agreement with this, transmission electron microscopy (TEM) analysis of *ttk* embryos revealed the presence of properly localised SJs (Fig. 7D). To further analyse SJ functionality, we assayed for the trans-epithelial diffusion barrier of the tracheal tubes by injecting 10 kDa rhodamine-labelled dextran (Baumgartner et al., 1996; Lamb et al., 1998). We detected diffusion of this dye into the trachea of *ttk* mutants (Fig. 6O). These results show that, although the assembly of SJs is not grossly affected, the complexes are not fully functional. This inefficient SJ activity might be responsible, at least in part, for the tube size defects observed in *ttk* mutants. Taken together, our results indicate that *ttk* regulates tube size by controlling chitin filament formation and its proper assembly via SJ activity.

Ttk is required for proper luminal cuticle formation

In *ttk* mutants, we found clear ultrastructural defects that were related to abnormal chitin deposition. At late stages of development, wild-type embryos display three distinguishable layers of cuticle (envelope, epicuticle and procuticle). In addition, the luminal cuticle is decorated by regular ridges known as taenidia. These taenidial folds are filled by the procuticle, loaded with lamellar chitin, which, at TEM resolution, can be recognised as a continuous and electron-

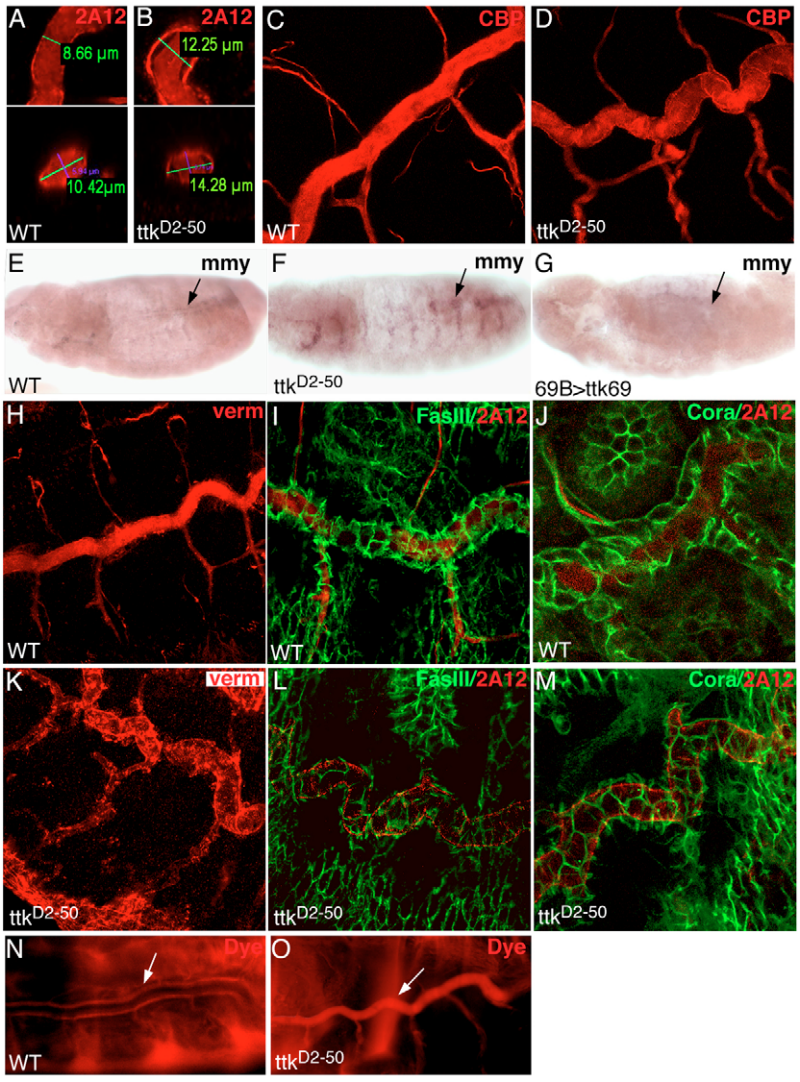

Fig. 6. Ttk controls tracheal tube size. (**A**,**B**) Portions of dorsal trunk (DT) in longitudinal views (upper panels) or in cross-sections (lower panels) of wild type (WT; A) and *ttk* mutants (B). Thickness of the tubes is measured. (**C**,**D**) Stage 16 embryos showing accumulation of CBP in the DT. *ttk* mutants show an abnormal intraluminal chitin filament (D). (**E-G**) Expression pattern of *mmy* in stage 14 embryos. Notice the increased levels of expression in *ttk* mutants (F) and the absence of expression when *ttk* is generally overexpressed (G). (**H**,**K**) Stage 16 embryos. Verm is abnormally accumulated in *ttk* mutants (K). (**I**,**J**,**L**,**M**) Stage 16 embryos. Accumulation of septate junction (SJ) markers (FasIII and Cora; green) in *ttk* mutants (L,M) is only slightly affected (L) or is comparable to wild type (M). (**N**,**O**) Stage 16 embryos injected with a 10 kDa rhodamine-labelled dextran. *ttk* embryos (O) are permeable to the dye, which fills the tracheal lumen (arrows), whereas the wild-type trachea is impermeable (N). All panels except E-G,N and O show projections of confocal sections of laterally viewed embryos.

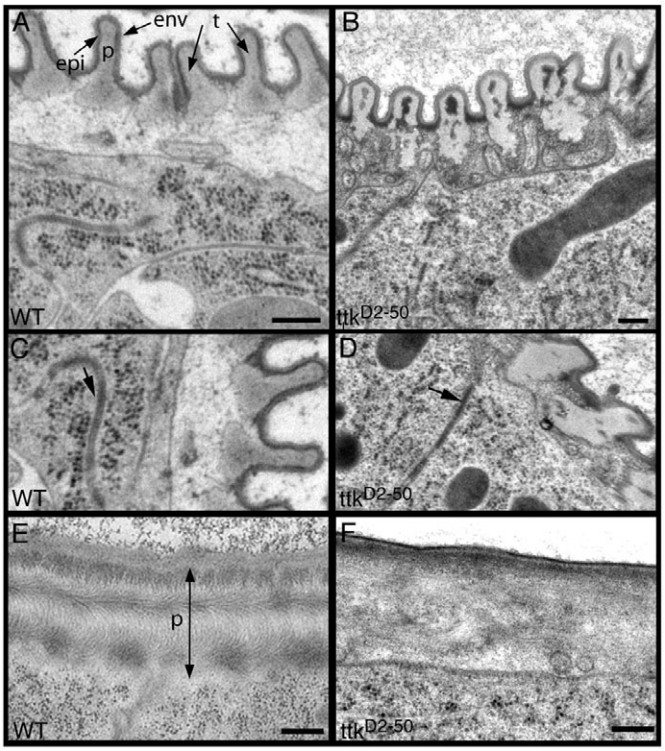

Fig. 7. Ttk is required for proper luminal cuticle formation. Transmission electron microscopy (TEM) micrographs of late-stage embryos. (**A**,**B**) Longitudinal sections of the dorsal trunk (DT). Notice the taenidial folds (t) and the cuticle layers – envelope (env), epicuticle (epi) and procuticle (p) – within each taenidium. (B) *ttk* mutants show a disorganised taenidial structure and density differences at the level of the procuticle. (**C**,**D**) Detail of septate junctions (SJs; arrows) between adjacent DT cells. As in wild type (C), SJs are correctly positioned in *ttk* mutants. (**E**,**F**) Detail of the epidermal cuticle. In the procuticle (p) of wild-type embryos (E), the chitin laminae are arranged in a typical helicoidal pattern. In *ttk* mutants (F) the procuticle is very disorganised. Scale bars: 200 nm.

dense material with an organised aspect (Fig. 7A) (Araujo et al., 2005; Locke, 2001). In *ttk* embryos, taenidia showed an irregular shape, size and pattern. In addition, the material filling the taenidia (procuticle) was disorganised, discontinuous and frequently contained inclusions of more electron-dense material (Fig. 7B). The cuticle of the larval epidermis was also affected in *ttk* mutants. Instead of the characteristic lamellar organisation of wild-type procuticle (Fig. 7E), *ttk* mutants showed an amorphous, unstructured layer (Fig. 7F). These observations indicate that *ttk* is required for both epidermal and tracheal cuticle formation.

DISCUSSION

Ttk is required for different events during tracheal development

In this study, we found that Ttk acts as a key gene for tracheal development by positively and negatively regulating multiple autonomous and non-autonomous targets.

In a similar fashion to the transcription factors Trh and Vvl (reviewed in Ghabrial et al., 2003), which are involved in orchestrating early events of tracheal development, Ttk plays a role in orchestrating several late tracheal events. Ttk69 has been found to act mostly as a repressor. Here we identify Ttk targets that appear to be negatively regulated (such as *mmy* and *esg*) whereas others appear to be positively regulated (such as *pyd* and *bnl*). In this latter case, Ttk might be converted into a positive regulator, as already described during photoreceptor development (Lai and Li, 1999).

We identified different tracheal requirements for Ttk. Interestingly, most of them depend on Ttk regulating events downstream of cell fate specification, at the level of cellular responses (see below). Additionally, a few other requirements depend on cell fate specification, as has been described for most other functions of Ttk in other developmental situations. For instance, Ttk regulates fusion cell specification by acting as a target and mediator of N, as occurs during sensory organ development (Guo et al., 1996; Okabe et al., 2001) and oogenesis (Jordan et al., 2006). Such regulation of Ttk by N might be post-transcriptional, as occurs during sensory organ development (Okabe et al., 2001). Remarkably, we found that, although Ttk is sufficient to repress *esg* expression in fusion cells, it might not be the only *esg*- and fusion fate-repressor, because absence of Ttk does not increase the number of Esg-positive cells, as does downregulating N (Ikeya and Hayashi, 1999; Llimargas, 1999; Steneberg et al., 1999). Other N targets might be redundant with Ttk, and such redundancy could reinforce N-mediated repression of fusion fate in positions in which inductive signals (such as Bnl, Dpp and Wg) (Ikeya and Hayashi, 1999; Llimargas, 2000; Steneberg et al., 1999; Chihara and Hayashi, 2000; Llimargas and Lawrence, 2001) are very high, particularly near the branch tips.

The role of Ttk during cell rearrangements

Cell rearrangements during development are common to most animals and ensure proper morphogenesis. During tracheal development, many branches grow and extend by cell intercalation (Neumann and Affolter, 2006; Pilot and Lecuit, 2005). Several cellular and genetic aspects of tracheal intercalation have been well described (Ribeiro et al., 2004). However, targets of Sal (which inhibits intercalation) are currently unknown.

Here, we identify Ttk as a new and positive regulator of intercalation. We found that Ttk is involved in cell junction modulation by transcriptionally regulating *pyd*, the only junctional protein shown, so far, to affect intercalation (Jung et al., 2006). In fact, modulation of AJs has been proposed to play a role during intercalation (Neumann and Affolter, 2006). However, Pyd cannot be the only Ttk effector of intercalation, because the *pyd* mutant phenotype is much weaker than that of *ttk* mutants. Accordingly, we found that, in *ttk* mutants, cells in branches that usually intercalate remain paired and cuboidal, and appear unable to change shape and elongate. Although other explanations could account for the impaired intercalation detected in *ttk* mutants, we propose that inefficient cell shape changes represent the main cause, and might prevent the proper accomplishment of several events, such as the sliding of cells, formation of a first autocellular contact and zipping up, thereby blocking intercalation. Hence, we propose that cell shape changes, particularly cell elongation, are an obligate requisite for different steps of intercalation. Other targets of Ttk might presumably be regulators or components of the cytoskeleton involved in cell shape changes. It is relevant to point out here that Ttk has also been proposed to regulate morphogenetic changes required for dorsal appendage elongation (French et al., 2003).

How does Ttk relate to the known genetic circuit (Sal-dependent) involved in intercalation? Being a transcription factor, Ttk initially appeared as an excellent candidate to participate in this genetic network by regulating *sal* and/or *kni* expression. However, we found both these genes to be normally expressed in *ttk* mutants, and we detected several differences in the intercalation phenotype of *ttk* loss

versus *sal* upregulation. For instance, although both situations block intercalation, cells expressing *sal*, unlike those lacking *ttk*, are still able to undergo a certain change in shape, from cuboidal to elongated (our unpublished observations). Therefore, our results fit a model in which Ttk acts in a different and parallel pathway to Sal during intercalation. Consistent with this model, we found that Ttk is not sufficient to promote intercalation on its own, because its overexpression cannot overcome the inhibition of intercalation imposed by Sal in the DT. Finally, genetic interactions (our unpublished results) also favour this model, because we found that: (1) *ttk* overexpression did not rescue lack of intercalation produced by *sal* overexpression (even though it rescued the intercalation defects of *ttk* mutants), and (2) absence of *sal* (by means of the constitutive activation of the Dpp pathway) does not overcome the intercalation defects of *ttk* mutants. Therefore, we propose that Ttk promotes intercalation by endorsing changes in cell shape, but absence of Sal is still required to allow other aspects of intercalation to occur.

Ttk in tube size

Tube size regulation is essential for functionality. We found that Ttk is involved in such regulation. Tube expansion and extension relies on a luminal chitin filament that assembles transiently in the tracheal tubes (reviewed in Swanson and Beitel, 2006). The metabolic pathway that leads to chitin synthesis involves several enzymes, among which are Mmy and Kkv (Araujo et al., 2005; Devine et al., 2005; Tonning et al., 2006; Tonning et al., 2005). In addition, other proteins are known to participate in the proper assembly and/or modification of the chitin filament, such as Knk, Rtv (Moussian et al., 2006), Verm and Serp (Luschnig et al., 2006; Wang et al., 2006). SJs are also required to regulate tube size (Behr et al., 2003; Hemphala et al., 2003; Llimargas et al., 2004; Paul et al., 2003; Wu et al., 2004) and it was proposed that they exert this activity, at least partly, via the control of the apical secretion of chitin modifiers (Wang et al., 2006). Our results revealed that *ttk* acts as a key gene in tube size control, playing at least two roles: it regulates chitin filament synthesis and SJ activity.

SJ regulation by Ttk appears functional rather than structural: we detected mild defects in the accumulation of only some SJ markers and there was a loss of the transepithelial diffusion barrier, whereas accumulation of other markers and SJ localisation remained apparently unaffected. We speculate that Ttk transcriptionally controls one or several SJ components that contribute to maintain the paracellular barrier and to control a specialised apical secretory pathway. As a result, chitin binding proteins such as Verm or Serp are not properly secreted.

We also found that *mmy* is transcriptionally regulated by Ttk. *mmy* tracheal expression positively depends on a mid-embryonic peak of the insect hormone 20-hydroxyecdysone (Tonning et al., 2006). Therefore, we propose that Ttk and ecdysone exert opposing effects on chitin synthesis. Excess of *mmy* mRNA results in the abnormal deposition of the chitin filament (S.J.A., unpublished), as occurs in *ttk* mutants. Defects in chitin deposition might lead to the irregular organisation of taenidia and the faint larval cuticle observed in *ttk* mutants (our unpublished observations). Strikingly, Ttk is also required for normal chorion production (French et al., 2003), which represents another specialised secreted layer.

Ttk is required for intracellular tube formation downstream of cell fate specification

ttk mutants are defective in the formation of terminal and fusion branches. These defects are due, in part, to non-autonomous, secondary and/or pleiotropic effects of *ttk*. For instance, *ttk* mutants exhibited a dorsal closure defect, which prevented the approach and fusion of contralateral DBs. Additionally, terminal and fusion branches depend on correct cell type specification, which did not reliably occur in *ttk* mutants. For instance, DSRF was missing in some presumptive terminal cells of *ttk* mutants, impairing terminal branch formation (Guillemin et al., 1996). These tracheal cell identity specification defects might be related to non-autonomous requirements of *ttk*. For instance, DSRF is not properly expressed in *ttk* mutants because of an abnormal expression of its regulator, Bnl (Sutherland et al., 1996).

It is important to note that, in spite of these non-autonomous and cell fate specification defects, two pieces of evidence indicate that *ttk* also plays a specific and autonomous role in the formation of terminal and fusion tubes. First, markers for fusion and terminal cell specification were expressed in many tracheal cells of *ttk* mutants, but yet most of these cells did not form terminal or fusion branches. Second, only the tracheal expression of *ttk* in *ttk* mutants (but not the constitutive activation of the *btl* pathway, which regulates the terminal and fusion identity) (Samakovlis et al., 1996a) was able to restore the formation of terminal branches.

A common feature of terminal and fusion branches is that they both display intracellular lumina that lack detectable junctions. The cellular events that precede the formation of fusion and terminal branches differ, but the mechanisms by which their intracellular lumina form has been proposed to be comparable (Uv et al., 2003). We found that, in *ttk* mutants, terminal and fusion cells engage in the correct cellular changes before intracellular lumen formation. However, neither of these two cell types finalised the cellular events leading to tube formation. It has been proposed that the lumen of terminal and fusion branches forms by the coalescence of intracellular vesicles that use a 'finger' tip provided by the neighbouring stalk cell as a nucleation point (Uv et al., 2003). Interestingly, we found that vesicles containing luminal material are less abundant in *ttk* mutants. These observations suggest a new role for Ttk in the formation of intracellular lumina in distinct cell types. Intracellular lumen formation also occurs in other branched tubular structures, such as in vertebrate endothelial cells (Kamei et al., 2006) and in the excretory cell of *Caenorhabditis elegans*, presumably by the coalescence of vesicles (Buechner, 2002). Importantly, a crucial role for vesicle formation and their fusion during intracellular tube formation has been demonstrated (Kamei et al., 2006).

To our knowledge, *ttk* is the first gene described to be involved in intracellular lumen formation during tracheal development. Possible targets of Ttk might be genes related to the apical surface and the underlying cytoskeleton, because several of these genes are involved in *C. elegans* excretory canal formation (Buechner, 2002; Gobel et al., 2004). Additionally, genes involved in intracellular vesicle trafficking might also be good candidates, as has been recently reported for *C. elegans* (Liegeois et al., 2007). In this respect, we have detected several abnormalities in *ttk* mutants that might reflect defects in vesicle trafficking.

We are grateful to J. Casanova for all the support and advice given throughout this work. We thank A. Travers and C. Murawsky for help and involvement during the first steps of the project; C. López-Iglesias and her group from the Serveis Cientificotècnics of the Universitat de Barcelona for the excellent electron microscopy work; L. Bardia for the confocal microscopy technical support and advice; C. Klambt, A. Travers and the Bloomington Stock Center for providing some of the flies used in this study; many members of the *Drosophila* community and the Developmental Studies Hybridoma Bank for various reagents. We are grateful to all members of the Llimargas and Casanova laboratories for the continuous support and discussions. We specially thank R. Méndez for the technical help. We thank J. Casanova, M. Furriols, R. Hampson and D. Shaye for critically reading the manuscript. S.J.A.

acknowledges an I3P postdoctoral contract from the Consejo Superior de Investigaciones Científicas (CSIC) and a Beatriu de Pinós fellowship in J. Casanova's lab; C.C. was supported by a fellowship from the Ministerio de Educación y Ciencia; and M.L. acknowledges a contract from the 'Ramón y Cajal' programme. This work was supported by the Ministerio de Ciencia y Tecnología de España (BMC2002-00359, BFU2005-02187/BMC) and the CIRIT of the Generalitat de Catalunya.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/20/3665/DC1

References

Affolter, M., Bellusci, S., Itoh, N., Shilo, B., Thiery, J. P. and Werb, Z. (2003). Tube or not tube. Remodeling epithelial tissues by branching morphogenesis. *Dev. Cell* **4**, 11-18.

Araujo, S. J., Aslam, H., Tear, G. and Casanova, J. (2005). mummy/cystic encodes an enzyme required for chitin and glycan synthesis, involved in trachea, embryonic cuticle and CNS development-Analysis of its role in Drosophila tracheal morphogenesis. *Dev. Biol.* **288**, 179-193.

Audibert, A., Simon, F. and Gho, M. (2005). Cell cycle diversity involves differential regulation of Cyclin E activity in the Drosophila bristle cell lineage. *Development* **132**, 2287-2297.

Badenhorst, P. (2001). Tramtrack controls glial number and identity in the Drosophila embryonic CNS. *Development* **128**, 4093-4101.

Baonza, A., Murawsky, C. M., Travers, A. A. and Freeman, M. (2002). Pointed and Tramtrack69 establish an EGFR-dependent transcriptional switch to regulate mitosis. *Nat. Cell Biol.* **4**, 976-980.

Baumgartner, S., Littleton, J. T., Broadie, K., Bhat, M. A., Harbecke, R., Lengyel, J. A., Chiquet-Ehrismann, R., Prokop, A. and Bellen, H. J. (1996). A Drosophila neurexin is required for septate junction and blood-nerve barrier formation and function. *Cell* **87**, 1059-1068.

Behr, M., Riedel, D. and Schuh, R. (2003). The claudin-like megatrachea is essential in septate junctions for the epithelial barrier function in Drosophila. *Dev. Cell* **5**, 611-620.

Brown, J. L. and Wu, C. (1993). Repression of Drosophila pair-rule segmentation genes by ectopic expression of tramtrack. *Development* **117**, 45-58.

Brown, J. L., Sonoda, S., Ueda, H., Scott, M. P. and Wu, C. (1991). Repression of the Drosophila fushi tarazu (ftz) segmentation gene. *EMBO J.* **10**, 665-674.

Buechner, M. (2002). Tubes and the single C. elegans excretory cell. *Trends Cell Biol.* **12**, 479-484.

Campos-Ortega, A. J. and Hartenstein, V. (1985). *The Embryonic Development of Drosophila melanogaster*. New York: Springer-Verlag.

Chen, C. K., Kuhnlein, R. P., Eulenberg, K. G., Vincent, S., Affolter, M. and Schuh, R. (1998). The transcription factors KNIRPS and KNIRPS RELATED control cell migration and branch morphogenesis during Drosophila tracheal development. *Development* **125**, 4959-4968.

Chihara, T. and Hayashi, S. (2000). Control of tracheal tubulogenesis by Wingless signaling. *Development* **127**, 4433-4442.

de Celis, J. F., Llimargas, M. and Casanova, J. (1995). Ventral veinless, the gene encoding the Cf1a transcription factor, links positional information and cell differentiation during embryonic and imaginal development in Drosophila melanogaster. *Development* **121**, 3405-3416.

Devine, W. P., Lubarsky, B., Shaw, K., Luschnig, S., Messina, L. and Krasnow, M. A. (2005). Requirement for chitin biosynthesis in epithelial tube morphogenesis. *Proc. Natl. Acad. Sci. USA* **102**, 17014-17019.

French, R. L., Cosand, K. A. and Berg, C. A. (2003). The Drosophila female sterile mutation twin peaks is a novel allele of tramtrack and reveals a requirement for Ttk69 in epithelial morphogenesis. *Dev. Biol.* **253**, 18-35.

Ghabrial, A., Luschnig, S., Metzstein, M. M. and Krasnow, M. A. (2003). Branching morphogenesis of the Drosophila tracheal system. *Annu. Rev. Cell Dev. Biol.* **19**, 623-647.

Giesen, K., Hummel, T., Stollewerk, A., Harrison, S., Travers, A. and Klambt, C. (1997). Glial development in the Drosophila CNS requires concomitant activation of glial and repression of neuronal differentiation genes. *Development* **124**, 2307-2316.

Gobel, V., Barrett, P. L., Hall, D. H. and Fleming, J. T. (2004). Lumen morphogenesis in C. elegans requires the membrane-cytoskeleton linker erm-1. *Dev. Cell* **6**, 865-873.

Guillemin, K., Groppe, J., Ducker, K., Treisman, R., Hafen, E., Affolter, M. and Krasnow, M. A. (1996). The pruned gene encodes the Drosophila serum response factor and regulates cytoplasmic outgrowth during terminal branching of the tracheal system. *Development* **122**, 1353-1362.

Guo, M., Bier, E., Jan, L. Y. and Jan, Y. N. (1995). tramtrack acts downstream of numb to specify distinct daughter cell fates during asymmetric cell divisions in the Drosophila PNS. *Neuron* **14**, 913-925.

Guo, M., Jan, L. Y. and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron* **17**, 27-41.

Harrison, S. D. and Travers, A. A. (1990). The tramtrack gene encodes a Drosophila finger protein that interacts with the ftz transcriptional regulatory region and shows a novel embryonic expression pattern. *EMBO J.* **9**, 207-216.

Hemphala, J., Uv, A., Cantera, R., Bray, S. and Samakovlis, C. (2003). Grainy head controls apical membrane growth and tube elongation in response to Branchless/FGF signalling. *Development* **130**, 249-258.

Hogan, B. L. and Kolodziej, P. A. (2002). Organogenesis: molecular mechanisms of tubulogenesis. *Nat. Rev. Genet.* **3**, 513-523.

Ikeya, T. and Hayashi, S. (1999). Interplay of Notch and FGF signaling restricts cell fate and MAPK activation in the Drosophila trachea. *Development* **126**, 4455-4463.

Jazwinska, A., Ribeiro, C. and Affolter, M. (2003). Epithelial tube morphogenesis during Drosophila tracheal development requires Piopio, a luminal ZP protein. *Nat. Cell Biol.* **5**, 895-901.

Jiang, L. and Crews, S. T. (2003). The Drosophila dysfusion basic helix-loop-helix (bHLH)-PAS gene controls tracheal fusion and levels of the trachealess bHLH-PAS protein. *Mol. Cell. Biol.* **23**, 5625-5637.

Jordan, K. C., Schaeffer, V., Fischer, K. A., Gray, E. E. and Ruohola-Baker, H. (2006). Notch signaling through tramtrack bypasses the mitosis promoting activity of the JNK pathway in the mitotic-to-endocycle transition of Drosophila follicle cells. *BMC Dev. Biol.* **6**, 16.

Jung, A. C., Ribeiro, C., Michaut, L., Certa, U. and Affolter, M. (2006). Polychaetoid/ZO-1 is required for cell specification and rearrangement during Drosophila tracheal morphogenesis. *Curr. Biol.* **16**, 1224-1231.

Kamei, M., Saunders, W. B., Bayless, K. J., Dye, L., Davis, G. E. and Weinstein, B. M. (2006). Endothelial tubes assemble from intracellular vacuoles in vivo. *Nature* **442**, 453-456.

Kuhnlein, R. P. and Schuh, R. (1996). Dual function of the region-specific homeotic gene spalt during Drosophila tracheal system development. *Development* **122**, 2215-2223.

Lai, Z. C. and Li, Y. (1999). Tramtrack69 is positively and autonomously required for Drosophila photoreceptor development. *Genetics* **152**, 299-305.

Lamb, R. S., Ward, R. E., Schweizer, L. and Fehon, R. G. (1998). Drosophila coracle, a member of the protein 4.1 superfamily, has essential structural functions in the septate junctions and developmental functions in embryonic and adult epithelial cells. *Mol. Biol. Cell* **9**, 3505-3519.

Lee, M., Lee, S., Zadeh, A. D. and Kolodziej, P. A. (2003). Distinct sites in E-cadherin regulate different steps in Drosophila tracheal tube fusion. *Development* **130**, 5989-5999.

Liegeois, S., Benedetto, A., Michaux, G., Belliard, G. and Labouesse, M. (2007). Genes required for osmoregulation and apical secretion in Caenorhabditis elegans. *Genetics* **175**, 709-724.

Llimargas, M. (1999). The Notch pathway helps to pattern the tips of the Drosophila tracheal branches by selecting cell fates. *Development* **126**, 2355-2364.

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

Llimargas, M. and Lawrence, P. A. (2001). Seven Wnt homologues in Drosophila: a case study of the developing tracheae. *Proc. Natl. Acad. Sci. USA* **98**, 14487-14492.

Llimargas, M., Strigini, M., Katidou, M., Karagogeos, D. and Casanova, J. (2004). Lachesin is a component of a septate junction-based mechanism that controls tube size and epithelial integrity in the Drosophila tracheal system. *Development* **131**, 181-190.

Locke, M. (2001). The Wigglesworth Lecture: Insects for studying fundamental problems in biology. *J. Insect Physiol.* **47**, 495-507.

Lubarsky, B. and Krasnow, M. A. (2003). Tube morphogenesis: making and shaping biological tubes. *Cell* **112**, 19-28.

Luschnig, S., Batz, T., Armbruster, K. and Krasnow, M. A. (2006). serpentine and vermiform encode matrix proteins with chitin binding and deacetylation domains that limit tracheal tube length in Drosophila. *Curr. Biol.* **16**, 186-194.

Manning, G. and Krasnow, M. A. (1993). Development of the *Drosophila* tracheal system. In *The Development of Drosophila melanogaster*. Vol. I (ed. M. Bate and A. Martínez-Arias), pp. 609-685. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Moussian, B., Tang, E., Tonning, A., Helms, S., Schwarz, H., Nusslein-Volhard, C. and Uv, A. E. (2006). Drosophila Knickkopf and Retroactive are needed for epithelial tube growth and cuticle differentiation through their specific requirement for chitin filament organization. *Development* **133**, 163-171.

Neumann, M. and Affolter, M. (2006). Remodelling epithelial tubes through cell rearrangements: from cells to molecules. *EMBO Rep.* **7**, 36-40.

Okabe, M., Imai, T., Kurusu, M., Hiromi, Y. and Okano, H. (2001). Translational repression determines a neuronal potential in Drosophila asymmetric cell division. *Nature* **411**, 94-98.

Oshima, K., Takeda, M., Kuranaga, E., Ueda, R., Aigaki, T., Miura, M. and Hayashi, S. (2006). IKK epsilon regulates F actin assembly and interacts with Drosophila IAP1 in cellular morphogenesis. *Curr. Biol.* **16**, 1531-1537.

Paul, S. M., Ternet, M., Salvaterra, P. M. and Beitel, G. J. (2003). The Na+/K+ ATPase is required for septate junction function and epithelial tube-size control in the Drosophila tracheal system. *Development* **130**, 4963-4974.
Pilot, F. and Lecuit, T. (2005). Compartmentalized morphogenesis in epithelia: from cell to tissue shape. *Dev. Dyn.* **232**, 685-694.
Read, D. and Manley, J. L. (1992). Alternatively spliced transcripts of the Drosophila tramtrack gene encode zinc finger proteins with distinct DNA binding specificities. *EMBO J.* **11**, 1035-1044.
Ribeiro, C., Ebner, A. and Affolter, M. (2002). In vivo imaging reveals different cellular functions for FGF and Dpp signaling in tracheal branching morphogenesis. *Dev. Cell* **2**, 677-683.
Ribeiro, C., Neumann, M. and Affolter, M. (2004). Genetic control of cell intercalation during tracheal morphogenesis in Drosophila. *Curr. Biol.* **14**, 2197-2207.
Samakovlis, C., Hacohen, N., Manning, G., Sutherland, D. C., Guillemin, K. and Krasnow, M. A. (1996a). Development of the Drosophila tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. *Development* **122**, 1395-1407.
Samakovlis, C., Manning, G., Steneberg, P., Hacohen, N., Cantera, R. and Krasnow, M. A. (1996b). Genetic control of epithelial tube fusion during Drosophila tracheal development. *Development* **122**, 3531-3536.
Steneberg, P., Hemphala, J. and Samakovlis, C. (1999). Dpp and Notch specify the fusion cell fate in the dorsal branches of the Drosophila trachea. *Mech. Dev.* **87**, 153-163.
Sutherland, D., Samakovlis, C. and Krasnow, M. A. (1996). branchless encodes a Drosophila FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell* **87**, 1091-1101.
Swanson, L. E. and Beitel, G. J. (2006). Tubulogenesis: an inside job. *Curr. Biol.* **16**, R51-R53.
Tanaka-Matakatsu, M., Uemura, T., Oda, H., Takeichi, M. and Hayashi, S. (1996). Cadherin-mediated cell adhesion and cell motility in Drosophila trachea regulated by the transcription factor Escargot. *Development* **122**, 3697-3705.
Toba, G., Ohsako, T., Miyata, N., Ohtsuka, T., Seong, K. H. and Aigaki, T. (1999). The gene search system. A method for efficient detection and rapid molecular identification of genes in Drosophila melanogaster. *Genetics* **151**, 725-737.
Tonning, A., Hemphala, J., Tang, E., Nannmark, U., Samakovlis, C. and Uv, A. (2005). A transient luminal chitinous matrix is required to model epithelial tube diameter in the Drosophila trachea. *Dev. Cell* **9**, 423-430.
Tonning, A., Helms, S., Schwarz, H., Uv, A. E. and Moussian, B. (2006). Hormonal regulation of mummy is needed for apical extracellular matrix formation and epithelial morphogenesis in Drosophila. *Development* **133**, 331-341.
Uv, A., Cantera, R. and Samakovlis, C. (2003). Drosophila tracheal morphogenesis: intricate cellular solutions to basic plumbing problems. *Trends Cell Biol.* **13**, 301-309.
Wang, S., Jayaram, S. A., Hemphala, J., Senti, K. A., Tsarouhas, V., Jin, H. and Samakovlis, C. (2006). Septate-junction-dependent luminal deposition of chitin deacetylases restricts tube elongation in the Drosophila trachea. *Curr. Biol.* **16**, 180-185.
Wilk, R., Weizman, I. and Shilo, B. Z. (1996). trachealess encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in Drosophila. *Genes Dev.* **10**, 93-102.
Wu, V. M. and Beitel, G. J. (2004). A junctional problem of apical proportions: epithelial tube-size control by septate junctions in the Drosophila tracheal system. *Curr. Opin. Cell Biol.* **16**, 493-499.
Wu, V. M., Schulte, J., Hirschi, A., Tepass, U. and Beitel, G. J. (2004). Sinuous is a Drosophila claudin required for septate junction organization and epithelial tube size control. *J. Cell Biol.* **164**, 313-323.
Xiong, W. C. and Montell, C. (1993). tramtrack is a transcriptional repressor required for cell fate determination in the Drosophila eye. *Genes Dev.* **7**, 1085-1096.