**LETTERS**

*Drosophila* Src regulates anisotropic apical surface growth to control epithelial tube size

Kevin S. Nelson1, Zia Khan2,5,6, Imre Molnár4, József Mihály4, Matthias Kaschube2,5,6 and Greg J. Beitel1,7

Networks of epithelial and endothelial tubes are essential for the function of organs such as the lung, kidney and vascular system. The sizes and shapes of these tubes are highly regulated to match their individual functions. Defects in tube size can cause debilitating diseases such as poly cystic kidney disease and ischaemia1,2. It is therefore critical to understand how tube dimensions are regulated. Here we identify the tyrosine kinase Src as an instructive regulator of epithelial-tube length in the *Drosophila* tracheal system. Loss-of-function *Src42* mutations shorten tracheal tubes, whereas *Src42* overexpression elongates them. Surprisingly, Src42 acts distinctly from known tube-size pathways and regulates both the amount of apical surface growth and, with the conserved formin dDaam, the direction of growth. Quantitative three-dimensional image analysis reveals that *Src42*- and *dDaam*-mutant tracheal cells expand more in the circumferential than the axial dimension, resulting in tubes that are shorter in length—but larger in diameter—than wild-type tubes. Thus, Src42 and dDaam control tube dimensions by regulating the direction of anisotropic growth, a mechanism that has not previously been described.

The *Drosophila* tracheal system is a gas-exchange organ that arises from clusters of epithelial cells that invaginate, branch and interconnect to form a network of tubes. After network formation, tracheal lumens markedly increase their diameters and lengths3. Importantly, these expansions result exclusively from changes in cell shape and apical surface area (ASA), because tracheal cell number does not change during expansion4,4.

Multiple cellular processes are known to control tracheal-tube size. Overexpression of the apical determinant Crumbs (CRB) increases both the length and diameter of late-stage tracheal tubes5, and loss of the basal polarity protein Scribble (SCRIB) increases tube length5,6.

Mutations in planar cell polarity (PCP) genes also cause modest tube over elongation resulting from increased apical cell surface7. The best-characterized size-control pathway involves the apical extracellular matrix (aECM), whose assembly depends on a cell–cell junction termed the septate junction. After tracheal metameres fuse, a chitin-based aECM is secreted apically into the tracheal lumen8,10. Putative chitin deacetylases Verm and Serp are secreted into the lumen to regulate aECM organization and are required to prevent over elongation of tracheal tubes11,12. Basolateral septate junctions direct apical secretion of Verm and Serp through an uncharacterized pathway12,13. Consequently, mutations in almost all septate-junction components also cause tube over elongation11,13,15,16. So far, mutations that markedly shorten the length of late-stage embryonic tracheal tubes have not been reported. Further, with the possible exception of CRB, the size-control proteins identified so far act as ‘permissive’ rather than ‘instructive’ factors, because their overexpression does not cause phenotypes opposite to their loss-of-function phenotypes12,14,16.

Src-family kinases17 have been implicated in controlling the size of mammalian epithelial and endothelial tubes, though the mechanisms by which they do so have not been elucidated18,19. We therefore examined the tracheal system of *Drosophila* embryos homozygous for null mutations in either of the two known Src-family kinase genes, *Src42* (also known as *Src42A*) and *Src64* (also known as *Src64B*). Neither zygotic nor maternal/zygotic *Src64* mutants showed tracheal defects or genetic interactions with a mutation in ATP6 that elongates tracheal tubes15, and *Src64* was not detected in tracheal cells using immunohistochemistry (Supplementary Fig. S1). In contrast, by late embryogenesis (stage 16), zygotic loss of *Src42* caused the dorsal trunk, the largest and most easily analysed tracheal tube, to be about 27% shorter than in wild-type controls (Fig. 1b,d,q) and to have a ‘stretched’ appearance, in which the angle of the anterior transverse connective 1 relative to the dorsal trunk was significantly increased (Fig. 1e,f) and the normal ventral
Src42 acts autonomously to control tracheal tube length. (a–d) The dorsal trunk (DT, arrow) of Src42 null mutant embryos seems normal at early embryonic stage 14 (a,c), but is markedly shorter than the wild type by stage 16 (b,d). Asterisks indicate endpoints for dorsal-trunk measurements. Lumens in a–o and p are visualized by staining for 2A12 antigen. (e,f) Dorsal-trunk shortening increases the angle between the anterior transverse connective (TC) and the dorsal trunk in Src42 mutants (n = 5, P = 6 × 10⁻⁹). (q) The angle of the posterior ventral curve is also increased in Src42 mutants (n = 5, P = 1 × 10⁻³). (i,j) Src42 kinase activity is required for dorsal-trunk elongation, because tracheal-specific expression (btl-Gal4; ref. 24) of a wild-type Src42 transgene (Src42 WT) but not a kinase-dead form of Src42 (Src42 KM) results in tracheal tubes that are comparable in length to those with null alleles. For q and r, error bars represent s.d., all P values are determined using Student t-tests and n = 6 for all samples. Scale bars, 20 μm for a–d,i–j,o–p; 10 μm for e–h; 5 μm for k–n.

Figure 1 Src42 acts autonomously to control tracheal tube length. (a–d) The dorsal trunk (DT, arrow) of Src42 null mutant embryos seems normal at early embryonic stage 14 (a,c), but is markedly shorter than the wild type by stage 16 (b,d). Asterisks indicate endpoints for dorsal-trunk measurements. Lumens in a–o and p are visualized by staining for 2A12 antigen. (e,f) Dorsal-trunk shortening increases the angle between the anterior transverse connective (TC) and the dorsal trunk in Src42 mutants (n = 5, P = 6 × 10⁻⁹). (q) The angle of the posterior ventral curve is also increased in Src42 mutants (n = 5, P = 1 × 10⁻³). (i,j) Src42 kinase activity is required for dorsal-trunk elongation, because tracheal-specific expression (btl-Gal4; ref. 24) of a wild-type Src42 transgene (Src42 WT) but not a kinase-dead form of Src42 (Src42 KM) results in tracheal tubes that are comparable in length to those with null alleles. For q and r, error bars represent s.d., all P values are determined using Student t-tests and n = 6 for all samples. Scale bars, 20 μm for a–d,i–j,o–p; 10 μm for e–h; 5 μm for k–n.

curve of the posterior dorsal trunk metamere 10 was absent (Fig. 1g,h). Measurements revealed that Src42 elongation defects become apparent after tracheal metameres fuse midway through embryogenesis (Figs 1a,c and 2j) as the dorsal trunk begins elongating. Importantly, the elongation defect of Src42 mutants is independent of cell number, because Src42 and wild-type dorsal trunks have the same number of cells. (l) Src42 mutants completely lack Src42 labelling. (m,n) The Src42 KM and Src42 WT transgenes are expressed at similar levels in the trachea of Src42 mutants. (o) Src42 KM acts as a dominant negative in the tracheal system, as its overexpression inhibits dorsal-trunk elongation. (p) Conversely, overexpression of Src42 WT in the tracheal system of Src42 heterozygotes overelongates tracheal tubes. (q) Quantification of dorsal-trunk length in Src42 rescue experiments reveals statistically significant differences between Src42 WT and Src42 KM. (r) An allelic series using loss- and gain-of-function alleles and transgenes reveals that tracheal length correlates with Src42 levels. Note that overexpression of Src42 WT results in tracheal tubes that are comparable in length to those with null alleles. For q and r, error bars represent s.d., all P values are determined using Student t-tests and n = 6 for all samples. Scale bars, 20 μm for a–d,i–j,o–p; 10 μm for e–h; 5 μm for k–n.

As Src-family kinases can mediate cellular functions independently of their tyrosine kinase activity, we expressed either a wild-type (Src42 WT; ref. 22) or a kinase-dead form of Src42 (Src42 KM; ref. 23)
Figure 2  Src42 acts independently of the aECM-based tube-size-control pathway. (a–c) Activated Src42 (pSrc) co-localizes with DE-cad at the adherens junction. (d–f) pSrc shows some partial overlap with CRB at the subapical membrane. (g,h) Activated Src localizes apical to the septate junction, which is labelled with Coracle (Cora), a canonical septate-junction marker. (i–j) Src42 mutants show essentially no pSrc labelling, indicating that the pSrc antibody is specific for activated Src42. (k,l) Overexpression of Src42 results in a marked increase in pSrc. (m–p) Labelling for pTyr shows that most tyrosine-phosphorylated proteins also localize apical to septate junctions (m,n). Src42 is required for much of the tyrosine phosphorylation at the apical membrane (m–p) and Src42 overexpression increases apical pTyr (q,r). (s–v) Src42 is epistatic to the aECM/septate-junction pathway, because Src42 mutants fully suppress tracheal overelongation in aECM/septate-junction mutants, such as nrv2. (w–z) Src42 mutations do not disrupt septate-junction organization (note localized red Cora staining at the apical region of the lateral membranes, arrowheads in w and x) or Verm secretion (green luminal staining), and suppress nrv2 tube elongation without restoring septate-junction organization (note diffuse Cora staining) or Verm secretion (note the lack of Verm staining) (y,z). Dashed lines mark the apical surface. (aa) The dorsal trunks of Src42 nrv2 double mutants are the same length as those of Src42 single mutants. The length of the dorsal trunk between transverse connectives 5 and 10 was measured because the overelongation of aECM/septate-junction mutants is most apparent in the posterior dorsal trunk. Error bars, s.d. P values are from Student t-tests. For all samples, n = 5. (bb–ee) nrv2 null mutants do not show obvious changes in the levels or localization of pSrc (bb,cc), Src42 (dd) or pTyr (ee), indicating that the aECM does not directly regulate Src42. See Fig. 2 for wild-type comparisons. (ff–ii) Overexpression of Src42 in a nrv2 background results in a loss of luminal 2A12 and tracheal tubes that are cystic (ii). The enhanced phenotype indicates that Src42 and nrv2 are unlikely to function in the same genetic pathway. Dashed lines mark the apical cell surface. (j) Measurement of dorsal-trunk length in wild-type, Src42 and nrv2 animals at two-hour intervals shows that the dorsal trunks of wild type, nrv2 and Src42 are the same length at 10 h, just after tracheal metanemes fuse (P = 0.46 for wild type and Src42, P = 0.78 for wild type and nrv2). However, by 12 h, Src42 dorsal trunks are shorter than wild type (P = 0.044), whereas nrv2 mutants are not different from wild type (P = 0.69). nrv2 mutants do not show significantly increased tube length when compared with wild-type embryos until 14 h. Error bars, s.d. P values are from Student t-tests. For hour 14 (stage 16), n = 5 for each genotype. For all other times, n = 3 for each genotype. Scale bars, 10 μm.

Specifically, these results indicate that the kinase activity of Src42 is required autonomously in trachea for dorsal-trunk elongation. Overexpression of Src42WT in the tracheal system resulted in severe dorsal-trunk fusion defects that precluded analysis of dorsal-trunk length (data not shown). However, overexpressing Src42WT in a Src42
heterozygote did not disrupt fusion and increased tracheal dorsal-trunk length by about 8% (Fig. 1p,r). Combinations of loss-of-function Src42 alleles and overexpression constructs produced an allelic series in which the tracheal length ranged from about 27% too short to about 8% too long (Fig. 1r). Thus, Src42 acts instructively to regulate tube size.

Because the kinase activity of Src42 is required for regulating tracheal length, we determined the subcellular localization of activated (phosphorylated Tyr 400) Src42 (pSrc) in tracheal cells. As reported previously23, whereas endogenous Src42 localizes throughout the plasma membrane of all dorsal-trunk cells (Fig. 1k), activated Src42 largely co-localizes with DE-cadherin (DE-cad, also known as SHG) at the adherens junction (Fig. 2a–c, arrows) and partially co-localizes with CRB at the subapical membrane (Fig. 2d–f, arrowheads). Activated Src42 does not co-localize with septate junctions (Fig. 2g,h). Notably, a large fraction of phosphorytrosine (pTyr) immunostaining that normally localizes to the adherens junction and subapical membrane (Fig. 2m,n) is absent from Src42 mutants (Fig. 2o,p), whereas Src42 overexpression results in a marked increase in pTyr content (Fig. 2q,r). Thus, Src42 seems to be either directly or indirectly required for the majority of tyrosine phosphorylation in the tracheal system. Given the critical role pTyr is thought to play in junctional and cytoskeletal assembly and turnover23,26,27, Src42 mutations have unexpectedly mild effects on tracheal morphology.

The elongated-tracheal phenotype caused by Src42 overexpression is highly similar to that caused by polarity, septate-junction and aECM loss-of-function mutations (Fig. 2u and refs 5,6,11,12,14,16). To investigate whether Src42 acts in these pathways, we carried out genetic epistasis experiments using null alleles of Src42 and several polarity, septate-junction and aECM genes. Unfortunately, double-mutant combinations of Src42 and the polarity genes scrb, yrt and lgl resulted in severe defects in embryogenesis, making clear analysis of dorsal-trunk length difficult. However, the short-tracheal phenotype of Src42 was epistatic to all tested septate-junction and aECM mutations (Fig. 2s–v,aa, Supplementary Fig. S2m–p and Table S1a).
Importantly, Src42 suppressed overelongation without improving aECM or septate-junction organization (Fig. 2w–z and Supplementary Fig. S2a–p). Consistent with this, Src42 overexpression resulted in dorsal-trunk overelongation without significantly disrupting septate-junction integrity or aECM organization (Supplementary Fig. S2q–t). These results indicate that Src42 acts either downstream of the septate-junction/aECM tube-size-control pathways or independently in a parallel pathway.

To distinguish between ‘downstream’ and ‘in parallel’, we assessed Src42 activity in septate-junction, aECM and polarity mutants. If Src42 acts downstream of the septate-junction/aECM or polarity pathways, loss-of-function mutations in septate-junction, aECM or polarity genes should increase Src42 activity. However, we were unable to detect any changes in the levels or localization of pSrc, pTyr or total Src42 in any tube-expansion mutants (Fig. 2b–e and Supplementary Fig. S2u–bb), although elevated pSrc was readily detected when Src42 was overexpressed in the tracheal system (Fig. 2k,l). Moreover, overexpression of Src42 in a nrv2 null background (an essential septate-junction gene) resulted in a markedly enhanced tracheal phenotype consisting of loss of lumenal 2A12 and a cystic lumen (Fig. 2f–i), indicating that Src42 and nrv2 do not function in the same linear pathway. Consistent with this, Src42 and nrv2 act at separable developmental times. Src42 is required to initiate elongation between hours 10 and 12 of development, whereas nrv2 is required to restrict tube length after hour 12 (Fig. 2j).

We also asked whether Src42 acts in the canonical PCP pathway to control tracheal length. However, in contrast to Src42, mutations in the PCP genes dsh, fz and sano cause elongated trachea (Supplementary Fig. S3m and ref. 7), and the short phenotype of Src42 was completely epistatic to all tested PCP mutants (Supplementary Table S1a). Further,
Figure 5  dDaam functions with Src42 to control tube size. (a–c) dDaam co-localizes with pSrc at the subapical membrane of dorsal-trunk cells (arrows, b, c). However, dDaam is not expressed in fusion cells (asterisks). (d) Src42 co-immunoprecipitates with dDaam from embryo lysates. The blot is representative of three independent experiments (the entire blot is shown in Supplementary Fig. S2g). Anti-GFP (green fluorescent protein) antibody was used as a negative control. IP, immunoprecipitate; WB, western blot. (e–j) dDaam is enriched in apical circumferential rings (arrows in e and h) that run orthogonally to the length of the tube (e). In Src42 mutants, dDaam still localizes to circumferential rings, but the rings are coarser and more broadly spaced (h). Images in e and h are superficial sections of the dorsal trunk. Localization of Src42 is not affected in early stage 17 dDaam mutants (f,j). Nonetheless, early stage 17 dDaam mutants show a reduction in the levels of pSrc (g,j). Note that pSrc is not affected in fusion cells, where dDaam is not expressed (asterisks, j).

sano and dsh mutants did not show any changes in the levels of activated Src42 (Supplementary Fig. S2a–x and data not shown) and Src42 mutations did not alter the planar polarization of wing hairs, thorax bristles or denticile belts (Supplementary Fig. S3a–l). Specific expression of a dominant negative Src42 construct in the thorax also did not affect planar polarization (Supplementary Fig. S3h) and caused only very subtle effects when expressed in a specific wing region (Supplementary Fig. S3d). Finally, whereas DSH1 and other PCP gene products have a planar-polarized localization in other epithelia such as the epidermis, their distribution seems uniform in the trachea (Supplementary Fig. S3q–t and ref. 7). Thus, Src42 acts either downstream or in parallel to PCP genes to control tube size. Together, these data indicate that Src42 acts in a pathway separate from known genes that control tracheal-tube size.

To understand the cellular functions of Src42 in tube-size control, we investigated the morphology of wild-type and Src42 dorsal-trunk cells. We reasoned that short trachea could result either from a general failure to expand apical surface or from a failure to direct surface expansion anisotropically along the length of the tube, which would result in shorter but fatter tubes that were inappropriately expanded along the circumferential axis (Fig. 3a). To distinguish between these models, we measured both the length and diameter of wild-type and Src42 metamere 8 lumens. Interestingly, whereas Src42 lumens were about 37% shorter than wild-type controls (Fig. 3b–d), they were also about 11% larger in diameter (Fig. 3e). We calculated that the total ASA of Src42 metameres is reduced by about 30% (Fig. 3f and Methods), indicating that Src42 is required for increasing apical surface. However, the increased lumen diameter of Src42 mutants indicates that Src42 is also required for directing available surface expansion along the axial dimension.

To quantify these results at the single-cell level, we determined ASA, axial length, aspect ratio and cell orientation using three-dimensional reconstructions of individual wild-type and Src42 tracheal cells (Fig. 3g–i and Methods). As observed for the whole metamere, the median ASA and axial length of individual Src42 cells were smaller than those of wild-type cells (Fig. 3j,k). Interestingly, despite the smaller size of Src42 cells, the median aspect ratios of wild-type and Src42 cells were not significantly different (Fig. 3l). However, the median angle of the longest cell dimension relative to the longitudinal tube axis was closer to 90° in Src42 than in wild-type dorsal-trunk cells (Fig. 3m).

Together, these data confirm that Src42 is required for controlling the amount of apical surface expansion, and for directing available expansion anisotropically along the longitudinal axis of the tube. These results establish that epithelial tube size can be controlled by regulated anisotropic growth of the apical surface (Fig. 3a, model 2).

To define the molecular mechanisms by which Src42 acts, we tested loss-of-function mutations in candidate Src42 interactors for a short-tracheal phenotype (Supplementary Table S1b). We found that dDaam (also known as DAAAM, Dishevelled associated activator of morphogenesis), a conserved Diaphanus-related formin that has been shown to bind vertebrate Src (ref. 28), showed a mild, but significant, short-tracheal phenotype near the end of embryogenesis (stage 16; Figs 4m, S1i). Shortening was more apparent after hatching (Fig. 4a–c), raising the possibility that the weaker embryonic phenotype was due to maternal dDaam (refs 29,30; failure of dDaam maternal/zygotic

Stage 17 dDaam embryos were imaged to account for maternal dDaam. (k) Quantification of apical pSrc and Src42 levels in wild-type (WT) and dDaam early stage 17 dorsal-trunk cells shows that the levels of activated Src42 are reduced in dDaam mutants. (l) Measurement of dDaam and Src42 mutant dorsal trunks shows that both are shorter than wild type. dDaam; Src42 double mutants do not show a more severe elongation defect than Src42 single mutants. For k and l, error bars represent s.d., P values are from Student’s t-test and n = 5 for all samples. (m) A model for the interactions of aECM and Src42/dDaam in controlling lumen size. Src42 is required for apical surface growth and both Src42 and dDaam direct growth along the longitudinal axis. aECM restricts tube length. Note that Src and dDaam are shown at the adherens junction (AJ), but the locations of the pools of Src and dDaam that control the amount and direction of growth are not known. Scale bars, 10 μm for a–f, i–j; 5 μm for e, h.

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embryos to cellularize precludes a direct test of this possibility). Similarly to Src42, dDaam acts autonomously in the tracheal system, because tracheal expression of Flag-tagged dDaam fully rescued the shortened dorsal trunk of dDaam mutants (Fig. 4c,f). Remarkably, despite only causing modest reductions in dorsal-trunk length at embryonic stage 16, zygotic dDaam mutations were still able to completely suppress the overelongation of septate-junction, aECM, apico-basal and PCP mutants (Fig. 4g–l and Supplementary Table S1a). Further, whereas Src42; scrib mutants had gross embryonic defects, dDaam; scrib mutants underwent largely normal development and had trachea with the dDaam short-tracheal phenotype (Fig. 4n,o and Supplementary Table S1a). Thus, dDaam seems to act downstream of or in parallel to all characterized control pathways for tracheal–tube size.

Using quantitative imaging, we determined that, as in Src42 mutants, the apical surfaces of dDaam tracheal cells were misoriented, overexpanding in the circumferential direction to produce lumens that were shorter but larger in diameter (Fig. 4p–r,t–y). However, unlike Src42 mutants, the apical surface of metamere 8 and the median apical surface of individual tracheal cells were not markedly altered in dDaam mutants (Fig. 4s,v). Thus, dDaam is required to orient apical surface growth, but either it is not required to control the amount of expansion or there is sufficient maternal dDaam to enable normal apical surface expansion.

The similar tracheal phenotypes of Src42 and dDaam mutants indicated that they may act in the same pathway to control anisotropic surface growth. Consistent with this possibility, the short-tracheal phenotype of the dDaam; Src42 double mutant was not enhanced when compared with that of the Src42 single mutant, which is shorter than that of the dDaam mutant (Fig. 5l). In addition, dDaam co-localizes with pSrc at the adherens junction and subapical membrane of dorsal-trunk cells (arrows in Fig. 5a–c) and Src42 co-immunoprecipitates with dDaam from embryo lysates (Fig. 5d). Further, loss of dDaam decreased apical pSrc levels in dorsal-trunk cells (Fig. 5g,i,j,k), indicating that dDaam acts upstream of Src42 in regulating tube size. This is consistent with work in mammalian systems showing that DIA-related formins activate Src by binding to the auto-inhibitory SH3 domain28,31,32. Interestingly, though there were no obvious changes in the localization or levels of Src42 in dDaam mutants (Fig. 5i,j,k), the apical circumferential rings of dDaam were coarser and more broadly spaced in Src42 mutant trachea (Fig. 5e,h), further demonstrating functional interactions of these proteins.

Importantly, dDaam mutations do not completely eliminate pSrc staining. This result indicates that dDaam does not regulate all pools of Src42 in tracheal cells, which could explain why Src42 mutants cause defects in both surface growth and orientation of growth, whereas dDaam mutations affect only orientation of growth. Consistent with this possibility, overexpression of Src42 in dDaam trachea caused marked excess growth in the circumferential direction, markedly increasing tube diameter without increasing length (Supplementary Fig. S4). Together, these results support a model in which Src42 regulates surface expansion in a process that does not require dDaam, and dDaam and Src42 act together in a complex that directs expansion along the longitudinal axis of the tube (Fig. 5m).

Given that Src42 and dDaam control directionality of cell growth in what is essentially a planar epithelium, we further investigated whether Src42 and dDaam act in the canonical PCP pathway. Although Src42 and dDaam do not act in PCP pathways outside the trachea (Supplementary Fig S3a–l), PCP genes could act upstream of Src42 and dDaam to control anisotropic growth in tracheal cells. As a diagnostic feature of aberrant anisotropic growth is an increase in tube diameter when tube length is decreased, we determined tracheal diameter in embryos mutant for fi, a PCP gene whose loss causes short trachea. Tube diameter was unaltered (Supplementary Fig. S3o,p), indicating that canonical PCP genes act distinctly from Src42 and dDaam.

Surprisingly, loss of Src42 or dDaam did not cause isotropic (equal in all dimensions) growth of apical surface. Instead, apical surface growth was markedly reoriented along the circumferential tube axis (Figs 3l,m; 4x,y). Thus, Src42 and dDaam are required for orienting anisotropic growth rather than being essential for anisotropic growth per se. Interestingly, homologues of both Src42 and dDaam have been shown to be required for mitotic-spindle orientation13,34, suggesting that Src and DAAM may have more broadly conserved roles in cellular anisotropy than have been previously suspected.

Our results reveal that Drosophila trachea use a mechanism of epithelial tubelike-size control in which anisotropic growth of the apical surface drives increases in tube length or diameter. To our knowledge, this mechanism has not previously been described. Given that mammalian Src-family kinases have been implicated in control of tube size in the mammalian kidney19 and vascular system18, and the general conservation of basic cellular processes, these results have important implications for control of tube size in other systems.

METHODS

Methods and any associated references are available in the online version of the paper at www.nature.com/naturecellbiology

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AUTHOR CONTRIBUTIONS

K.S.N. carried out all experiments, Z.K. generated and analysed individual dorsal-trunk cell quantifications, I.M. generated the UAS–Flag–dDaam construct and the dDaam antibody, K.S.N., Z.K., M.K. and G.J.B. designed and interpreted the experiments and K.S.N., Z.K., J.M. M.K. and G.J.B. wrote the paper.

COMPETING FINANCIAL INTERESTS

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METHODS

Fly stocks and genetics. A complete list of the loss-of-function alleles used in this study is in Supplementary Table S1a and b and they are described in FlyBase (www.flybase.org). For all dorsal-trunk measurements, either wild-type or w1118, btl > GFP animals served as wild-type controls as specified. The specific alleles used for images are as follows: Src42Δm, Src64Δm, dDaam118, Actin5C::GFP, and Atsp42. The upstream activating sequence (UAS)–Src42Δm and UAS–Src64 lines are described in ref. 22. The UAS–Src42Δm transgene contains a K276M mutation that abolishes catalytic activity as described in ref. 23. The UAS–Flag–dDaam is derived from the RE67944 complementary DNA clone. The btl-Gal4 driver33 was used for all tracheal-specific expression. The FM7 dfl–YFP (yellow fluorescent protein), FM7 act-GFP, CyO dfl–YFP, CyO act–Z, TM6 dfl–YFP and TM6 act–Z balancers were used for genotyping.

Drosophila immunohistochemistry and dextran dye injections. Immunohistochemistry and dextran dye injections were carried out as described previously41,42. Images are from stage 16 embryos unless otherwise indicated. The following antibodies were used: rabbit anti-GFP 1:1,000 (Invitrogen, A11122), mouse anti-GFP 1:1,000 (Invitrogen, A11120), mouse anti-Atp42 1:300 (ref. 11), mouse anti-Src42 1:1,000 (ref. 25), rabbit anti-Src64 1:500 (ref. 35), rabbit anti-pSrc1:1,000 (Invitrogen 44660G), mouse anti-pSrc1:250 (Assay Designs 905-669), rabbit anti-pTyr 1:500 (Invitrogen 61-5800), rabbit anti-dDaam 1:1,000 (ref. 29), rat anti-DE-cad 1:120 (DSHB, DCAD2), guinea pig anti-Cora 1:10,000 (ref. 36), rabbit anti-Verm 1:300 (ref. 11), guinea pig anti-Verm 1:500 (ref. 12), rabbit anti-Serp 1:300 (ref. 11), mouse anti-Aiptet 1:20 (DSHB, a5), mouse anti-Flag 1:1,000 (Sigma, F1804-1MG) and mouse anti-CRB1 1:20 (DSHB, Cq4). Alexa Fluor 488, 546 and 647 (Invitrogen) were used for secondary antibodies.

Whole dorsal-trunk image acquisition and analysis. Confocal sections (embryos) and differential interference contrast images (larva) were used to measure the length of the embryonic dorsal trunk as described previously43. The length of metamere 8 was assessed from the anterior fusion point to the posterior fusion point. The diameter of metamere 8 was measured at three consistent points: just posterior to the dorsal branch and 3 μm in from each fusion point. Metamere 8 measurements were not normalized because it is not clear that diameter correlates with embryo length. As the metamere 8 lumen closely approximates a cylinder for wild-type, Src42 and dDaam animals, ASA for metamere 8 was calculated using the formula ASA = π × length × diameter.

To assess pSrc intensity, the average fluorescence intensity of apical pSrc in dorsal-trunk cells was divided by the pSrc levels in the corresponding fusion cells. This provided an internal control because dDaam is not expressed in fusion cells43. Apical Src42 intensity was also measured and normalized to the levels of Src42 in fusion cells. Average fluorescence intensity was determined from a region of interest along the apical surface of wild-type and dDaam sections using ImageJ (ref. 37).

Single-cell image acquisition and analysis. For quantitative analysis of individual metamere 8 dorsal-trunk cells, we used confocal sections of stage 17 fixed whole-mount embryos that express cytoplasmic GFP through the btl-Gal4 driver. For all samples, embryos were labelled for GFP and Cora, which delineate cell boundaries. The cytoplasmic fraction of each cell was collected using an image voxel size of 0.092 μm × 0.092 μm × 0.162 μm. Initially, we scaled image volumes to a 1:1:1 aspect ratio (0.162 μm × 0.162 μm × 0.162 μm). The resulting volume was median filtered using a spherical filtering component of foreground regions were found, and the connected components were individually dilated a further ∼0.7 μm.

Each connected component was triangulated to obtain a surface triangle mesh. The triangulation was obtained using the Marching Cubes algorithm34. We selected the three-dimensional surface mesh corresponding to the tube manually. The eigenvector corresponding to the largest eigenvalue of the covariance matrix of the vertices in the tube triangle mesh was designated the axial direction vector.

Each tube-surface-mesh triangle was intersected with Cora-labelled voxels and assigned the average intensity of intersected voxels. This operation created a two-dimensional image on the surface of the mesh from which a difference of Gaussian image was computed. Values in this image were thresholded to obtain a binary image on the surface mesh where foreground triangles outlined cell–cell junctions. The binary image was dilated and then inverted to obtain central markers for a segmentation using the marker-assisted Watershed Transform on the original surface intensity image44. Apical surface meshes were obtained from individual segmented regions. Any meshes outside fusion cells and incorrectly segmented meshes were manually removed.

Surface-area measurements were obtained by adding together the surface areas of the triangles of apical surface meshes. The eigenvectors corresponding to the largest and second largest eigenvalues of the covariance matrix computed from vertices of the apical surface mesh were used to designate the long and short directions of the mesh respectively. The axial direction vector was projected on the plane defined by these two vectors to obtain a local, projected tube direction. We computed the angle relative to the long apical surface dimension and the projected tube direction.

Next, we computed a centroid of the surface mesh using the vertices of each mesh triangle. We designated the centroid of the triangle closest to the mesh centroid as the centre of the apical surface mesh. The length of a of the vector from the surface mesh centre to a vertex on the edge of the surface oriented closest to the short direction was used to approximate the length of the short apical surface dimension. The long apical surface dimension b was estimated using the surface area A and an ellipse approximation of the surface mesh shape b = A/(πa) enabling computation of an aspect ratio b/a corrected for the curvature of the surface. Last, the lengths of the vectors between the surface-mesh centre and an edge vertex oriented in the same and the opposite direction as the projected tube direction were used to approximate the axial length.

The median ASA for individual dorsal-trunk cells matched closely with the values expected by dividing the average whole-metamere ASA by the average cell number (wild type, 28.2 μm2 versus 24.8 μm2; Src42, 17.1 μm2 versus 17.3 μm2), demonstrating consistent values from two independent approaches.

Immunoprecipitation. 100 μl of 12–18 h wild-type embryos were lysed in 1 ml of solution containing PBS at pH 7.6, 1% Triton X-100, and mini protease inhibitor tablets (Roche, 04693124001). Lysates were centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was divided in two and added to 30 μl of Protein A beads containing either rabbit anti-GFP (Invitrogen, A11122) or rabbit anti-dDaam antibodies45. Lysates and beads were rotated at 4 °C for 8–12 h and washed four times in lysis buffer. Bound proteins were eluted by the addition of ×1 loading buffer. Src42 was assayed using chick anti-Src42 1:5,000 (ref. 25) in a standard western blot.

Statistics. For whole Drosophila dorsal-trunk measurements, two-tailed Student t-tests assuming unequal variance were used. For individual Drosophila dorsal-trunk cell measurements, two-tailed Mann–Whitney tests were used, which do not assume normal distributions and are less likely to indicate significance owing to the presence of outliers. P < 0.05 was considered statistically significant for all analyses.

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Figure S1. Src64 does not regulate tracheal tube size. (a, b) Src64 maternal/zygotic (M/Z) null mutations do not show obvious tracheal phenotypes. (c, d) However, forced expression of Src64 in the tracheal system using UAS-Src64 and the btl-gal4 driver can cause over-elongation of tracheal tubes in an otherwise WT background (c), and can rescue the short DT of Src42 mutants (d). (e-i) Src64 is not detected in the tracheal system (e, f, i, j). Src64 can be found in the midgut, demonstrating the specificity of the anti-Src64 antibody (g, h, k, l). Note that embryonic hindguts lack SJs and thus show diffuse Na,K-ATPase staining (magenta in k,l). (m-p) Src64 mutants cannot suppress the DT over-elongation or other defects in the aECM/SJ pathway mutant, Atpα. Dashed white lines indicate the tracheal apical surface. (q, r) Quantification of DT length reveals that Src64 mutant DTs are not different than WT DTs (q) and that Src64 cannot suppress the over-elongation of Atpα trachea (r). For q and r, error bars represent standard deviation, p values are from Student’s t-tests. For q, n = 5 for all samples. For r, n = 4 for all samples. Scale bars: 20µm for a-d; 10µm for e-p.
**Figure S2** Src42 controls tube size independent of the aECM pathway. 

(a, b) Lumenal secretion of Serpentine (Serp) is not affected in Src42 mutants. 

(c, d) Src42 suppresses the nrv2 tube elongation defect without restoring Serp secretion. Note the absence of Serp staining and the diffuse Cor localization. 

(e, f) Lumenal chitin organization is normal in Src42 mutants, with a clear gap between the apical surface (Crb) and the lumenal chitin fibrils (arrow, inset). 

(g, h) In aECM/SJ mutants like nrv2, the chitin is more diffuse and the gap is missing (inset, g). 

(i, j) The SJ barrier function is normal in Src42 mutants, as assessed by the exclusion of a 10kDa fluorescent dye injected into the body cavity from the tracheal lumen. 

(k, l) Dye readily leaks into the SJ mutant nrv2 (k). Src42 suppresses tube elongation without restoring SJ function (l). 

(m-p) Src42 mutations also suppress the DT over-elongation of the aECM mutant verm without restoring aECM organization. 

(q-t) Over-expression of Src42 does not dramatically alter the lumenal accumulation of Verm (q) or Serp (r), the organization of lumenal chitin (s), or the SJ barrier function (t), indicating that Src42-dependent tube expansion acts independent of the aECM/SJ pathway. 

(u-x) The levels and localization of activated Src42 (pSrc) are not different from WT in sano mutants. 

(y-bb) verm mutants do not show an obvious change in the levels or localization of pSrc (y, z), Src42 (aa), or pTyr (bb). See Fig. 2 for WT comparisons. Scale bars: 10μm for a-bb.
Figure S3 Src42 and dDaam do not act in canonical PCP pathways to regulate tube dimensions. (a-d) In WT animals, wing hairs are all oriented such that they point toward the distal end of the wing (a, a'). Mutations in the PCP gene dsh cause characteristic swirls in wing hair orientation (b, b'). In contrast, homozygous Src42JP45 adult escapers do not show defects in hair PCP (c, c'). Similarly, expression of the dominant negative Src42KM along the anterior-posterior border of the wing (asterisk in d) with the ptc-Gal4 driver does not dramatically disrupt wing hair orientation (d'). In some ptc>Src42KM wings, very subtle defects in orientation were present (arrow in d'), though the hairs still pointed towards the distal end of the wing. The subtle effects are likely non-specific as expression of Src42KM in the entire wing using ms1096-Gal4 resulted in extensive growth and morphogenesis defects (data not shown). (e-h) Though disrupted in PCP mutants (f), thorax bristle orientation was normal in Src42JP45 adults (g) and in ms1096>Src42KM adults (h). The ms1096-Gal4 line drives expression in the thorax and the entire wing. (i-l) In WT animals, an enrichment of F-actin localizes to the posterior edge of cell in the denticle belts of embryos (i, white arrows). Src42 and dDaam null alleles do not disrupt the orientation of the F-actin (j, k). In contrast, loss of the core PCP gene Van Gough (Vang) causes some F-actin accumulations to localize to the anterior edge of epidermal cells (l, yellow arrows). For i-l, all images are ventral views with anterior to the left. (m, n) The DT of dsh1 embryos is not short like Src42 and dDaam mutants, but rather slightly convoluted (a). Over-expression of Dsh in the tracheal system did not alter tube dimensions (n). (o, p) Quantitative measurement of embryonic stage 17 metamere 8 lumens indicates that only ft mutants show a significant change in length from WT (asterisk in c, p < 0.05). Neither dsh nor ft mutants show any changes in tube diameter, indicating that canonical PCP components do not regulate anisotropic surface orientation. Error bars represent standard deviation. p values are from Student’s t-tests. For all samples, n = 5. (q-t) In epidermal cells of stage 16 embryos, GFP-tagged Dsh localizes to the lateral cell junctions (arrows indicate the absence of Dsh-GFP on junctions along the A-P axis, q and r). In contrast, Dsh-GFP is uniformly expressed throughout DT cells in the tracheal system and does not show a planar polarized distribution (g and h). Scale bars: 200µm for a-h; 25µm for a’-d’; 5µm for i-l; 20µm for m, n; 10µm for q-t.
Figure S4 Src42 can increase apical surface expansion in dDaam mutants, but cannot correct orientation of growth. (a-d) As compared to WT (a), dDaam mutants have shorter but wider tubes, indicating that growth of the apical surface is misoriented (b). Over-expression of Src42 increases apical surface, resulting in longer tracheal tubes (c). When Src42 is over-expressed in dDaam mutants, apical surface still expands, but only in the circumferential axis (d). For a-d, images are of metamere 8 and the dashed line highlights the length of the metamere. Arrowheads in (a) point to the transverse connectives. (e, f) Quantitative measurements of metamere 8 reveal the length of the DT is the same in dDaam embryos and dDaam embryos over-expressing Src42 (e). However, diameter increases dramatically (f). All measurements are from stage 16 embryos. Error bars represent standard deviation. p values are from Student’s t-tests. For all samples, n = 5. (g) Uncropped image of the immunoprecipitation gel from Figure 5d. Black arrowhead indicates IgG band and red arrowhead indicates Src42 band. Scale bar: 5 µm for a-d.
Table S1 Tracheal phenotypes of examined mutants. a. Tracheal length, Verm secretion, and aECM organization for all loss-of-function single and double mutants. All phenotypes are from stage 16 embryos, except for the trachea length of the indicated dDaam alleles, which are from early first instar larvae (L1).

*Qualitative assessment of trachea length. --, very short; -, short; 0, wildtype; +, slightly long; ++, long; +++, very long. Numbers in parentheses indicate the length of measured trachea, (average ± standard deviation, n ≥ 5.) as a percent of WT trachea length.

†Tracheal length percentages shown are measured from transverse connectives 5 to 10.

‡Double mutant combinations between Src42 and polarity mutations resulted in head involution defects and dorsal closure defects, which produce shortened bodies and convoluted trachea. Due to these defects, proper staging of the embryos and correct normalization of trachea length are impossible.

ND, not determined.

b. Tracheal phenotypes of embryos mutants for candidate Src-interacting genes.

z, test zygotic for short trachea; e, epistasis test using SJ or aECM mutant; m, test m/z for short trachea; s, test for changes in localization or levels of gene product in Src42 mutants; DT, tracheal Dorsal Trunk.