

Genetic control of epithelial tube size in the *Drosophila* tracheal system

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SUMMARY

The proper size of epithelial tubes is critical for the function of the lung, kidney, vascular system and other organs, but the genetic and cellular mechanisms that control epithelial tube size are unknown. We investigated tube size control in the embryonic and larval tracheal (respiratory) system of *Drosophila*. A morphometric analysis showed that primary tracheal branches have characteristic sizes that undergo programmed changes during development. Branches grow at different rates and their diameters and lengths are regulated independently: tube length increases gradually throughout development, whereas tube diameter increases abruptly at discrete times in development. Cellular analysis and manipulation of tracheal cell number using cell-cycle mutations demonstrated that tube size is not dictated by the specific number or shape of the tracheal cells that

constitute it. Rather, tube size appears to be controlled by coordinately regulating the apical (luminal) surface of tracheal cells. Genetic analysis showed that tube sizes are specified early by branch identity genes, and the subsequent enlargement of branches to their mature sizes and maintenance of the expanded tubes involves a new set of genes described here, which we call tube expansion genes. This work establishes a genetic system for investigating tube size regulation, and provides an outline of the genetic program and cellular events underlying tracheal tube size control.

Key words: *Drosophila*, Tracheal system, Tubulogenesis, Epithelial tube, Morphogenesis, Epithelial maintenance

INTRODUCTION

Animals depend upon epithelial and endothelial tubes to transport gases and liquids in the body, and the sizes of the tubes are critical for their transport function. Tube size must be controlled not only during tube formation but throughout development in order to maintain functionality as the animal grows. For example, the human aorta expands approx. 100-fold between embryonic and adult life to keep pace with increased circulatory demand: the adult aorta has a diameter larger than the entire embryo at the time of its formation. Most fundamental questions about tube size regulation, however, remain unanswered. How are the sizes of tubes specified? How are the sizes and shapes of the constituent cells coordinated to construct tubes of the correct size? How do cells measure the diameter and length of the tubes that they form? What cytoskeletal and extracellular-matrix components are used to form and maintain cells in tubular shapes? How are these components regulated to adjust tube size during development?

The *Drosophila* tracheal (respiratory) system provides a tractable model system for investigating these questions. The tracheal system arises from clusters of ectodermal cells that invaginate to form epithelial sacs from which branches sequentially sprout, extend and interconnect. This creates a ramifying network of approx. 10^4 tubes that transports oxygen throughout the larval body (reviewed by Manning and Krasnow, 1993). Substantial progress has been made in

identifying and characterizing the genetic programs governing epithelial sac formation, sprouting and growth of the primary, secondary and terminal branches, and tracheal tube fusion (reviewed by Shilo et al., 1997; Metzger and Krasnow, 1999). Also, branch identity genes that specify differences among primary branches have been characterized (Samakovlis et al., 1996a; Kuhnlein and Schuh, 1996; Vincent et al., 1997; Wappner et al., 1997; Chen et al., 1998). However, little is known about the genetics and cell biology of tracheal tube formation and tube size regulation.

Several features of the tracheal system make it an excellent system for investigating the mechanisms of tubulogenesis and tube size control. First, the tubes are simple in structure. They are an epithelial monolayer and hence lack complexities introduced by the multilayer structure of most vertebrate tubes including blood vessels and the bronchial tubes of the lungs. Second, each tube is composed of only a small number of cells, each of which can be followed during development (Samakovlis et al., 1996a). Finally, powerful genetic and genomic approaches can be applied to identify and analyze tubulogenesis genes. Because fundamental mechanisms controlling epithelial tube outgrowth and branching are similar in *Drosophila* and vertebrates (Metzger and Krasnow, 1999), it seems likely that other aspects of the process, including tube formation and size regulation will also be conserved.

In this report, we describe morphometric, cellular and genetic studies of tracheal tube growth during embryonic and

larval life, as well as a screen for mutants with tracheal tube size defects. The results establish a genetic system for investigating tube size regulation, and provide an outline of the genetic program and cellular events underlying tracheal tube size control.

MATERIALS AND METHODS

Fly strains

l(2)k13717, *l(2)k11012*, *l(2)k04223*, *l(2)k06507*, *l(2)03953* and *l(3)06524* were obtained from the Berkeley Drosophila Genome Project, *megatrachea*^{VE896} and *mega*^{EA97} from Norbert Perrimon (Perrimon et al., 1989 and personal communication), and *gnarled*^{ex59} from Nir Hacohen (N. Hacohen and M. K., unpublished), *blistered* (*pruned*)^{ex84} (Guillemin et al., 1996), *pointed*^{Δ88} (Scholz et al., 1993), and *escargot*^{G66} (Whiteley et al., 1992) are null alleles. *armadillo*^{YD35} is a null allele and P[*arm*^{S14-C}] is a mutant *arm* transgene that provides *arm* signaling but not junctional function (Orsulic and Peifer, 1996). In *cycA*^{neo0114} animals, nuclear cycle 16 is converted to an endocycle resulting in half the normal number of cells with twice normal ploidy and size (Sprenger et al., 1997). *dacapo*^Δ and *dacapo*^{Δ544} are null alleles and cause many cells to undergo an extra round of division without commensurate cell growth (de Nooij et al., 1996; Lane et al., 1996). The N722 *bt1*-GAL4 driver expresses GAL4 throughout the tracheal system (Shiga et al., 1996) and P127::GAL4 driver expresses GAL4 in tracheal fusion cells (Steneberg et al., 1998). The UAS::srcGFP responder expresses a membrane-associated GFP fusion protein (Kaltschmidt et al., 2000). UAS-DPP has been described (Wappner et al., 1997). *I-eve-1* is a P[*lacZ*] insertion in *trachealess* that expresses β-galactosidase in tracheal cell bodies and nuclei (Perrimon et al., 1991). Canton-S was the wild-type strain.

Histology and immunohistochemistry

Antisera 2A12 and TL1, and embryo fixation, staining and staging procedures are described in Samakovlis et al., (1996a). Other antisera used were: AS55 (Reichman-Fried et al., 1994), anti-crumbs mAb Cq4 (Wodarz et al., 1995), anti-DSRF(Blistered) mAb 2-161 (from M. Gilman), anti-β-galactosidase (Capel), anti-green-fluorescent protein (anti-GFP) mAb GFP-20 (Sigma), anti-Trachealess antiserum (Wilk et al., 1996), and anti-Coracle antiserum (Fehon et al., 1994). Confocal images were captured and analyzed using an MD2010 confocal microscope and ImageSpace software (Molecular Dynamics).

Morphometric analysis

To measure embryonic tracheal tube sizes, wild-type or *I-eve-1/+* embryos were stained with TL1 or mAb 2A12 and anti-β-galactosidase or anti-Trachealess, mounted in Permout (Fisher Scientific), and imaged by confocal microscopy. Mounting in Permout or other non-aqueous media caused an approx. 30% reduction in size compared to live embryos, however, it did not appear to affect relative tube sizes or otherwise alter tracheal morphology. Cell numbers were determined by counting Trachealess-positive nuclei in confocal images. Lumen diameters and lengths were measured in anti-β-galactosidase-stained *I-eve-1/+* embryos (stage 12-14, 17 wild-type embryos) or in 2A12-stained embryos (all others). Because mutant phenotypes progressively worsen during development, all comparisons of mutants to wild type were done on carefully staged stage 16 embryos (four in-line gut lobes). Tube length was determined by tracing the three-dimensional path of the tube through confocal sections. Segmental DT length was measured between adjacent transverse connectives (see Table 1 for branch names and abbreviations). Total DT length was measured from the anterior DT in tracheal segment 1 (Tr1) to the junction of DT and transverse connective (TC) in Tr10. Regression analysis showed that

total DT length strongly correlated ($P < 10^{-4}$) with embryo length in wild-type stage 14 and 16 embryos and in the tube expansion mutants; total DT length was therefore normalized to embryo length to reduce the effect of variability in embryo size. Tube diameter and wall thickness were determined from confocal images using a 100× objective and 0.5-0.7 μm steps between confocal sections. For longitudinal sections, dimensions were determined in the section with the maximal lumenal diameter at the point halfway between the DB and the TC of the next segment anterior. For tangential sections, dimensions were also determined at the halfway point, but to avoid overestimation minimal lumen diameters and corresponding wall thicknesses were used.

Measurements of larval tracheal tube sizes were made on air-filled tracheal tubes in living larvae anesthetized with ether and imaged using DIC optics. Images were captured and analyzed using IP Lab Spectrum (Signal Analytics Corporation). To determine tube lengths, larvae were manipulated so that the tube to be measured was aligned in a single horizontal plane; no corrections were made for minor out-of-plane deviations. Staged larvae were obtained by selecting individuals from developmentally synchronized populations (siblings from a 1 hour egg lay) that had just hatched, entered or exited a molt, or begun pupating. Hypoxic L3 larvae were obtained by rearing freshly hatched L1 larvae in 5% O₂ until they reached L3.

Tube diameters are lumenal diameters except where noted. Reported values are means and standard error of the mean (s.e.m.). In many cases, the s.e.m. is small and not visible in the figures.

Screen for tracheal tube mutations and characterization of phenotypes

Approximately 1100 P[*lacZ*] insertion lines (Torok et al., 1993) were screened for tracheal defects and *lacZ* expression by staining embryos with 2A12 and anti-β-galactosidase antibodies. Also, selected mutants from the P[*lacZ*] screen of Samakovlis et al., (1996a) were examined for tracheal tube formation and size defects. Lines with tracheal tube size or shape defects were rebalanced with marked balancer chromosomes, and tracheal phenotypes of homozygotes were analyzed after staining with 2A12 and TL1 antisera. Salivary gland and hindgut morphologies were visualized by TL1 staining. Gross embryo and gut morphologies were normal in all mutant lines described here except *l(2)k13717*, which occasionally failed dorsal closure, and *l(2)03953* which had abnormal gut constrictions and looping.

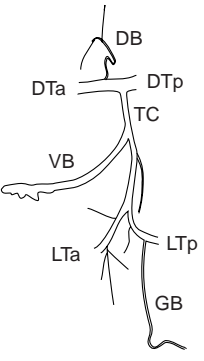
Cytological positions of the mutants were determined by complementation tests for viability and tracheal phenotype with deficiencies on chromosomes II and III (Bloomington Drosophila Stock Center). Non-complementing deficiencies were: *l(2)03953b* – Df(2L)TW161, Df(2L)TW84; *l(2)04223* – Df(2L)Dwee-delta5; *l(2)k11012b* – Df(2R)CX1 (hemizygous tracheal phenotype weaker than homozygous); *l(3)06524* – Df(3L)ZN47; *gnarled*^{ex59} – Df(3R)by62. The tube expansion mutations complemented the following alleles: *l(2)03953* – *diaphanous*^[2,5]; *l(2)k11012b* – *short stop*^[k3010, k5405, 2, 5], *mastermind*^[k3751, 2]; *l(3)06524* – *drifter*^[B129, E82-2D], *KLP64D*^[92, 6b, N72, L733.27, L733.34, A8N123]; *gnarled*^{ex59} – *hyperplastic discs*¹⁵, *l(3)s2681*, *dalmatian*¹⁸⁴, *twins*^[01436, 02414].

RESULTS

Characterization of tracheal tube growth during development

To begin to understand how tracheal tube size is regulated, we measured the sizes of the primary tracheal branches at different stages in development and under different environmental conditions. At the end of embryogenesis, just before the tracheal system becomes functional, each branch has a

Table 1. Tracheal tube sizes

	Branch [∞]	Diameter (μm) ⁺			Length (μm) ⁺			Aspect Ratio [‡]		Cells
		Embryo	Larva	$\frac{\text{Larv.}}{\text{Emb.}}$	Embryo	Larva	$\frac{\text{Larv.}}{\text{Emb.}}$	Embryo	Larva	
	Dorsal trunk anterior (DTa)	4.4 ±0.4	32 ±2	7	31 ±2*	430 ±20*	13.8	6.3*	13*	23*
	Dorsal trunk posterior (DTp)	5.5 ±0.4	36 ±2	7						
	Dorsal branch (DB)	0.8 ±0.1	3.5 ±0.1	4	50 ±3	330 ±60	6.5	63	93	3 [†]
	Transverse connective (TC)	1.7 ±0.2	13 ±1	8	57 ±3	490 ±50	8.5	34	38	12
	Visceral branch (VB)	1.2 ±0.1	14 ±1	11	49 ±4	n.d.	n.d.	41	n.d.	7 [†]
	Lateral trunk anterior (LTa)	0.7 ±0.1	7.1 ±0.2	10	34 ±2	300 ±60	8.7	49	42	4 [†]
	Lateral trunk posterior (LTp)	0.6 ±0.1	6.9 ±0.4	12	19 ±2	150 ±20	7.8	32	22	3 [†]

[∞]Branch locations are shown in schematic (Tr5 segment, embryonic stage 16).

⁺Diameter and length values (mean ± s.e.m., n > 5) for segment Tr5 at embryonic stage 17 and the end of the third larval instar.

[‡]Length/diameter

*Combined value for Tr4 DTp + Tr5 DTa

[†]From Samakovlis et al., 1996a

characteristic size and shape (Table 1). The luminal diameters of different branches ranged from 0.6 to 6 μm, their lengths ranged from 20 to 60 μm, and their aspect ratios (length/diameter) ranged from 6 to 60. The size variation for each branch from individual to individual was typically 15% or less of the average value, indicating that branch size is precisely specified. That the size of each branch is regulated independently is emphasized by the finding that tube diameter

can change abruptly at the boundaries between branches. For example, the diameter of the dorsal trunk (DT) in tracheal segment Tr5 was seven times bigger than that of the dorsal branch (5.5 μm versus 0.8 μm), and the transition occurred precisely at their junction. Although branch diameter was relatively constant along the length of most branches, some showed a significant and characteristic taper. For example, DT diameter decreased by approx. 20% across each segment.

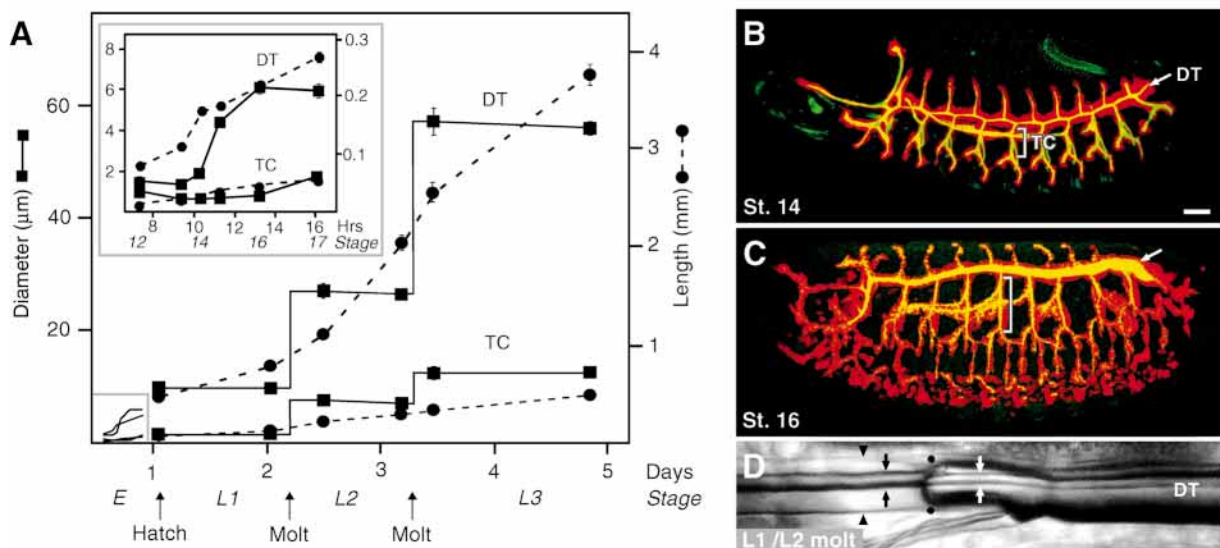


Fig. 1. Tracheal tube growth during development. (A) Plot of dorsal trunk (DT) diameter (in segment Tr9), DT length (total) and transverse connective (TC) diameter and length (in Tr5) during embryonic (E, see inset) and larval (L1, 2, 3) development. Tube lengths increase continuously while diameters expand only during brief intervals. The apparent increases at hatching are an artifact resulting from embryo shrinkage during mounting (see Materials and Methods). Error bars are mean ± s.e.m. for n = 5–21. (B,C) Projections of confocal images of stage 14 and 16 embryos (*I-eve-1/+*) with tracheal cells labeled in red (*I-eve-1* β-galactosidase marker) and tracheal lumen in yellow/green (TL1 antigen in B; 2A12 antigen in C). Note the large increase in DT diameter (arrow) between stages 14 and 16, and the increase in TC length (bracket). (D) Close up of a portion of the DT during the L1/L2 molt. Note the small, gas-filled L1 cuticular tube (arrows) inside the bigger L2 cuticular tube (arrowheads), showing the stepwise increase in DT diameter at the molt. The L2 tube is in the process of filling with gas from posterior (right) to anterior (left); dots mark the gas-liquid interface. The L1 cuticle will be removed at the end of the molt. Bar: 20 μm (for B,C), 10 μm (for D).

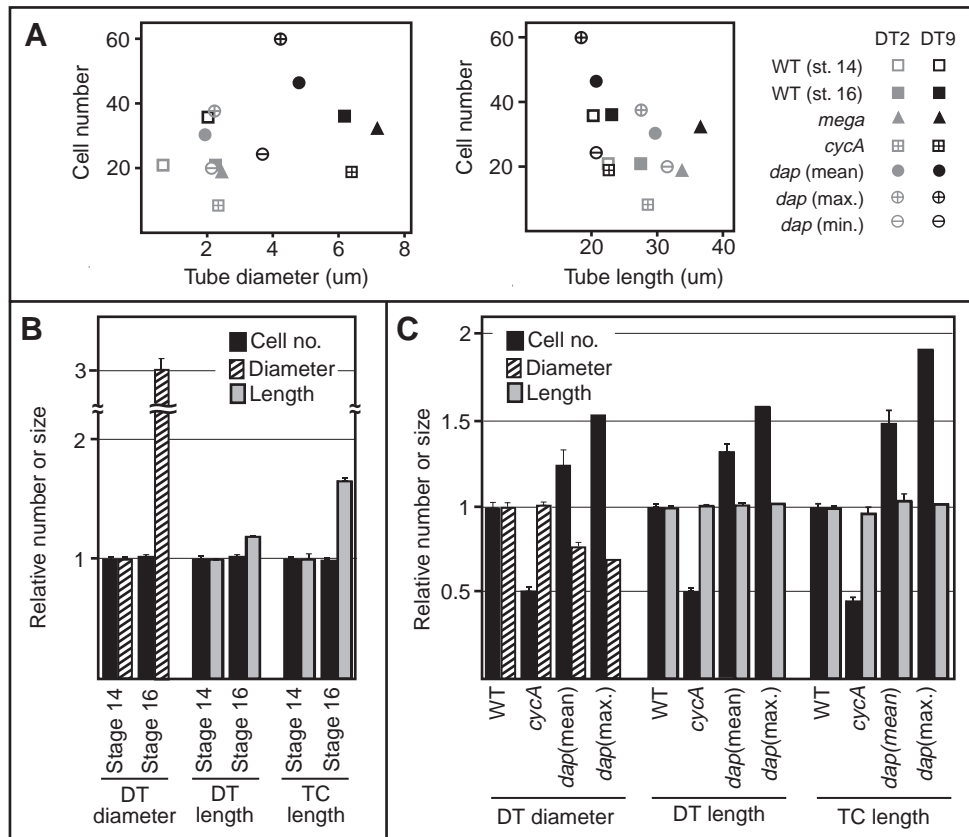


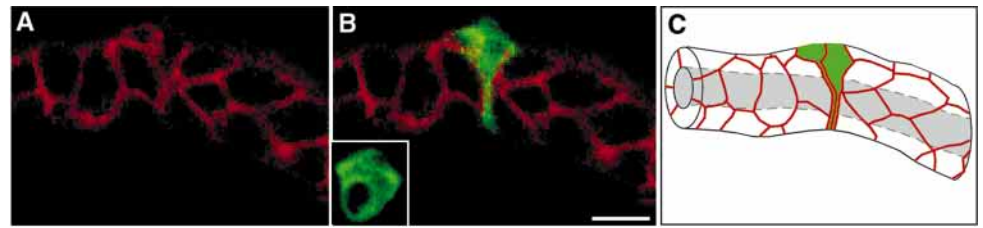
Fig. 2. Independence of tracheal tube size and cell number. (A) Scatter plots comparing cell number to tube diameter (left) or length (right) in DT segment Tr2 (DT2) and Tr9 (DT9) at different developmental stages and genotypes as indicated. Note that neither tube length nor diameter correlates with cell number. WT (stage 14) and WT (stage 16), mean for stage 14 and stage 16 wild-type and *l-eve-1/+* embryos; *mega*, mean for stage 16 *megatrachea^{EA97}* mutants. *cycA*, mean for the approx. 50% of stage 16 *cyclinA^{ne0114}/Df(3L)vin3* mutants in which primary branching occurred; failure of primary branching in the others is presumably an indirect effect because many of these embryos were grossly abnormal. *dap* (mean), *dap* (max.) and *dap* (min.), mean, maximum and minimum number of cells in stage 16 *dacapo⁴* and *dacapo⁴⁴⁵⁴* mutants. For all measurements, $n=9-11$ and s.e.m. values (not shown) were $\leq 10\%$ of the mean. (B) Comparisons of cell number to tube size for the DT and TC in stage 14 and 16 wild-type embryos. Note that although cell number remains constant between stages 14 and 16, tube diameter and lengths increase. Values shown are means and s.e.m. for DT9 (for DT diameter), for the sum of cell counts in DT2, DT5, and DT9 and for total DT length (for DT length), and for TC5 (for TC length). All values have been normalized to those at stage 14 (DT9 cell number, 37 ± 2 ($n=10$); DT9 diameter, 2.1 ± 0.1 μm ($n=7$); DT 2+5+9 cell number, 83 ± 2 ($n=7$); total DT length, 194 ± 1 μm ($n=17$); TC5 cell number, 15 ± 1 ($n=10$); TC length, 28 ± 0.4 μm ($n=16$)). Total DT length measurements were further normalized to embryo length (stage 14, 369 ± 4 μm ($n=17$); stage 16, 361 ± 4 μm ($n=21$)) to reduce the effect of variability in embryo size (see Materials and Methods). Length and diameter values at stage 16 were all significantly different ($p < 10^{-10}$) from those at stage 14 by two-tailed Students T tests. (C) Comparisons of cell number to tube size in the cell-cycle mutants *cycA* and *dap* at stage 16. Genotypes are described in A and values were normalized as described in B except that stage 16 WT was the reference value (embryo lengths: *cycA*, 366 ± 4.9 μm ($n=9$); *dap*, 364 ± 6.2 μm ($n=9$)). Note that tube diameter and length do not vary with cell number. Although *dap* mutations caused a reduction in DT diameter, this effect was not due to the increased cell number since DT diameter was reduced even in mutant DT segments with fewer than normal cells (e.g. *dap* (min.) in A). Because *dap* mutations affect virtually all tissues, the reduction in DT diameter may be indirect.

The tracheal system grows extensively during development. All primary branches are ≤ 2 μm in diameter when they first bud in the embryo at stage 12 (data not shown), but are up to 40 times bigger in the mature larva (Table 1). The kinetics of tube growth during embryonic and larval life were analyzed for two branches, the DT and transverse connective (TC) (Fig. 1A). There were three important findings. First, branches did not gradually increase in diameter but instead underwent discrete periods of expansion at specific times during development. DT diameter was constant during the 3 hours of primary branch budding and outgrowth (embryonic stages 11 to 13), but tripled during a 3 hour period in midembryogenesis

(stages 14-16; Fig. 1A-C). Diameter then remained constant for the rest of embryonic life and throughout the first larval period, but increased abruptly again at each larval molt (Fig. 1A,D). The same discrete periods of dilation were observed for the TC except that the embryonic expansion occurred 3 hours later than for the DT (Fig. 1A and data not shown). DT diameter increased a total of 40-fold, from 1.5 to 57 μm in Tr9, and TC diameter increased 16-fold, from 0.8 to 13 μm in Tr5 (Fig. 1A).

Second, growth in tube length occurred with distinctly different kinetics than growth in diameter. The lengths of the DT and TC steadily increased throughout embryonic and larval

Fig. 3. Tracheal cells with different shapes form tubes of the same diameter. Lateral view of a portion of the DT at a fusion joint in a P127::GAL4; UAS::srcGFP embryo double labeled to show cell junctions (Coracle protein; red) and the two fusion cells that compose the fusion joint (GFP; green). (A,B) Montage of confocal sections, with A showing the red channel (cell junctions) and B showing both red and green channels (cell junctions and fusion cells). Inset in B is a sagittal view of a fusion junction showing that fusion cells completely surround the lumen. (C) Schematic representation of B. DT lumen (see Fig. 4A,B) is shown gray. Despite the very different structure of fusion cells (doughnut-shaped) from other DT cells (cobblestone-shaped), they form a tube of the same diameter. Bar, 5 μm (for A, B) and 7.5 μm (for inset).



life, in contrast to the discrete periods of diameter expansion (Fig. 1A). Thus, tube length and diameter are independently regulated.

Third, tracheal tubes did not grow proportionately during development. The length and diameter of each branch expanded to different extents, so the shape of each branch changed as it grew (Table 1). For example, the aspect ratio of the DT increased from 6 to 13 during the larval period, while the aspect ratio of the posterior lateral trunk (LTp) decreased from 32 to 22. Thus, both the size and shape of each branch undergo programmed changes during development.

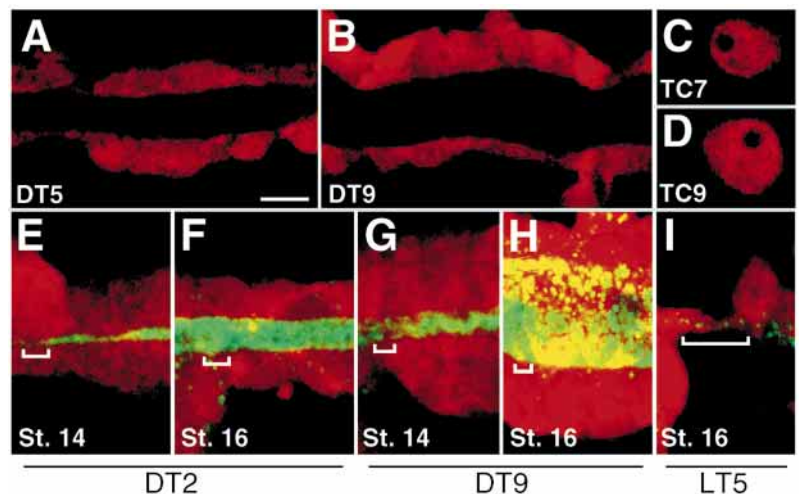
The complex and stereotyped changes in tracheal tube size suggest that tube size is controlled by a hardwired developmental program. We tested whether oxygen physiology can influence the program because it is known to regulate another important aspect of tracheal development, the sprouting of tracheal terminal branches (Jarecki et al., 1999). We compared DT and TC branch size between larva reared in normal (21%) or low (5%) oxygen, a condition that stimulates terminal branching. There was little effect of oxygen on tube size: the diameters of the DT in Tr9 and TC in Tr5 were not significantly changed by hypoxia (52 ± 2 and 13 ± 1 μm versus 57 ± 2 and 13 ± 1 μm in normoxia), and there was only a small (approx. 16%) reduction in DT and TC length (3200 ± 60 and 380 ± 10 μm versus 3800 ± 120 and 460 ± 20 μm in normoxia)

that paralleled the reduction in overall body size seen under the hypoxic condition. Similarly, in larvae with an approx. 50% reduction in body size due to a mutation in the *chico* insulin receptor substrate gene (Bohni et al., 1999), DT and TC diameters were unchanged from wild type (55 ± 2 and 12 ± 1 μm in *chico*² versus 57 ± 2 and 13 ± 1 μm in wild type) while their lengths were reduced by 27% (2800 ± 80 and 330 ± 20 μm in *chico*² versus 3800 ± 120 and 460 ± 20 μm in wild type). Thus, differences in oxygen physiology and general (*chico*-dependent) growth control have little or no effect on tube diameter but can influence tube length.

Tube size is not controlled by the number, size or overall shape of the constituent cells

To begin to elucidate the cellular mechanisms of tracheal tube size control, we examined the relationship between cell number and tube size. One obvious means of controlling tube size would be to regulate the number of cells that constitute the tube. Indeed, each major tracheal branch is composed of a characteristic number of cells (Samakovlis et al., 1996a). However, cell number was not tightly correlated with tube length or diameter (Fig. 2A; Table 1). Furthermore, because no significant cell division or death is detected in the tracheal system after embryonic stage 11 (Fig. 2B; Samakovlis et al., 1996a), the dramatic expansions of the tracheal tubes that occur

Fig. 4. Regulation at the apical surface of tracheal cells during tube growth. (A–D) Confocal images of stage 16 embryos (*I-eve-1/+*) showing longitudinal sections of the DT in segment Tr5 (A) and Tr9 (B), and cross-sections through the TC in Tr7 (C) and Tr9 (D). Tracheal cell bodies are stained red (*I-eve-1* β -galactosidase marker). Note apical (luminal) surfaces of the cells are regular and form even tubes while basal surfaces are irregular, and lumens are not symmetrically located with respect to the cells. (E–H) Projections of confocal images of longitudinal sections through the DT in segments Tr2 and Tr9 at stages 14 and 16 as indicated. Tracheal cells are stained red (*I-eve-1* marker) and lumens are stained green/yellow (TL1 antigen in stage 14, 2A12 antigen in stage 16). Note that as the DT expands from stage 14 to 16, DT luminal diameter increases substantially whereas diameter at the basal surface changes little, and cells become thinner. Cell thickness decreases from 3.5 ± 0.3 μm (range 1.3–5.3, $n=14$) to 1.9 ± 0.2 μm (range 1.0–3.3, $n=28$) in DT2, and from 4.0 ± 0.02 μm (2.9–5.7, $n=14$) to 3.1 ± 0.3 μm (range 1.6–4.8, $n=28$) in DT9. (I) Fusion joint in the lateral trunk in Tr5, stained as in E–H. The internal diameter of the tube formed by the LT fusion cells (< 0.7 μm) is 10 times smaller than the tube formed by DT fusion cells in H. Brackets (—), locations of fusion junctions. Bar, 4 μm (for A,B,E–I), 5 μm (for C and D).



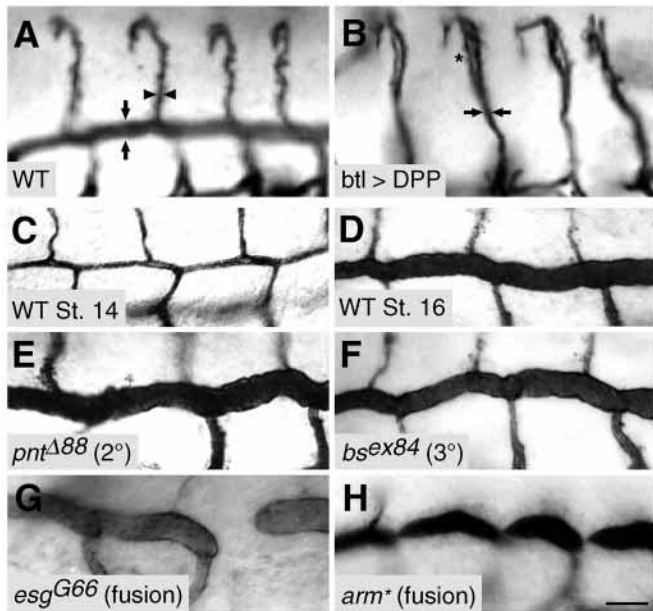


Fig. 5. Tracheal tube size is altered by changing branch identity but not by changing branch sprouting or fusion. (A) Lateral view of a portion of the wild-type tracheal system (stage 16) stained for the 2A12 luminal antigen. Note DT diameter (arrows) is much greater than that of DBs (arrowheads). (B) Similar view of an *N722 btl-GAL4; UAS-DPP* embryo in which DPP was misexpressed throughout the tracheal system. This changes the identity of the presumptive DT cells and causes them to migrate with the DB cells and form narrow diameter tubes (arrows). The ends of these hybrid branches show anomalous branching (*). (C,D) Region of wild-type trachea stained for TL1 (C) or 2A12 (D) luminal antigens. DT diameter increases dramatically between stages 14 and 16. (E-H) Similar views of the trachea at stage 16 in mutants that disrupt (E) secondary branching (Samakovlis et al., 1996a), (F) terminal branching (Guillemin et al., 1996), or (G,H) branch fusion (*esg*, Samakovlis et al., 1996b; *arm**, this work). None of the mutations significantly affect DT dilation or the sizes of other primary branches, even when DT segments are disconnected as in G and H. Most DT segments are also disconnected in *pnt^{Δ88}* embryos, and these also dilated approximately normally (not shown). C-F and H show Tr4-6; G shows segments Tr7-9 (with a DT break in Tr9). *arm**, *arm^{YD35/Y}*; *P[arms14-C]/+* (see Materials and Methods). Bar: 7 μm (for A,B), 10 μm (for C-H).

during embryonic and larval life must take place without change in cell number.

To verify that cell number does not dictate tube size, we manipulated cell number using mutations in cell cycle genes *cyclin A* (*cycA*) and *dacapo* (*dap*) (de Nooij et al., 1996; Lane et al., 1996; Sprenger et al., 1997). *cycA* mutations arrest the cell cycle before the last embryonic cell division, decreasing cell number by half. Tracheal branches in many *cycA* embryos formed with half the normal number of cells yet had normal diameters and lengths (Fig. 2C). In *dap* mutants, embryonic cells undergo an extra round of division and there were nearly twice the normal number of tracheal cells in some branches (Fig. 2C). Despite the increased cell number, primary branching occurred normally and branch sizes did not increase (Fig. 2A,C). In addition, we found that tube size was increased up to 60% without increasing cell number in the tube expansion

mutants described below. We conclude that tracheal tube size is controlled independently of cell number.

The *cycA* and *dap* results also imply that tube size is not controlled by rigidly specifying the size and shape of each tracheal cell. Because varying cell number using these cell cycle mutations causes reciprocal effects on cell size (de Nooij et al., 1996; Lane et al., 1996; Sprenger et al., 1997) but no corresponding effects on tube size, tracheal cells of different sizes and shapes must be able to form tubes of similar size. This is also evident in wild-type trachea at the regions where DT branches fuse to generate a long continuous tube. Each segment of DT is composed of multiple cobblestone-shaped cells (Fig. 3A), whereas each fusion joint consists of two toroidal cells (Fig. 3B; Samakovlis et al., 1996b). Despite these very different cell shapes, both form tubes of precisely the same diameter (see Fig. 4). Fusion cells at other joints in the tracheal system also have a toroidal structure, but form tubes with diameters 10 times smaller than those of DT fusion cells (compare Fig. 4H to 4I). Together, these results show that tracheal tube size is not dictated by the number, size or overall shape of the cells that compose the tube.

Tube size is controlled at the apical surface of tracheal cells

To gain further insight into the cellular basis of tracheal tube growth, we examined some of the structural changes that occur in tracheal cells during the embryonic dilation of the DT. Several observations suggest that the critical focus for tube size control is the apical (luminal) surface of the cells. First, the apical surface of the tracheal cells was smooth and regular throughout the period of DT dilation, implying a high degree of control, whereas the basal surface was very irregular (Fig. 4A,B). Second, the apical surface of tracheal cells, although itself highly regular, did not occupy a consistent position with respect to the basal surface or cell bodies and was often seen displaced to one side of the epithelium (Fig. 4A-D). Third and most importantly, during DT dilation, while the inner diameter of the tube (apical surface) increased dramatically, there was little change in the outer diameter of the tube (basal surface) (compare 4E to 4F, and G to 4H). The thickness of the epithelium changed, decreasing by 20-40%, from approx. 3.8 μm to approx. 2 μm (see Fig. 4 legend). Thus, there appears to be precise and coordinate control of the apical surface of tracheal cells during DT dilation, but little regulation of the basal surface.

Genetic control of tracheal tube size

The characteristic sizes and growth kinetics of each primary branch suggest that tube size is regulated as part of the overall identity of each branch. Previously, the DPP and EGF signaling pathways and several downstream transcription factors have been shown to be expressed and active in different primary branches and to control certain aspects of primary branch fate such as cell migratory and adhesive behavior (Vincent et al., 1997; Wappner et al., 1997). We found that misexpressing DPP throughout the tracheal system not only caused DT cells to adhere to and migrate with dorsal branch cells as previously shown, but also caused them to form narrow-diameter tubes like the dorsal branch (Fig. 5B). Similar effects are seen upon misexpression of *knirps* and *knirps-related*, downstream targets of DPP signaling (Chen et al., 1998). Thus, DPP

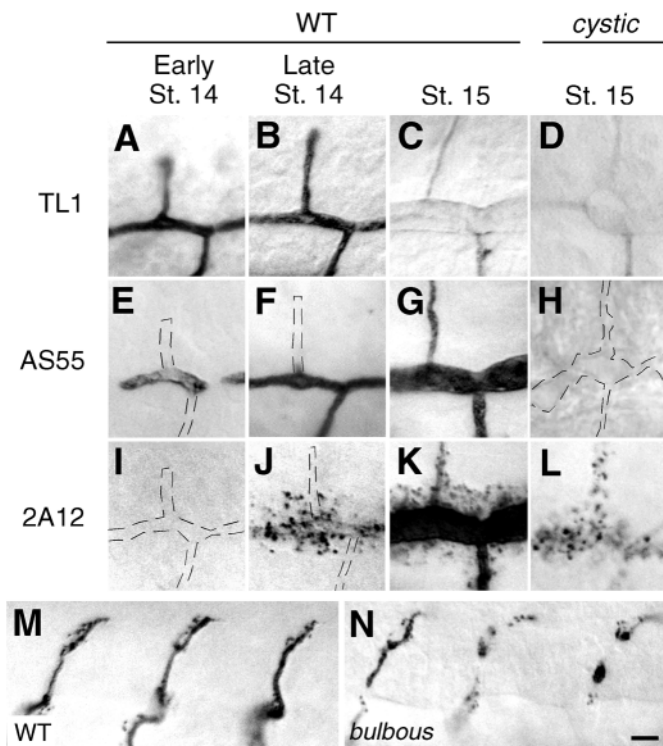


Fig. 6. Expression of tube expansion markers in wild type and tube expansion mutants. (A-L) Expression of TL1, AS55 and 2A12 tracheal luminal antigens during stages 14 and 15 in wild-type and *cystic* mutant embryos. The expression of the markers changes dramatically during this 2 hr period, as DT dilation begins. (A-C) TL1 expression is strong from stage 11 (not shown) through stage 14 and weak or off from stage 15 onwards. (D) TL1 turns off normally in the *cystic* tube expansion mutant. (E-G) AS55 expression begins at early stage 14 in the DT, and is strongly expressed in all branches by stage 15. Dashed lines outline tracheal tubes not expressing the marker. (H) In the *cystic* mutant, AS55 fails to be expressed. (I-K) 2A12 expression begins in late stage 14, initially as punctate cytoplasmic staining in the DT. It is strongly expressed in the lumens of all branches by stage 15. (L) In the *cystic* mutant, early punctate expression of 2A12 is normal but the later luminal expression is absent. (M,N) Expression of 2A12 antigen in ganglionic branches of stage 16 wild-type embryo (M) and the *bulbous* mutant (N). Note the irregularities and gaps in 2A12 staining in the mutant. Bar: 10 μ m (for A-N).

signaling can specify the sizes as well as migratory and adhesive properties of tracheal branches.

Once the early signaling pathways specify branch fates, how is this information translated into tubes of particular sizes? The previously defined genetic programs of primary (Samakovlis et al., 1996a; Sutherland et al., 1996), secondary (Hacohen et al., 1998) and terminal branch outgrowth (Guillemin et al., 1996) and branch fusion (Samakovlis et al., 1996b) are unlikely to be directly involved because they show different spatial and temporal patterns of activity than the tube size changes. Furthermore, primary branch sizes were largely unaffected in mutants in the secondary, terminal, or fusion programs (Fig. 5E-H). For example, in fusion mutants, disconnected DT segments still expanded normally (Fig. 5G,H). Thus, tube size and expansion appear to be controlled by a program that operates largely in parallel to the branch outgrowth and fusion programs.

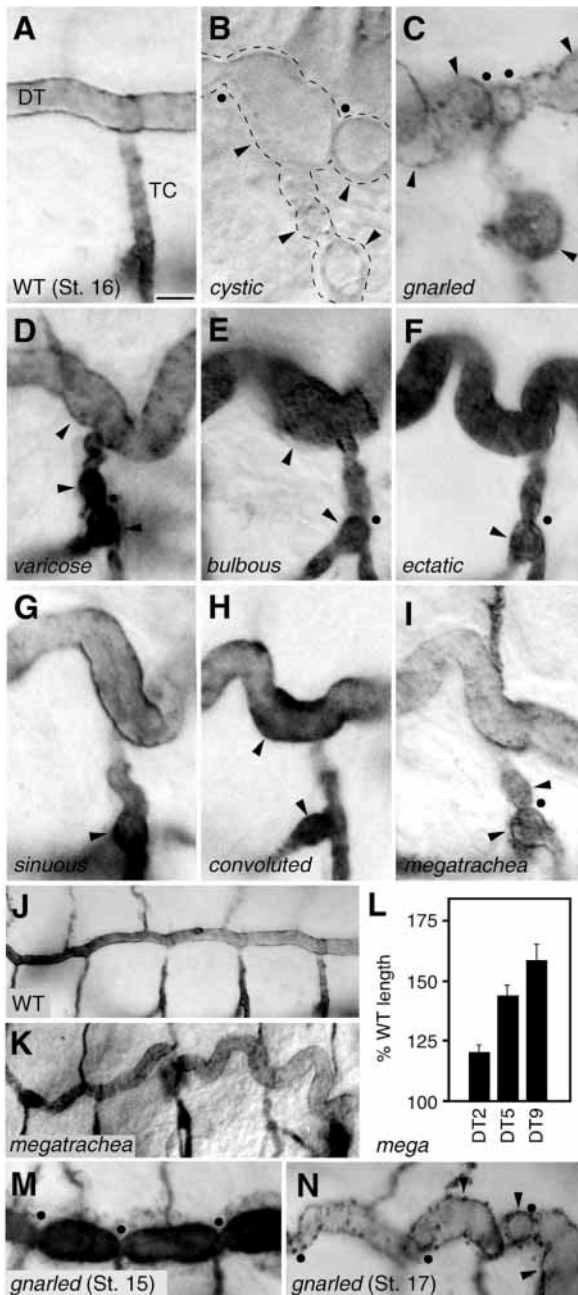
We identified specific molecular markers that change expression when the embryonic tubes start expanding. The luminal antigen TL1 is expressed throughout the developing tracheal system as primary branches bud and grow out, but expression turns off at the end of stage 14. This is when primary branching is completed and the branches begin to dilate and steadily elongate (Fig. 6A-C). About this time, two other luminal antigens, 2A12 and AS55, begin to be expressed throughout the tracheal system (Fig. 6E-G,I-K). In the next section, we describe a set of mutants defective in expansion and expression of some of these expansion markers, providing evidence that tube expansion is a genetically distinct phase of the tube size control program.

A set of genes required for tracheal tube expansion and maintenance

To identify additional genes involved in tube size control, we screened several large collections of P[lacZ] transposon insertion lines for mutations that altered tracheal tube size and shape without disrupting general embryonic morphology (see Materials and Methods). Nineteen mutations were found. Three additional mutations that cause tracheal tube size defects were provided by N. Perrimon (*megatrachea*^{EA97, VE896}) and N. Hacohen (*gnarled*^{ex59}). We focus here on the nine mutations that appear to be specific for tube size because they did not affect other aspects of tracheal development including primary branch budding and outgrowth or branch fusion. All nine mutations were recessive, and complementation tests for their tracheal functions showed that they define eight different genes. We assigned chromosomal locations to five of the genes by complementation tests with chromosomal deficiencies (Table 2). The tracheal phenotypes of these five mutations when hemizygous over deficiencies were not significantly stronger than when homozygous, indicating that these mutations are strong loss-of-function or null alleles. None of these mutations had any obvious effect on the morphology of two other tubular epithelial organs, the salivary gland and hindgut (data not shown).

The tracheal phenotypes of the nine mutants are summarized in Table 2. All of the mutations caused irregular and variable tracheal tubular morphologies, with local constrictions and/or dilations (expansions beyond the normal dimensions) in all major branches (Fig. 7B-I). The defects were most severe in *cystic* mutants, where regions of the tubes more than twice normal diameter alternated with severe constrictions (Fig. 7B). For all of the mutants, the lumen near the end of some of the smaller branches, particularly the ganglionic branches, appeared to be discontinuous due to failure of the associated tracheal cells to form or maintain a lumen or express the 2A12 luminal antigen (compare Fig. 6M to N).

Developmental analysis of the mutants using the TL1 and 2A12 markers showed that early events in tracheal morphogenesis including tracheal sac formation, budding and outgrowth of primary branches, lumen formation, and branch fusion occurred normally (Table 2). The expression of the terminal branching gene *blistered* (Guillemin et al., 1996) was also normal. The first morphological defects occurred at the end of embryonic stage 14 as tube expansion begins. DT fusion cells commonly failed to expand their lumens as much as the DT segments they connect, resulting in periodic constrictions along the DT that gave it a characteristic 'sausage link'



appearance (Fig. 7M). The phenotypes became progressively more severe as the animals developed and, by stage 16, the DT and other branches showed irregularities in diameter all along their lengths (Fig. 7B-I,N). However, not all branches were affected equally by all mutations. For example, the DT in *megatrachea* mutants had relatively few constrictions or dilatations, whereas other primary branches showed marked abnormalities (compare the DT and TC in Fig. 7I). Also, although the mutations caused defects throughout the tracheal system, none of the mutants completely abolished tube size regulation because many branches or portions of branches retained their normal diameter.

Most of the mutations affected tube length as well as diameter, and in some (*megatrachea* and *convoluted*) it was the more prominent effect (Table 2). DT length was normal in all

Fig. 7. Phenotypes of tracheal tube expansion mutants. (A-I) Close up of a representative region of the DT and TC (Tr5, 6 or 7) in wild type and eight tube expansion mutants. Trachea were stained for 2A12 antigen except the *cystic* mutant (B), which fails to stain with 2A12 and other tracheal-specific luminal antisera but retains some reactivity to mab Cq4 against the general apical epithelial marker Crumbs. Note the dilatations (arrowheads) and constrictions (dots) in the tubes. Also note that in most mutants the DT is too long and follows a tortuous path. (J,K) DT segments Tr2-6 in stage 16 wild-type and *mega*^{EA97} embryos. The DT is long and tortuous in *mega* mutants. (L) Quantitation of the increase in DT length in segments Tr2, Tr5, and Tr9 of stage 16 *mega*^{EA97} embryos. Posterior segments are affected more than anterior segments. (M,N) Dorsal trunk (Tr7-9) in *gnarled*^{ex59} embryos stained for 2A12 antigen. Early tracheal development is normal (not shown), but the phenotype progressively worsens from stage 15 onward. At stage 15 (M), the DT tubes are slightly enlarged with constrictions at each fusion joint (dots), giving it a 'sausage link' appearance. By stage 17 (N), the DTs are too long and have dilatations and constrictions all along their lengths. Bar: 5 μ m (for A-D), 10 μ m (for J,K,M,N).

mutants at the beginning of stage 15 (data not shown). However, in all of the mutants except *cystic*, the DT lengthened more than normal over the next 3 hours and, by stage 16, followed a long and convoluted path (e.g. compare Fig. 7J and K). There was no accompanying increase in cell number (see Table 2 legend). Again, not all branches were affected equally by the mutations. For example, none of the mutations substantially increased the length of TC (Table 2) and, in mutants with increased DT length, the posterior DT was affected significantly more than the anterior (Fig. 7K, L and data not shown).

We examined the effect of the mutations on expression of the tracheal expansion markers described above. The downregulation of the TL1 antigen at the beginning of the expansion period occurred normally in all mutants, as did the transient early cytoplasmic expression of the 2A12 antigen (Fig. 6D,L; data not shown). However, mutations in seven of the eight genes caused defects in the subsequent luminal accumulation of 2A12 antigen (Fig. 6L,N; Table 2), and three affected expression of the AS55 antigen (Table 2). The defects were most severe in *cystic* mutants, which lacked almost all AS55 staining and all 2A12 luminal staining. The other mutants showed poor or delayed staining primarily in the ganglionic and dorsal branches. Thus, AS55 and 2A12 antigens lie genetically downstream of most of the identified genes.

The above analyses indicate that the eight genes defined by this group of mutations are specifically required for proper tracheal tube expansion and maintenance of the expanded tubes, and most are required for normal expression of expansion markers. We refer to these genes as tube expansion genes.

DISCUSSION

To establish a tractable genetic system for investigating epithelial tube size control, we analyzed the growth of *Drosophila* tracheal tubes using morphometric, cellular and genetic approaches. Our major conclusions are: (i) tracheal tubes undergo reproducible changes in size during development, and their diameters and lengths are regulated

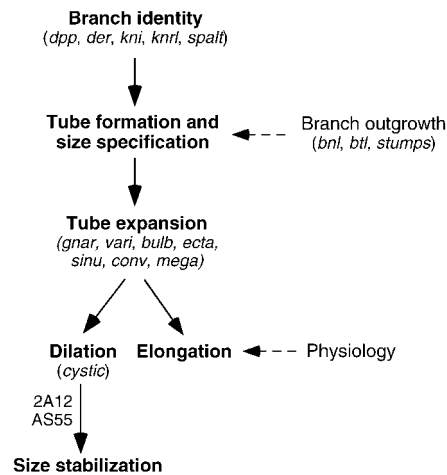


Fig. 8. Model for tracheal tube size control. Genes in the pathway may be organized hierarchically, with early genes regulating genes that act later in the pathway. Branch identity genes like *dpp* might control the expression or activity of one or more expansion genes. Most expansion genes are in turn required for proper expression of the 2A12 and AS55 antigens, which are therefore placed downstream in the hierarchy. There must be additional inputs to the hierarchy that coordinate branch expansion with branch budding and outgrowth events and other events in the animal, such as the hormonal signals that trigger the larval molts and associated tracheal dilations, and the physiological signals that regulate overall growth of the animal. *bnl*, *branchless*; *btl*, *breathless*.

separately, (ii) there is a specific genetic program governing these changes that is distinct from the previously characterized tracheal branching programs, and (iii) tube size appears to be controlled by coordinate regulation of the apical tracheal cell surfaces, rather than by specifying the number or overall structure of tracheal cells. We discuss the evidence for and implications of each of these, and propose an outline of the tube size control program along with molecular models for how it may regulate tube size.

Tracheal tubes undergo programmed changes in size and shape during development

Like other tubular organs, tracheal tubes grow dramatically as the animal grows during development, expanding up to 40 times their initial size by the end of larval life. Our morphometric analysis revealed a high degree of regulation and modularity in the control of this growth. Although all tracheal branches are interconnected and form a continuous epithelium, each branch behaves as an independent unit, growing at its own rate and to its own extent during development. Furthermore, the diameter and length of each branch are separately regulated: diameter increased abruptly at discrete times in development, whereas length increased gradually throughout development. Diameter and length also expanded to different extents, so the shape of the branches changed as they grew. Thus, the larval tracheal network is not simply an enlarged version of the one that initially forms in the embryo, but rather each branch in the network is specifically remodelled during development to create the mature structure. The magnitude and timing of the changes were highly reproducible, implying that they are developmentally programmed.

A genetic pathway for tube size control

To begin to elucidate the tube size control program, we carried out genetic studies that included both an investigation of the roles of known tracheal genes and a screen for new genes involved in the process. These studies indicate that tube size is controlled by a genetic program that is distinct from, and functions largely in parallel to, the previously characterized programs controlling branch outgrowth and fusion. The genetic analysis combined with our morphometric results suggests that the tube size control program can be divided into two major phases: an early phase in which tube size is specified and tracheal cells assemble into small tubular structures, and an expansion phase in which the diameter and length of the tubes grow to their mature sizes (Fig. 8). There may also be a stabilization phase that fixes tube diameter for the extended periods between dilations.

The evidence that tube size is specified early as part of the positional identity of each branch is as follows. First, the timing, rate and extent of tube size changes were characteristic for each primary branch, and small differences between branches were apparent as soon as branches formed. Second, the environmental and physiological variables tested had little effect on branch diameter. Third, branch diameter can be substantially altered by misexpressing early tracheal genes that specify other aspects of primary branch identity. Misexpressing DPP in the developing tracheal system (Fig. 5B), or misexpressing its downstream targets *knirps* and *knirps-related* (Chen et al., 1998), not only switched DT adhesive and cell migratory behavior as previously shown (Vincent et al., 1997; Wappner et al., 1997), but also caused the cells to form small diameter tubes like the dorsal branch. Thus, branch identity genes such as *dpp* and its targets appear to specify tube size along with other characteristics that distinguish different primary branches.

Several lines of evidence support the proposition that expansion is a second distinct phase of tracheal tube size control. Expansion is temporally distinct from and has different kinetics than the initial budding and tube formation events. During budding, small groups of tracheal cells migrate toward Branchless FGF signaling centers, assembling into small diameter tubes as they migrate (Sutherland et al., 1996). The expansion to larger diameter tubes does not begin until four hours later when the migrating branches reach the Branchless FGF signaling centers and expression of the signal ceases. At the same time there are marked changes in the rate of branch elongation as branches begin lengthening by a mechanism that does not involve directed cell migration. There are also changes in expression of specific molecular markers. The 2A12 and AS55 markers turn on at the onset of expansion, as does the Notch-ligand Serrate (Ikeya and Hayashi, 1999), while the TL1 marker turns off. Most importantly, we identified genes specifically required for expansion. In all the identified mutants, primary branch outgrowth and initial tube formation were normal, but all of the mutants showed defects in tube size and shape that manifested as expansion began, and progressively worsened. The similar phenotypes and timing of the defects suggests that these gene products act together in a process(es) that is specifically required for expansion and/or maintenance of the expanded tubes. We speculate on what that process might be and how it might be regulated in the next section.

Table 2. Summary of tube expansion mutant phenotypes[†]

Gene (alleles)	Location ¹	Mutagen ²	DT length ³	TC length ³	DT diameter defects	Diameter defects in other primary branches	2A12 staining defects ⁴	AS55 staining defects ⁴	Onset of phenotypes
<i>cystic</i> (<i>l(2)k13717b</i>)	II	P*	103±1%	86±1%	+++	+++	+++	+++	st. 14
<i>gnarled</i> (<i>ex59</i>)	85E11-F16	P*	125±4%	108±5%	++	++	++	++	st. 15
<i>varicose</i> (<i>l(2)03953b</i>)	38A6-39E1	P*	132±3%	101±2%	+	++	++	—	st. 15
<i>bulbous</i> (<i>l(2)k11012b</i>)	49B2-50D2	P*	136±4%	97±2%	+	++	++	—	st. 15
<i>ectatic</i> (<i>l(2)k04223</i>)	27A-27C9	P (27B1-2)	130±6%	96±3%	+	++	++	+	st. 15
<i>sinuous</i> (<i>l(3)06524</i>)	64C13-65A	P (64D3-4)	114±4%	98±3%	+	+	++	—	st. 15
<i>convoluted</i> (<i>l(2)k06507b</i>)	II	P*	124±6%	96±4%	+	+	++	—	st. 15
<i>megatrachea</i> (<i>EA97, VE896</i>) ⁵	X	EMS	137±7%	106±2%	+/-	++	—	—	st. 15

[†]Phenotypes of stage 16 embryos. For all mutants, early tracheal development, including primary branch budding and outgrowth, lumen formation and morphology, and TL1 antigen expression occurred normally during stages 11-14. Expression of the terminal branching marker Blistered (DSRF) was also normal at all stages and tracheal branches fused at the correct stages to form the contiguous tubular networks in all of the mutants. —, normal; +, mild; ++, moderate; +++, severe.

¹Cytological positions determined by deficiency mapping.

²EMS, ethyl methanesulfonate; P (map position), P element insertion at indicated position; P*, mapped P insertion site not at location of mutation.

³Values shown (mean ± s.e.m., n > 5) have been normalized to stage 16 wild-type values as described in Fig. 2. Values different than WT (p < 0.005) are in bold. Values for DT cell number, TC5 cell number, and embryo length (see Fig. 2 legend; mean ± s.e.m., n = 5-10) were: *cyst* 62±5, 13±0.6, 360±6 µm; *gnar* 77±2, 14±0.5, 350±3 µm; *vari*: 75±1, 13±0.4, 340±3 µm; *bulb* 70±3, 12±4, 360±4 µm; *ecta* 72±2, 12±0.8, 334±8 µm; *sinu* 76±3, 15±0.7, 340±5 µm; *conv* 66±2, 12±1, 330±3 µm; *mega* 73±1, 14±0.3, 390±4 µm.

⁴Defects included non-staining regions of ganglionic branches and/or delayed or reduced staining in the dorsal or other branches.

⁵EA97 and VE896 phenotypes were indistinguishable.

The tube expansion program consists of at least two subprograms, one controlling tube length and the other tube diameter. This is implied by our morphometric analysis, which showed that tube diameter and length expand at different times and with different kinetics. Furthermore, branch length, but not diameter, was influenced by alterations in environmental oxygen levels and by a mutation in the *chico* insulin-pathway growth control gene. Conversely, *cystic* mutations caused gross abnormalities in DT diameter without affecting DT length. Together, these results imply that there are separate mechanisms and genetic controls for tube length and diameter. Although each dimension is regulated separately, the genetic results imply that diameter and length control have many common components because mutations in seven of the eight tube expansion genes affected both tube length and diameter.

Cellular mechanism of tube expansion and the role of tube expansion genes

An important conclusion from our cellular analysis is that

tracheal tube size is not dictated by cell number or the specific shape of the cells that compose the tubes. There was not a tight correlation between the size of a tracheal tube and the number of cells in it, and altering cell number using cell cycle mutations did not affect tube size. Furthermore, the dramatic expansions of the tracheal tubes in the embryo and larva occurred without changing cell number, and tube sizes were altered in tube expansion mutants without affecting cell number. Because tracheal tubes of identical size were formed by different numbers of cells with very different shapes and sizes, tracheal tube size is not controlled by preprogramming the overall shapes of the constituent cells. Instead, tracheal cells in a given branch must collectively adjust their shapes to form a tube of the specified size.

A second important implication of our cellular analysis is that tracheal tube size appears to be controlled by coordinate regulation of the apical cell surface. The apical surface formed an even luminal surface, whereas the cell bodies and basal surface were irregular and not highly organized. During the

tracheal expansion in the embryo, tube diameter at the apical surface increased greatly whereas the diameter at the basal surface expanded little and remained irregular throughout the expansion. Furthermore, the luminal surface maintained an even circular profile but was not centered with respect to the cell bodies and basal surface. These observations suggest that the tube formed by the apical cell surface acts as an independent structure that is functionally isolated from other parts of the cells. This in turn predicts that some of the gene products controlling tracheal tube size will act on, or be located at, the apical surface. Although many molecular mechanisms for regulating the apical surface are possible, we suggest three plausible models.

In the first model, the apical cytoskeleton of the tracheal cells is the critical structure that determines tube size. An appealing paradigm for this is the ring canals that interconnect the nurse cells and oocyte during *Drosophila* oogenesis (reviewed by Cooley, 1998). Ring canals are annular actin-based structures that assemble underneath the plasma membrane at sites of incomplete cytokinesis. During a 3-day period of oogenesis, the actin rings dilate from 1 to 10 μm in diameter, similar in scale to some of the tracheal tube dilations. Ring canal expansion is an active process requiring the Src64 and Tec29 kinases (Guarnieri et al., 1998; Roulier et al., 1998). It is possible that equivalent cytoskeletal structures underlie the apical surface of the tracheal cells and control the diameters and lengths of tracheal tubes. For example, the addition or removal of actin filaments, or the sliding of filaments relative to one another, could both regulate and provide the motive force for dilating or constricting the tubes. Alternatively, the radius of curvature of the constituent filaments could be regulated to adjust ring diameter. An important difference from the oocyte is that, whereas oocyte ring canals are fully intracellular structures, the large tracheal tubes have multiple cells surrounding the lumen, so there would need to be some way of connecting and coordinating the apical cytoskeleton between tracheal cells. There would also have to be some means of regulating longitudinally oriented filaments separately from circumferential filaments to allow independent control of tube length and diameter. In this model, tube expansion genes might encode components or regulators of the actin cytoskeleton, or molecules that mediate the communication between cells.

In the second model, the apical plasma membrane and extracellular matrix that lines the lumen would dictate tracheal tube size and shape. A paradigm for this model is the system that controls the shape of *C. elegans*. Mutations that cause worms to be shorter and fatter, or too long, have been isolated (reviewed by Kramer, 1997), and many of these affect genes encoding collagens found in the extracellular matrix and cuticle surrounding the worm. The matrix is stiff and thought to act structurally as an exoskeleton. In the tracheal tubes, the extracellular matrix would form an internal scaffold, moulding the apical surface of the epithelium surrounding it into a tube. There would have to be ways to modify the structure of the matrix to form different size rings to generate different diameter tubes, and the matrix would have to be easily remodeled to allow rapid adjustment in tube size during the tracheal dilations. After each dilation, tracheal tubes become lined with cuticle; the conversion of an easily remodeled matrix to stiff cuticle would presumably fix tube diameter until the

next molt. Remarkably, tracheal tube elongation continues unabated after cuticle deposition, so there are apparently mechanisms that allow tracheal cuticle to increase in length without perturbing its radius of curvature. In this model, expansion genes might encode components or regulators of the extracellular matrix and its attachment to the cell surface.

In the third model, expansion would be driven by secretion or a pressure-generating process inside the lumen. The balance between this expansive force and inward constraining forces would determine the rate and extent of expansion of each tube. The constraining forces could be provided by the plasma membrane or an apical actin ring. In this model, expansion genes might encode the secretory machinery or pressure-generating system, or components of the restraining apparatus or other molecules that maintain tube integrity during expansion. Clearly, other models and combinations of the above models are possible. Molecular characterization of the expansion genes may help distinguish among them.

Tube size control in other organs and its implications for medicine

The characteristic sizes and shapes of the epithelial tubes in vertebrate organs suggest that, as in the *Drosophila* tracheal system, tubes sizes are carefully controlled. This regulation must be maintained throughout development because the tubes of most organs, including the lung, vascular system and kidney, expand dramatically in length and diameter as animals grow from embryos to adults. Although the cellular, genetic and molecular mechanisms that underlie tube size specification and growth have not been systematically investigated for vertebrate organs, Fankhauser (1945) found that the size of salamander kidney tubules is independent of the number and size of the constituent cells, as we found for *Drosophila* tracheal tubes. Recently, a set of *C. elegans* mutants were described with renal cell tubule defects similar to the tracheal tube defects that we observed in the tube expansion mutants (Buechner et al., 1999). Whether these parallels extend to the molecular level is not known. It will be important to discover how many distinct cellular and molecular pathways there are for tube size control and the extent to which they have been conserved in different organs and animals.

Understanding and ultimately manipulating the cellular and molecular processes that control tube size has important medical implications. For example, elucidating the genetic control of blood vessel size could aid in development of agents that enlarge existing blood vessels and enhance circulation to ischemic tissues. Tube size control programs might also provide new targets for treatment of aneurysms or for anti-angiogenic therapies to keep tumor blood vessels or other unwanted vessels small. Given the conservation of most fundamental biological processes, homologues of *Drosophila* tube size control genes might provide an entry for such studies.

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