Tramtrack regulates different morphogenetic events during *Drosophila* tracheal development

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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*, 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, Tracheless (Tth) 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 fulfill 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...
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<sup>D2-50</sup>, ttk<sup>M730</sup>, ttk<sup>e11</sup>, UAS- ttk69, UAS-ttk88, vvl<sup>GA3</sup>, trh<sup>1051</sup>, Df(3LR)-XT1, esg<sup>87-22</sup>, UAS-H, UAS-N<sup>α</sup>, UAS-blt<sup>10</sup>, blt-moe GFP, UAS- srcGFP, UAS- tau GFP, bltGFP 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 w<sup>yw</sup>. Drosophila stocks and crosses were kept on standard conditions at 25°C. Overexpression experiments were conducted at 20°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. Fehorn, University of Alberta, Canada), anti-FasIII (7G10, Developmental Studies Hybridoma Bank, DSHB), anti-Verm (S. Lasnich, University of Bayreuth, Germany), anti-Lac (M. Strigini, IMBB, Crete, Greece), mAb2A12 (DSHB), anti-DSRF (2-161, Cold Spring Harbor Laboratory, CSHL), anti-Kai (developed by J. Reivitz and provided by M. Ruiz-Gomez, CBM, Madrid, Spain), anti-DE-cad (DCAD2, DSHB), anti-Trh (made by N. Martin 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<sub>110,000</sub>; Molecular Probes) into the body cavity of embryos (Lamb et al., 1998). In situ hybridization was performed according to standard protocols, with ribop-Ⅰ probe (gift from M. Neumann, University of Basel, Switzerland). ribo-bnl and ribo-mony 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 bltGAL4 driving GFP-fusion proteins (blt>GF P in Figures). bltGAL4 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 bltGAL4/UAS-srcGFP:ttk<sup>D2-50</sup> or bltGAL4/UAS-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 10% 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 IRB2 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. TIIFF 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<sup>α</sup> mutants and ttk<sup>e11</sup> mutants expressing ttk69 in the tracheal tissue (obtained from the cross bltGAL4/bltGAL4; ttk<sup>D2-50/TM3 lacZ</sup> X ttk<sup>D2-50</sup>/UAS ttk69/TM3 lacZ 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 autacellular 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 (bltGAL4) (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 UAS ttk69 line was crossed to bltGAL4, 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<sub>D2-50</sub>) whereas milder defects were observed in hypomorphic mutants (ttk<sup>M730</sup>) (Fig. 1E and data not shown). The ttk locus encodes ttk88 and ttk69 (Read and Manley, 1992). Mutants for ttk<sup>88</sup> (ttk<sup>e11</sup>) (Xiong and Montell, 1993) are viable and do not show a tracheal phenotype. In addition, overexpression of ttk<sup>88</sup> did not result in tracheal defects (data not shown). Conversely, mutants for ttk<sup>69</sup> (ttk<sup>e11</sup>) (Lai and Li, 1999) displayed a clear tracheal phenotype (Fig. 1F) and, as already indicated, overexpression of ttk<sup>69</sup> affected tracheal development (Fig. 1A). These results suggest a specific role for ttk<sup>69</sup> during tracheal development. In this work, we used either ttk<sup>D2-50</sup> or ttk<sup>e11</sup> to analyse ttk tracheal requirements.
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-UAS ttk
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 (Fig. 2G). Conversely, we did not find cells expressing low levels of Ttk69 protein, which appeared to be lower in the presumptive fusion cells and higher in the fusion-adjacent cells (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 fulfill 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. 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-
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
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 Flustain, 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, 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. *serpine (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-

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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.
ttk regulates tracheal development

**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 autacellular 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 localization 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 realised 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.

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Development regulates tracheal development.

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