Serrano (Sano) Functions with the Planar Cell Polarity Genes to Control Tracheal Tube Length

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Abstract

Epithelial tubes are the functional units of many organs, and proper tube geometry is crucial for organ function. Here, we characterize serrano (sano), a novel cytoplasmic protein that is apically enriched in several tube-forming epithelia in Drosophila, including the tracheal system. Loss of sano results in elongated tracheae, whereas Sano overexpression causes shortened tracheae with reduced apical boundaries. Sano overexpression during larval and pupal stages causes planar cell polarity (PCP) defects in several adult tissues. In Sano-overexpressing pupal wing cells, core PCP proteins are mislocalized and prehairs are misoriented; sano loss or overexpression in the eye disrupts ommatidial polarity and rotation. Importantly, Sano binds the PCP regulator Dishevelled (Dsh), and loss or ectopic expression of many known PCP proteins in the trachea gives rise to similar defects observed with loss or gain of sano, revealing a previously unrecognized role for PCP pathway components in tube size control.

Introduction

Multicellular animals employ tubular structures in organs to transport vital fluids and gases that sustain life. Examples of organs with prominent tubular architecture include the circulatory system, the lung and kidney in mammals, the secretory and respiratory organs in flies, and the excretory organ in worms. Proper development of tubular networks is critical for the function of several organs, evidenced by disruption of these networks being an underlying cause of common human diseases including cardiovascular disease, poly cyclic kidney diseases, and asthma.

The Drosophila trachea is a branched network of tubular epithelia that transports oxygen and other gases throughout tissues. The comparative simplicity and genetic tractability of this system has made it one of the most powerful model systems to dissect tubular epithelial morphogenesis. Tracheal formation begins as tracheal placodes invaginate from the epidermis during early embryogenesis. Through stereotypic cell migrations, cell shape changes, and rearrangements of cell-cell junctions, tracheal cells generate a tubular network that extends branches to all embryonic tissues [1–4].

Each tracheal branch assumes a specific size as a consequence of branch-specific signaling events [5–10]. Tube size control is mediated by changes in cell shape, cell arrangement, and possibly cell size, but does not involve changes in cell number [11]. One category of genes that affect tube size encodes components of septate junctions, as mutations cause overelongated trachea [12–17]. Defects in apical extracellular matrix (ECM) proteins - which modify the structure of the chitin matrix - also lead to overelongated trachea, indicating that a dynamic and highly patterned apical extracellular matrix (ECM) regulates epithelial cell shape and tube size [18–22].

In epithelia, cells are polarized along the apical/basal axis. In epithelial tubes, the apical surface of each cell faces the lumen, whereas the basal surface faces surrounding tissues and/or a basement membrane. In addition to apical/basal polarity, epithelial cells in most tissues require information about their orientation within the plane, orthogonal to the axis of apical/basal polarity, in order to generate polarized structures such as cilia, or to move or orient themselves in a directed fashion. This type of polarization is referred to as planar cell polarity (PCP). In vertebrates, PCP is involved in diverse patterning events, including convergence extension during gastrulation, neural tube closure, inner ear sensory hair morphogenesis, and hair follicle orientation [23]. In Drosophila, PCP biases cell orientation in several adult epithelial tissues and has been implicated in ovarian border cell migration [24–27]. In many contexts, both in vertebrates and in Drosophila, a conserved PCP pathway – the Frizzled (Fz) pathway - mediates local cell-cell interactions that instruct neighboring cells to adopt appropriate polarity [24–27].

In Drosophila, loss or overexpression of PCP proteins causes disorganization of wing hairs and bristles on the thorax and/or alteration in the orientation of ommatidia in the compound eye. Analysis of such phenotypes revealed an evolutionarily conserved set of genes that control planar polarity – the “core” PCP factors. These factors include: Fz, a seven-pass transmembrane receptor [28]; Dishevelled (Dsh), an adaptor protein that acts downstream of Fz [29–31]; Flamingo/Starry Night (Fmi/Stn), a cadherin-family member with a seven-pass transmembrane domain [32,33];
Author Summary

Tubular organ formation is a ubiquitous process required to sustain life in multicellular organisms. In this study, we focused on the tracheal system of the fruit fly, *Drosophila melanogaster*, and identified Serrano (Sano) as a novel protein expressed in several embryonic tubular organs, including trachea. *sano* loss results in over-elongated trachea, whereas Sano overexpression causes shortened trachea, suggesting that *sano* is required for proper tracheal tube length. Interestingly, Sano overexpression results in typical planar cell polarity (PCP) defects in many adult tissues and pupal wing cells. The PCP pathway is highly conserved from flies to mammals and it has been known to control cell polarity within the plane of epithelial tissues. Importantly, we found that Sano binds Dishevelled (Dsh), a key PCP regulator, and loss or ectopic expression of many known PCP proteins in the trachea give rise to similar defects observed with loss or gain of *sano*, suggesting a new role for the PCP genes in tube length control. Interestingly, the changes in tube length and PCP defects in the wing were linked to changes in apical domain size, suggesting that Sano and the PCP components affect either membrane recycling and/or the linkage of the membrane to the cytoskeleton.

Results

**sano** Encodes a Cytoplasmic Protein That Is Apically Enriched

An enhancer trap screen for lines with expression in the developing salivary gland and trachea identified *rp395*, a P-element insertion that expresses β-gal throughout the salivary gland, in trachea, and in several other embryonic tissues, including the hindgut, midgut endoderm, CNS midline, posterior spiracles, and epidermis (Figure 1A–1D). Cloning and characterization of the flanking region revealed that the *rp395* P element had inserted after nucleotide 14 of the *RC* and *RD* transcripts of *serrano* (sano; CG12739), two of five alternatively spliced transcripts, designated RA-RE (Figure 1M). Northern analysis revealed a single size transcript of 4.6 kb, first detected in 4–8-hour embryos and reaching peak levels in 8–12-hour embryos (Figure S1). The transcript was detected at all subsequent developmental stages, but was not detected in RNA isolated from cultured *Drosophila* Schneider (S2) cells. With minor exceptions, the endogenous *sano* transcripts recapitulate the pattern of *rp395* β-gal expression (Figure 1E–1H).

*Sano* expression requires the transcription factors Sex combs reduced (*Scr*) in the salivary gland, Trachealless (Trh) in the trachea, and Single-minded (*Sim*) in the CNS midline (Figure 1I–1L); *sano* expression was not affected by loss of transcription factors including fork head, huckebein, or *CehA* that are expressed early in salivary gland formation (data not shown). Early transient tracheal expression of *sano* was observed in *trh* mutant cells also deficient for programmed cell death (*Df(3L)H99*), suggesting that initial tracheal expression is in part *trh*-independent and complete loss of *sano* expression in *trh* mutants is due to tracheal cell death (Figure S2). Since other known regulators of tracheal development, including *ventral veinless*/*drifter*, *trachea defective/aponics*, *breathless*, *branchless*, and *rhomboid*, did not affect *sano* expression (data not shown), initial *sano* expression could be regulated by factors that initiate *trh* expression. Since Trh and Sim bind the same consensus DNA sequence [53,54], regulation of *sano* expression by these proteins could be direct.

All predicted *sano* splice forms encode the same 778-residue ORF (Figure 1M). *Sano* is highly conserved in arthropods (Figure 2A), and is a member of a largely uncharacterized family of proteins with members from cnidarians to mammals that includes the recently identified Themis protein (also known as Gasp). Themis/Gasp is a cytosolic thymocyte-adaptor protein that binds Grb2 and is required for positive selection of thymocytes [55–60]. Because we were unable to generate antisera that detected endogenous *Sano*, we cloned and expressed both untagged and C-terminally tagged (GFP or HA) *Sano* in flies under Gal4/UAS control [61]. In both tracheal and salivary gland cells, each version of overexpressed *Sano* localized diffusely in the cytoplasm, with enrichment at apical membranes, colocalizing with the apical membrane markers Crumbs (Crb) and Stranded at Second (SAS) (Figure 2B–2D; [62–64]). During late embryogenesis and in the third instar larval salivary gland, however, *Sano*-GFP also localized to nuclei (Figure 3F, 3P′, and 3G and data not shown). Neither untagged (detected with Sano antiserum) nor HA-tagged *Sano* could be detected in nuclei at any stage. Taken altogether, these experiments suggest that *Sano* is an apically enriched cytoplasmic protein that may also sometimes localize to nuclei, a localization similar to that reported for the mammalian Themis/Gasp protein [55–60].

*sano* Affects Tracheal Tube Length

Three independent loss-of-function knock-out *sano* alleles, *sano*KO1, *sano*KO2, and *sano*KO3, were generated by homologous recombination.
PCR analysis confirmed that exons common to all five splice forms were replaced with the mini-white gene (Figure S3A and S3B). sano mRNA was not detected in sano homozygotes or in embryos transheterozygous for each sano allele over a deficiency that removes sano and nearby genes, indicating that the sano alleles are null (Figure 1N and 1O; Figure S3C, S3D, S3E, S3F, S3G, and S3H; data not shown). Each sano allele is homozygous lethal, and lethal over the sano deficiency, with death occurring during the 2nd instar larval stage. The sano lethality was partially rescued by expression of the Sano ORF under the control of a heat-shock promoter (HS-Sano) induced during larval stages (11/45 viable adults when heat shocked at 58–70 hr AEL).

Most features of salivary gland and tracheal development appear normal in sano mutant embryos (data not shown). Interestingly, however, staining with 2A12, a marker of tracheal lumen after stage 13, revealed that the dorsal trunk (DT) in sano mutants is more elongated and convoluted than in wild type (WT; Figure 3A–3D). Measurements of DT lengths from confocal projections of 2A12 staining from lateral views of stage 16 embryos revealed that sano mutant DTs are significantly (~12%) longer than wild type (Figure 3E). Tracheal cell numbers in the dorsal trunk of sano mutants (14.7±0.6, N=5, metamere 4) were comparable to those of WT (15.2±0.4, N=5, metamere 4; p>0.5, t-test), indicating that the elongated DT phenotype is not due to increased cell numbers.

Conversely, Sano overexpression using btl-Gal4 caused shortened DTs with discontinuous staining with either 2A12 or SAS (Figure 3F and 3F'). The UAS-Sano-GFP distributions in tracheal cells revealed that cells connecting adjacent segments of the DT (fusion cells) contact each other basally, but that the tracheal lumens and apical membranes are discontinuous. Fusion cell markers including Dysfusion (Dys), a bHLH-PAS transcription factor [66], and Arf-like-3 (Arl3), a small GTPase [67], were normally expressed in the discontinuous region of the DTs, indicating that fusion cells are not transformed to another fate (Figure 3G; data not shown). No increase in apoptosis was detected in the Sano-overexpressing trachea (Figure S5), and tracheal cell numbers in the btl-Gal4:UAS-Sano trachea (16±0.7, N=5, metamere 4; p>0.1, t-test), indicating that the shortened DT phenotype is not due to reduced numbers of tracheal cells. At 25°C, 100% of btl-Gal4:UAS-Sano embryos showed apical disconnection of DTs in more than one metamere, whereas neither btl-Gal4 nor UAS-Sano alone had the shortened apical DT phenotype (Table 1). Sano overexpression also caused mismigration and/or failure of other tracheal branches to connect (data not shown).
Figure 2. Sano protein is highly conserved in arthropods and localizes to the cytoplasm and apical membrane domain. (A) Sequence alignment of Sano with that of D. virillis, A. gambiae, T. castaneum, N. vitripennis, and D. pulex. Cyan, completely conserved residues in all species; Blue, identical residues; Yellow, similar residues. (B) GFP-tagged Sano protein was overexpressed in the trachea using btl-Gal4. GFP signal (green) is observed in the cytoplasm and is enriched in the apical domain where Crb, an apical membrane marker, localizes (red). (C) α-Sano antibody (green) recognized overexpressed Sano protein in the cytoplasm with apical enrichment in the trachea. Red, SAS. (D) GFP-tagged Sano protein was overexpressed in the salivary gland using sage-Gal4. Sano-GFP (green) was observed in the cytoplasm and was enriched in the apical domain where Crb localizes (red). Scale bars: 20 μm. Embryos in (B–D) are st.14.
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Sano expression in the trachea of sano null mutants rescued the elongated DT phenotypes observed in sano mutants (Figure 3E) and alleviated the gain-of-function phenotype of shortened DTs (Table 1), suggesting that an optimal dose of Sano is critical for proper tube length and that tube length is inversely related to Sano levels. Sano overexpression also reduced salivary gland luminal length (88.7 ± 2.0 μm (WT) vs. 58.9 ± 5.4 μm (Sano-overexpressing glands), N = 5 for each genotype; p<0.01, t-test), suggesting that Sano has generalized effects on tube length (Figure 3H and 3I).

Tracheal tube size is controlled neither by the number nor the overall size of the individual cells [11]. Nonetheless, mutations in several genes have been discovered that, like loss of sano, lead to tracheal tube overelongation. Most of these known genes either regulate chitin synthesis or encode components of the septate

Figure 3. Sano affects tracheal tube length. (A–D) st.15 and st.16 WT (A, C) and sano mutant (B, D) embryos stained for 2A12. (E) Quantification of tracheal DT length in WT, sanoΔ5 mutant and rescued embryos by btl-Gal4-driven overexpression of Sano in sanoΔ5 mutant background. Error bars indicate standard deviation (SD) (*p<10^-3, t-test). (F, F') Overexpression of Sano results in shortened DTs (arrows) that fail to connect. Higher magnification of the boxed region is shown in F'. Green, sano-GFP; red, 2A12; blue, SAS. (G) A fusion marker is normally expressed in Sano-overexpressing trachea. Green, sano-GFP; blue, Tgo; red, Dys. (H, I) Sano overexpression causes shortened salivary gland lumen. Asterisks indicate salivary gland of WT (H) and sage-Gal4>UAS-Sano-GFP salivary gland (I). Scale bars: 50 μm in (A–D) and F, 10 μm for (F', G), 20 μm for (H, I). Embryos in (F–H) are st.16.

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junction, an invertebrate structure that has trans-epithelial barrier functions analogous to the vertebrate tight junction [16,18–22,68]. To test whether sano function is linked to either category of known genes affecting tube length, we analyzed luminal chitin using a fluorescent chitin binding protein (CBP) and a fluorescent chitin binding lectin (Wheat Germ Agglutinin; WGA) [21]. Both known genes affecting tube length, we analyzed luminal chitin polysaccharide deacetylase activity [12,18,22]. Verm staining in sano mutant trachea was indistinguishable from WT (Figure S4C, S4D). We conclude that tracheal length defects in sano mutants are not a consequence of detectable alterations in chitin biogenesis.

Septate junction proteins, including Coracle (Cor), Neurexin IV (NrxIV), and Fasciclin 3 (Fas3), localized normally to the basolateral domain of sano mutant tracheal cells, suggesting that septate junctions are intact (Figure S4A and S4B; data not shown). A 10 kDa dextran dye exclusion assay indicated that barrier function of sepaate junctions is intact in sano mutants (Figure S4G, S4H, and S4I). Thus, neither septate junction function nor chitin cable assembly is disrupted in sano mutants, suggesting another mechanism for the elongated tracheal phenotype.

**Sano as a PCP Regulator**

sano is dynamically expressed in larval imaginal discs, structures that give rise to much of the adult during metamorphosis (Figure S6). Overexpression of Sano using several imaginal disc-specific Gal4 drivers caused planar polarity defects. For example, in the wild-type adult thorax, bristles point posteriorly, whereas in Sano-overexpressing adult thoraces, the bristles displayed altered orientations (Figure 4A and 4B). In the WT wing, each cell produces a single distally-oriented, actin-rich protrusion (a trichome, a.k.a. a “hair”). All Sano-overexpressing wing cells exhibited swirling hair patterns (Figure 4C and 4D). Sano overexpression in the eye caused ommatidial polarity defects, including misoriented and symmetrical photoreceptor phenotypes, as well as abnormal photoreceptor numbers (Figure 4E and 4F; data not shown), with about 14.5% of the ommatidia showing defects (137/916, N = 5). Polarity defects observed with Sano overexpression are similar to those observed when PCP genes are mutated or overexpressed [33,36,37,43,69–73], suggesting that Sano perturbs PCP. Next we examined Sano-overexpressing pupal wing cells. Phalloidin staining of actin-rich prehairs at 32 hours after puparium formation (APF) revealed that hair formation is delayed in Sano-overexpressing cells (Figure 5A), as observed in dsh mutant clones or in ago pk double mutant clones [74]. Phalloidin staining of the slightly older pupal wings (at 33–34 hours APF) revealed Sano-overexpressing cells with prehairs the same size as surrounding wild-type hairs but with altered polarity (Figure 5B). Sano overexpression sometimes produced multiple wing hairs, another PCP phenotype (Figure 5B). Wild-type hairs near some Sano-overexpressing clones exhibited non-cell-autonomous polarity defects (Figure 5B, arrows; Figure S7C and S7D), distinct from those near fz or stbm/Vang mutant clones; wild-type cells proximal to fz clones or distal to stbm/Vang clones have reversed hair polarity [34,35,75], which was not observed with Sano overexpression. All Sano overexpression clones that produced nonautonomous phenotypes mapped either between veins 3 and 4, distal to the anterior crossvein, or between veins 4 and 5, distal to the posterior crossvein (Figure S7A; N > 100 clones examined), both regions of which are sensitive to PCP alteration [49].

Core PCP proteins are asymmetrically localized in pupal wing cells during prehair formation and show typical “zigzag” localization patterns on the apical surfaces of the pupal wings [33,36,37,39,40,42]. When a PCP gene is mutated or overexpressed, other PCP proteins are typically mislocalized. Sano overexpression in pupal wings through either ptc-Gal4-driven expression or in Sano-overexpressing clones resulted in the mislocalization of all PCP proteins examined. Fmi, normally localized to both the proximal and distal sides of wing cells during prehair formation, was observed around the entire perimeter (Figure 5C and 5D). A similar mislocalization was observed with Sano overexpression. Fmi, normally localized to the distal side of the apical surface, exhibited reduced apical membrane distribution with Sano overexpression (Figure 5E, Figure S8B).

sano loss-of-function mutant clones in cells giving rise to adult tissues such as thorax and wing did not result in PCP phenotypes (data not shown). Similarly, actin prehairs of sano mutant clones in pupal wing cells always pointed distally as in WT (Figure S9A and S9B). Since mutations of some PCP genes, such as fz, show polarity defects only in very large clones [46], we induced sano mutant clones at earlier time points to generate a range of sizes of clones missing sano function. Even very large clones did not exhibit PCP defects (data not shown). However, although it was rare, when we induced clones relatively early (36–48 hours after egg laying (AEL), we obtained only twin spots (<5%, N = ~70), suggesting that the sano mutant cells either died or were eliminated from the wing epithelium (Figure S9C and S9D). On the other hand, sano null eye clones had defects characteristic of loss of known PCP genes, including misoriented ommatidia and loss of asymmetry (Figure 4G; Table 2). sano null eye clones also often had abnormal numbers of photoreceptors (Figure 4G; Table 2). In 3rd instar eye discs, the expression of BarH1, a marker for the R1 and R6 photoreceptors [76], showed ommatidial misrotation, consistent with the adult phenotype (Figure 4H and 4I), and the expression of mbb.5-lacZ, a marker for the R4 photoreceptor, was absent or significantly reduced in sano null clones, consistent with a cell fate change of R4 to R3, which has been observed with some PCP mutants, including fz and dsh [Figure 4I and 4K; [77]]. Our data suggest that although sano overexpression disrupts PCP signaling in multiple tissues, loss of sano results in a range of defects that are limited to fewer tissues.

**PCP Mutants Have Elongated DTs**

To determine if sano affects tube length by altering PCP signaling, we asked if other PCP mutants have tracheal length

| Table 1. Overexpression of Sano in the trachea causes shortened DTs. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| stage  | shortened DTs (%) | normal DTs (%) | N |
|-------|------------------|----------------|---|
| btl-Gal4/UAS-Sano  | 15 100 0 127    | 16 98.4 1.6 63  | 16 0 100 146    | 15 0 100 121    | 16 0 100 162  |
| btl-Gal4/+  | 15 1.8 98.2 109  | 16 0 100 146  | 16 0 100 121  |
| UAS-Sano/+  | 15 0 100 121  | 16 0 100 162  |
| sanoKO3; btl-Gal4/UAS-Sano  | 15 60.8 39.2 130 | 16 71.1 28.9 83  |

*All crosses were done at 25°C. doi:10.1371/journal.pgen.1000746.t001
Figure 4. Overexpression of Sano causes PCP defects, and sano loss results in ommatidial defects. (A, B) Adult thorax of WT (A) and of MS1096-Gal4 > UAS-Sano (B). Sano overexpression causes misorientation of thoracic bristles. (C, D) Adult wing of WT (C) and of ptc-Gal4 > UAS-Sano (D). WT wing shows distal orientation of hairs, whereas overexpression of Sano causes a swirling hair pattern. (E-G) Adult ommatidia of WT (E), sev-Gal4 > UAS-Sano (F) near the dorsal/ventral boundary, the equator, and sano mutant clones in the dorsal compartment (G). sano mutant cells have w +/+ marker and they are distinguishable from the neighboring WT w−/− cells by the pigment around the cells. Schematic drawings are shown in the panels below the actual images with black and red shapes indicating the orientation of ommatidia normally found in the dorsal and ventral hemisphere of the eye, respectively, and blue shapes indicating a loss of ommatidial asymmetry. sano mutant cells are marked by the light green color in G and H. Green circles in H indicate ommatidia with abnormal photoreceptor number. (H, I) BarH1 expression in WT (H) and in sano mutant clones in the eye discs (I). BarH1 (blue), an R1 and R6 photoreceptor marker reveals ommatidial misrotation in the sano clones (arrows). Red, Elav. The absence of GFP signal indicates sanoKO2 mutant clones. Scale bar: 5 μm. (J, K) WT and sano mutant clones in the eye discs. m80.5-lacZ (red), a R4 photoreceptor marker is absent or significantly reduced in the sano clones (arrows). Blue, BarH1. The absence of GFP signal indicates sanoKO2 mutant clones. Scale bar: 5 μm.

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defects, including null mutants of the core PCP genes $fz$, $dsh$, $fmi$, $dgo$, $stbm$, and $pk$, the $ft$/$ds$ group of PCP regulator genes $fj$, $ft$, and $ds$, and the PCP downstream effectors $rhoA$, $Drok$, $zip$ and $sqh$. For $dsh$, a key hub in canonical Wingless (Wg)/Wnt signaling and in Fz-dependent PCP signaling, we used the $dsh^1$ allele, which is defective for only its PCP function [69,70]. Interestingly, many PCP mutants had tracheal length defects, exhibiting similar elongated DT phenotypes as loss of $sano$ (Figure 6A–6F and 6I). Among the core PCP genes, $fz$, $dsh$ and $fmi$ had elongated DTs, whereas $dgo$, $pk$ and $stbm$ mutant embryos had normal DTs. Among the $ft$/$ds$ PCP regulator group, $fj$ and $ds$ had elongated DTs. Among the PCP downstream effectors, $rhoA$ and $zip$ mutant embryos showed elongated DTs, revealing a potential role for the cytoskeleton in tracheal elongation. $Drok$ mutant embryos also

Table 2. $sano$ mutant eyes have various phenotypes including abnormal numbers of photoreceptors, loss of asymmetry, and misrotation of ommatidia.

| abnormal photoreceptor numbers (%) | symmetrical ommatidia (%) | misrotation of ommatidia (%) | number of ommatidia scored | number of clones examined |
|-----------------------------------|---------------------------|-----------------------------|----------------------------|--------------------------|
| 24.0                              | 5.8                       | 20.7                        | 208                        | 6                        |

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Figure 6. Many PCPm have tracheal length defects. (A–F) 2A12 staining in dsh1 (A), ds33K (B), rhoA72F (C), fmiE59/stan192 (D), ds33K/Df(2L)Exel8003 (E), rhoA72F/rhoA72O (F) mutant embryos. (G, G') btl-Gal4-driven overexpression of Dsh in the trachea results in a shortened DT phenotype similar to the Sano overexpression phenotype. Higher magnification of the boxed region is shown in G'. Red, 2A12; blue, SAS. (H) A nuclear fusion marker is normally expressed in Dsh-overexpressing trachea. Blue, Tgo; red, Dys. (I) Quantification of the length of DT of st. 16 PCP mutants. Error bars, SD (*, longer DT length, p<0.05, t-test; **, shorter DT length, p<0.05, t-test). (J) Yeast-two-hybrid assay using the full length and several fragments of the Dsh protein shows that the small fragment (~100 a.a.) of Dsh between the PDZ and DEP domains binds to Sano. (K) co-IP experiment using embryo extracts confirms the interaction between Sano-HA and Dsh-GFP. Scale bars: 20 μm for (A–G), 10 μm for (G', H). All embryos shown are st. 16.

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have convoluted trachea, but overall tracheal length was comparable to WT. ft and sph mutant embryos had shorter DTs than WT, but the DTs were contiguous (Figure 6I). Overexpression of Dsh or a constitutively-active form of RhoA in the trachea caused shortened DT defects with discontinuities similar to \textit{sano} overexpression (Figure 6G and 6G’; [78]), further implicating this pathway in apical cell surface elongation. Sano and Dsh are both cytoplasmic proteins, and Sano binds Dsh in yeast two-hybrid assays and co-immunoprecipitation (co-IP) (Figure 6J and 6K), providing a physical link between Sano and PCP proteins that is consistent with genetic interactions between \textit{dsh} and \textit{sano}; double mutants of \textit{sano} and \textit{dsh} have elongation defects similar to those of \textit{sano} or \textit{dsh} alone, suggesting that Sano and Dsh act in a common pathway (Figure 6I). Moreover, reduction of PCP function of Dsh (\textit{dsh}+/+) suppressed the Sano overexpression phenotype in the thorax, a finding also consistent with Sano acting through Dsh (Figure S10). The apical enrichment of Dsh in the late embryonic trachea and Fmi localization to the adherens junctions (Figure S11) is consistent with PCP proteins acting at the apical membrane. These data suggest that Sano affects tube length by impinging on Dsh activity, likely through its role in PCP signaling. Also consistent with this model is our finding that \textit{sano}\textit{ft} double mutant trachea have DT lengths that are intermediate between those of \textit{ft} and \textit{sano} mutants alone (Figure 6I).

**Sano Overexpression Results in Smaller Apical Domains**

Since wing epithelial cells become hexagonally packed prior to PCP proteins regulating hair formation [79], we examined cell shape in Sano-overexpressing wing cells. As observed with other PCP mutants, Sano-overexpressing cells often assume a pentagonal shape instead of the typical hexagonal shape (Figure 7A; [79]). Sano overexpressing cells also have smaller apical domains

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**Figure 7. Overexpression of Sano results in smaller apical domains.** (A) Sano-overexpressing pupal wing cells (green) are smaller than neighboring wild-type cells and often have a pentagonal rather than hexagonal shape (arrows). Red, E-Cad. (B) Quantification of the perimeter of three different clones of Sano-overexpressing single pupal wing cell and corresponding wild-type neighbors. Error bars, SD. *, p<0.05, t-test. (C–E) Adult wings. Sano overexpression at the anterior/posterior boundary with \textit{ptc-Gal4} results in a narrower region between veins 3 and 4 (arrows in C and D). Sano overexpression in the entire wing with \textit{MS1096-Gal4} results in a dramatic decrease in overall wing size. (F–H) E-Cad staining of tracheal cells (metamere 4). WT (F). \textit{btl-Gal4}-driven Sano-overexpressing trachea (G) reveal a significant decrease in apical domain size. The tracheal cells in \textit{rhoA} mutant embryos have larger apical domains (H). Arrowheads indicate apical domain of single cells in each allele. (I, J) E-Cad staining of tracheal cells (metamere 1). WT (I) and \textit{esg} mutant trachea that have fusion defects (J) have comparable apical domain size. All of the embryos shown are st.16. Scale bars: 5 µm in (A), 1 µm in the inset, 10 µm in (F–J). doi:10.1371/journal.pgen.1000746.g007
than surrounding wild-type cells (Figure 7A; here, we define the apical domain as the area circumscribed by the zonula adherens, where E-Cad localizes). Using E-Cad staining, we measured the apical domain perimeters from several examples of single Sano-overexpressing cells and found a 29–41% decrease in the perimeters of the Sano-overexpressing cells compared to their wild-type neighbors (Figure 7A and 7B).

A decrease in apical domain size with Sano overexpression was also seen in adult tissues. For example, ptc-Gal4-driven Sano expression at the anterior-posterior wing margin resulted in a decreased distance between wing veins L3 and L4 compared to wild-type wings (Figure 7C and 7D; 170.29±2.10 pixels vs. 210.91±4.58 pixels, N=3 for each genotype; p<0.005, t-test). Wing cell numbers in the area demarcated by veins L3, L4, the anterior crossvein, and an imaginary line starting from the tip of posterior crossvein and perpendicular to L3, did not reveal a significant difference in cell number between WT and ptc-Gal4/UAS-Sano wings (334.3±16.9 vs. 360±17.3, n=3 for each genotype; p>0.5, t-test), indicating that the decrease of the adult wing size is due to a decrease in apical domain size. Likewise, global wing expression of Sano, using the MS1096-Gal4 [90], resulted in a decrease in overall wing size (Figure 7E). To ask if Sano affects tracheal tube length through changes in apical domain size, we examined E-Cadherin staining of WT, sano mutant, and Sano-overexpressing trachea. We also examined E-Cadherin staining in PCP mutants with altered tracheal tube length. Although it was difficult to ascertain differences in apical domain size of individual cells between WT and sano mutant trachea, which are expected to be at most ~12% different, we observed a marked decrease in apical domain size in the Sano-overexpressing tracheal cells (Figure 7G). A similar decrease in apical domain size was observed in Dsh-overexpressing tracheal cells (data not shown). Moreover, the tracheal cells of thick, one of the PCP mutants, had larger apical domains than WT (Figure 7H), indicating that the changes in tube length observed with Sano and other PCP genes are due to altered cell geometry and not altered cell arrangement. In escargot (eg) mutant trachea, where infrequent DT breaks occur, apical domain size was comparable to WT, suggesting that the smaller apical domain size observed with sano and dsh overexpression is not due to a failure of adjacent DT segments to fuse (Figure 7I and 7J).

Discussion

Here, we report the discovery of Sano, a novel cytoplasmic protein enriched in the apical domains of developing tubular organs and other epithelia. Loss of sano results in overelongated tracheal tubes, whereas increased Sano shortens tracheal tubes, frequently leading to failures in dorsal trunk fusion. In adult tissues, Sano overexpression leads to characteristic PCP defects including misorientation of hairs on the adult wing, mislocalization of core PCP proteins in pupal wing cells, misorientation of thoracic bristles and loss of asymmetry in the rhabdomyeres of the ommatidia. Based on clonal analysis, loss of sano does not cause PCP defects in the wing but is required for normal ommatidial organization. Further support for Sano functioning as a PCP regulator is provided by our finding that mutations in both global and core PCP genes, as well as in the PCP downstream effectors, result in tracheal length defects similar to those seen with loss of sano. Based on comparisons of Sano-overexpressing and wild-type cells, Sano limits apical membrane domain size, suggesting that Sano and known PCP regulators control the linkage of the subapical cytoskeleton to the apical membrane and/or modulate apical membrane trafficking by regulating levels of endocytosis and exocytosis.

Identification of a Novel Protein Affecting PCP

Since the discovery that the Fz pathway controls PCP, many additional PCP components have been identified, including core factors, several PCP regulators, and general and tissue-specific downstream effectors [24,25,27]. Sano overexpression causes PCP defects in adult epithelial tissues as well as mislocalization of core PCP proteins. In wing cells, sano null cells appeared normal although we very occasionally obtained twin spot-only clones, suggesting a role for Sano in cell survival or in epithelial maintenance. It is unclear whether this function is related to PCP. On the other hand, sano loss in the eye gave rise to a range of defects, some of which are typical of PCP mutants, including loss of R4 cell specification, ommatidial misorientation, and loss of equatorial asymmetry.

The direct physical interaction between Dsh and Sano (Figure 6J and 6K) provides potential mechanistic insight into Sano function. The interaction between Dsh and Sano appears quite different from that between Dsh and Naked cuticle (Nkd), a Wingless (Wg) antagonist that also gives rise to PCP defects when overexpressed. Dsh participates both in canonical Wg/Wnt signaling and in Fz-dependent PCP signaling [29–31]. Like Sano, Nkd directly binds Dsh, and overproduced Nkd causes polarity defects and limits Wg signaling activity presumably by sequestering, degrading and/or modifying Dsh and thus blocking its participation in PCP [81]. Unlike Nkd, however, Sano overexpression does not cause defects typical of those seen when canonical Wg signaling is blocked. Moreover, whereas Nkd overexpression blocks Dsh activity, our studies of tube length control suggest that Dsh and Sano act in the same direction: gain or loss of Dsh mimics the gain or loss of Sano in the trachea. Similarly, dsh sano double mutants have the same tracheal length defects as each single mutant (Figure 6L). Likewise, overexpression of either Dsh or Sano in the eye using sev-Gal4 causes similar changes in ommatidial polarity and rotation [82]). Finally, we showed that reduced dsh function suppresses the Sano overexpression PCP phenotypes in the thorax (Figure S10). Overall, the interaction and genetic data suggest that Sano and Dsh act together in a common pathway.

A Role for PCP Signaling in Tube Size Control

It is intriguing that loss-of-function mutations in many, albeit not all, PCP genes result in similar tube elongation defects observed with loss of sano (Figure 6). PCP signaling can provide directional cues at the single cell level, such as directions on where to place the single hair within a Drosophila wing cell, or at the level of cell groups, such as controlling the organization of mechanosensory bristles in the Drosophila thorax and arrangement of photoreceptors in the Drosophila eye. PCP signaling also controls the behavior of cell populations undergoing extensive rearrangements, such as the dynamic morphogenetic changes that occur during body axis elongation in Drosophila and vertebrates and in ovarian border cell migration [23,27,83]. A recent study has implicated mammalian Fat4, a vertebrate homologue of the Drosophila global PCP protein Fat, in promoting renal tubule elongation through its effects on oriented cell divisions [51]. In those studies, loss of Fat4 led to shorter renal tubules, a defect exacerbated by simultaneous loss of one copy of Vang2, a vertebrate homologue of the core PCP protein Sbm/Vang. Consistent with this finding, our studies reveal that, in the trachea, mutations in the proteins that negatively regulate Fat (Dsh and Fj) and the Sbm/Vang complex (Fz, Dsh) have the opposite defect: longer tubes. In the case of the trachea, a tissue whose final cell divisions occur much earlier in development than when Sano affects tube length, the effects of the PCP pathway are on cell
shape rather than on the orientation of cell division. Whether the subcellular mechanisms by which PCP genes regulate oriented cell divisions in vertebrates and apical membrane elongation in flies are similar or distinct is not clear, but the parallels in the two systems provide evidence for evolutionarily conserved functions for PCP genes in tubular architecture.

The finding of a role for PCP genes in tube length control raises two crucial questions. (1) Are PCP proteins asymmetrically localized in tubular epithelia in the same way they are in wing, eye and border cells [23,26,27]? (2) How do PCP genes regulate tube length? We examined the subcellular localization of Dsh and Fmi in the tracheal cells, where Dsh localizes mainly in the cytoplasm and is enriched at the apical domain at later stages, and Fmi localizes to the adherens junctions (Figure S11). Unfortunately due to the irregular shape of tracheal cells and the three-dimensional structure of the tracheal tube, we could not determine with adequate resolution whether the PCP proteins are asymmetrically distributed. However, our data provides new insight into how PCP affects tracheal tube size. In the trachea, loss of sano or PCP function resulted in tubes that were 7–15% longer than WT based on apical domain measurements (2A12 staining). Since the sano trachea have the same number of cells as wild-type, each tracheal cell, on average, must have an apical domain that is approximately 12% longer than wild-type. Although an accurate measurement of apical dimensions in the trachea could not be obtained due to the shape and curvature of the tube, in rhoA mutant trachea, where the elongated DT defects were most obvious, the apical domains of the DT cells were consistently larger than WT (Figure 7H). Similarly, E-Cad were most obvious, the apical domains of the DT cells were of the secretory machinery [85,86].

Materials and Methods

Flies and Antibodies

Fly strains used in this study were: f(x211), f(x21), pk(+13), MS1096-Gal4 (P. Adler); Ser4, sh, sm, mpg, dsh, shn192, shn6, pk, nck1, nck2, Drok3, zp1, zp2, d1/2, fide-2, egzG3-1, Df(2L)Ej603, Df(2R)ED1076, Df(2R)ED18610, Df(2R)EJ271, sev-Gal4, (Bloomington stock center); Df(2R)Ej6068 (Exelixis); 1XGal4 (S. Hayashi); n2聆X (L. Luo); ptc-Gal4 (D. Pan); HS-Scr (M. Scott); dgo, md0.5-um (D. Strutt); fim-K69 (T. Uemura); sago-Gal4 (A. Vaninav and D.J.A., unpublished).

The primary antibodies used were mouse a—b-gal (Promega, 1:500), rabbit a—GFP (Molecular Probes, 1:10,000), mouse a—HA (Roche, 1:500), mouse 2A12 (DSHB, 1:10), rabbit a—Crb (DSHB, 1:10), rabbit a—SAS (D. Cavenag, 1:500), mouse a—Spec (DSHB, 1:1), rat a—DE-Cad (DSHB, 1:10), rat a—Fmi (DSHB, 1:10), rat a—Dsh (T. Uemura, 1:1,000), rabbit a—Stbm (T. Wolf, 1:200), rabbit a—Pk (J. Axelrod, 1:2,000), guinea pig a—Verm (C. Samakovlis, 1:500), CBF-FITC (New England BioLabs, 1:500), WGA-488 (Molecular Probes, 1:1,000), guinea pig a—Cor (R. Fehon, 1:2,000), rabbit a—NeuIV (H. Bellem, 1:2,000), mouse a—Fas3 (DSHB, 1:10), rabbit a—Dya (S. Greaves, 1:300), rabbit a—Ar3 (S. Hayashi, 1:2,500), rabbit a—Elav (DSHB, 1:250), and rat a—BarH1 (H. McNeil, 1:1000). Fluorescence-labeled secondary antibodies were used at a 1:500 dilution (Molecular Probes).

Immunohistochemistry

Embryo fixation and staining were performed as described [87] except for the a—E-Cad staining, for which embryos were fixed in 4% paraformaldehyde in PBS and dehydrilized with ethanol. 5th instar np392 larval discs were dissected and fixed in 2% paraformaldehyde in PBS for 20 minutes, incubated with primary antibody overnight (4°C) and then with the appropriate secondary antibody for two hours (RT).

Whole-Mount in situ Hybridization on Embryos and Imaginal Discs

In situ hybridizations were performed as described by [88]. The pB3 cDNA, isolated by screening a cDNA library provided by L. Kauvar, was used to generate an anti-sense digoxigenin-labeled sano RNA probe.

Identification of Sano

sano was identified in a P-element expression screen in Corey Goodman’s laboratory. We obtained the np392 line because of its salivary gland and tracheal expression. Sano was independently identified in an EP screen for genes that when misexpressed alter the eye phenotype of Dsh+ and Nkd overexpression (S. Silva, G. Celik, C.-C. C. and K.A.W., unpublished).

sano Null Alleles

Null sano mutants were generated by homologous recombination [65]. Genomic fragments upstream and downstream of the sano ORF were amplified by PCR and cloned into pW25, which carries white+, the recognition site for I-SceI endonuclease, and FRT sites. The construct was injected into embryos by Rainbow Transgenic Flies, Inc. Transformants were crossed to flies carrying hs-Lac and hs-Fip and progeny were heat shocked (37°C) for 1 hour 48–72 hours AEL.

Transgenic Flies

The sano ORF was PCR-amplified and cloned into the pUAST [61] or pBAl expression vector [89] to create UAS-Sano and
HS-Sano. UAS-Sano-GFP and UAS-Sano-HA were created using the Drosophila Gateway Vector system (Carnegie Institution).

Sano Antibody

A PCR fragment spanning the sano ORF was amplified and cloned into the pProEx expression vector (Life Technologies, Inc.). The construct was transformed into BL21-DE3 cells, from which Sano inclusion body preparations were made. Recombinant full-length protein was further purified from an SDS-polyacrylamide gel slice as described [90]. Rat polyclonal antibodies were generated by Covance, Inc. and used at a dilution of 1:50.

sano Mutant or Overexpression Clones in Pupal Wing

sano mutant or overexpressing clones were generated by the Flip-mediated recombination technique [91,92]. Clones were induced either 36–48 or 48–60 hours AEL by a one hour heat shock (37 °C). The genotype for sano mutant clones was hs-FLP/+; act-GFP FRT42/sanoKO3 FRT42. The genotype for Sano flip-out clones was either act>y>Gal4/+; UAS-Sano-GFP/hs-FRT or act>y>Gal4/+; arm-fl-GFP/+; UAS-Sano-HA/hs-FLP.

Phalloidin/Antibody Staining of Pupal Wings

Pupae were fixed 32–34 hours APF in 4% paraformaldehyde in PBS overnight (4 °C). Pupal wings were dissected and washed several times in 0.5% PBST (0.5% Triton X-100 in PBS) and incubated with phalloidin-568 (Molecular Probes, 1:1000) for one hour (ice). For antibody staining, pupae were fixed at 28 hours APF in 4% paraformaldehyde in PBS for one hour (4 °C). Pupal wings were dissected and washed in 0.1% PBST. Wings were incubated in primary antibodies overnight (4 °C) and then in secondary antibodies for two hours (ice).

Eye Imaginal Disc Staining

sano mutant eye clones were generated using eyeless-FLP (ey-FLP). sano mutant cells were distinguished by the absence of the GFP signal. The 3rd instar larvae were dissected in the PBS, fixed with fixation buffer (0.1M PIPES (pH6.9), 1mM EGTA (pH6.9), 1.0% Triton X-100, 2mM MgSO4, 1% formaldehyde), blocked in a solution (50mM Tris[pH6.8], 150mM NaCl, 0.1% Triton X-100, 5mg/ml bovine serum albumin (BSA)). The discs were incubated in primary antibodies in a washing/incubation solution (50mM Tris[pH6.8], 150mM NaCl, 0.1% Triton X-100, 1mg/ml BSA) overnight at 4 °C and then in secondary antibodies for two hours (ice).

Adult Eye Sections

sevenless-Gal4 (sev-Gal4) was used to overexpress Sano in the eye. sevenless mutant eye clones were generated using sev-FLP. sevenless mutant cells were w+/w−, which can easily be distinguished from w+/w+ heterozygous cells and from w−/w− twin spots in whole eyes. Since it is difficult to distinguish w+/w+ versus w+/w− in thin sections, however, we chose only eyes with large mutant clones and adjacent w−/w− twin spots for sectioning. Fixation and semi-thin sectioning of the adult eyes were slightly modified from [93]. Sections from at least five independent eyes were analyzed for each genotype.

Dorsal Trunk (DT) Length and Pupal Wing Cell Perimeter Measurements

Embryos were stained with 2A12 and projections from lateral views of confocal sections of the DT lumen of st. 16 embryos (at the four equal-compartment midgut stage) were traced from the starting point of metamere one to the point where the last transverse connective (TC) meets the DT in metamere nine using the Image J program (NIH). At least ten samples were measured and normalized to the length of the embryo for each genotype. An average length from three independent measurements of each sample was calculated.

Pupal wings were stained for E-Cadherin and the perimeter of pupal wing cells overexpressing Sano-GFP and of their wild-type neighbors were measured by Image J.

Co-Immunoprecipitation and Western Blotting

du-Gal4/UAS-Sano-HA; dsh-GFP/+ embryos were used for co-IP, and du-Gal4/UAS-Sano-HA and dsh-GFP embryos were used as controls. The embryos were collected and homogenized in radioimmunoprecipitation (RIPA) buffer (Cell Signaling) including protease inhibitor cocktail (Roche). A small aliquot of the cleared supernatant was used for the Western to check the protein input with α-GFP and α-HA. Dynabeads Protein G (Invitrogen) was incubated with mouse α-HA (Roche) or rabbit α-GFP (Molecular Probes) for 10 minutes at RT. After several washes with PB/Tw (0.01% Tween-20 in 1x PBS), the remaining supernatant was incubated with antibody-bound Dynabeads Protein G for 20 minutes at RT. The beads were washed three times with RIPA buffer, and boiled in SDS sample buffer to elute the proteins. Bound antigen was detected by enhanced chemiluminescence (GE Healthcare). The antibodies for Western blotting were used at the following concentrations: rat α-HA (Roche, 1:2,000), mouse α-GFP (Roche, 1:2,000). Co-IPs were repeated three times with the same results.

Northern Hybridization

The developmental Northern blot was prepared as described [94] and hybridized with a Bgl II/Nor I fragment from pPB3 cDNA labeled by random priming.

Dextran Injections

Fluorescence-labelled 10kDa dextran (Molecular Probes) injections were performed as described [22,95], using wild-type embryos as a negative control and the mega mutant as a positive control.

ApopTag Staining

Embryos were dechorinated and fixed in the fixative for 20–30 min at RT. The fixative includes 800µl 5x buffer B (50mM KPO4, pH6.8, 225mM KCl, 75mM NaCl, 65mM MgCl2), 800ml 37% formaldehyde, 2.5ml dH2O and 8ml heptane. Antibody staining was performed with α-Tgo antibody to mark the tracheal nuclei. After secondary antibody labelling, the embryos were treated with 10µg/ml proteinase K for 1 min and post-fixed with 3.7% formaldehyde in 0.1% Tween20 in 1x PBS (PBT). ApopTag staining was performed using ApopTag Plus Peroxidase In Situ Apoptosis Kit (Millipore, S7101), and the cells undergoing apoptosis were labeled with rhodamine-conjugated α-Dig antibody (1:10, Roche).

Supporting Information

Figure S1  Developmental Northern of sano. (A) sano transcript is most abundant in 8–12 hour embryos and is not maternally contributed (note absence of expression in 0–2 hour embryos), sano is not expressed in Drosophila S2 cells (last lane of gel, S). Found at: doi:10.1371/journal.pgen.1000746.s001 (0.62 MB TIF)

Figure S2  sano expression in trhH99 mutants. (A–F) sano mRNA expression in st.10–12 wild-type (A, C, E) and trhH99 mutant (B, D, F) embryos. Weak tracheal expression is observed in trhH99
mutants at early stages (arrows in B, D, F, G and H) *sano* expression in st.13 wild-type (G) and *thl* H99 mutant (H) embryos. *sano* mRNA is completely absent in the trachea (arrows in G and H), whereas the midline expression is still strong in the *thl* H99 mutant (arrowheads in G and H).

**Figure S3** Generation of *sano* null alleles by homologous recombination. (A) Schematic diagram for *sano* knockout and the primers used for diagnostic PCRs. (B) Genomic PCRs for the three knockout mutants KO1, KO2 and KO3. The original transgenic fly line carrying the knockout transgenic construct is used as a negative control. (C–H) *sano* mRNA expression in wild-type embryos (C, E, G) and in embryos transheterozygous for a *sano* null mutant over a deficiency that removes *sano* (D, F, H). *sano* expression is absent in *sano*KO1/Df(2R)Exel6088 (D), *sano*KO2/Df(2R)Exel6088 (E), and *sano*KO3/Df(2R)Exel6088 (H) embryos.

**Figure S4** Known pathways affecting tracheal tube length are unaffected in *sano* mutant embryos. (A–D) Chitin cable and a chitin-modifying enzyme show normal levels and distribution in *sano* mutants. Chitin-binding protein (CBP; A, B), α-Vermiform (Verm; C, D), (E and F) Septate junction marker α-Coracite (Cor) shows normal distribution. (G–I) Barrier function is intact in *sano* null trachea. Dye exclusion assay in wild-type, *sano* null trachea showing colocalization with E-Cad. Red, Fmi; Blue, E-Cad; Green, Dsh; Yellow, SAS. (C–E) Fmi localization in the WT tracheal cells. During tracheal morphogenesis, Fmi localizes in the cytoplasm (A), and at later stages, it shows enrichment at the apical membrane (B). Green, Dsh; Red, SAS. (C–E) Fmi localization in the WT tracheal cells. During tracheal morphogenesis, Fmi is detected at the adherens junction in the trachea showing colocalization with E-Cad. Red, Fmi; Blue, E-Cad; Green, SAS.

**Figure S5** No significant increase of apoptosis is detected in *sano*-overexpressing trachea. (A, B) Compared to WT (A), no significant increase of apoptosis was detected in the *sano*-overexpressing tracheal cells even when a huge gap was seen (B). The images are merameter 2–4 of the st.16 embryos. Green, Tgo; red, Apoptag. Scale bars: 20 μm.

**Figure S6** *sano* expression in the imaginal discs. (A–C) β-gal expression of rp395 in the imaginal discs of 3rd instar larvae. (D–F) *sano* mRNA expression in wild-type imaginal discs. 

**Figure S7** Examples of *sano*-overexpressing clones in the pupal wings. (A) Cartoon image indicates regions where *sano* overexpression causes non-autonomous polarity defects in adjacent wild-type cells. (B) *sano* overexpression sometimes causes multiple wing hairs (arrows), another typical PCP phenotype. (C and D) Examples of nonautonomous PCP defects in *sano*-overexpressing clones. (C) Unlike the other genes showing nonautonomy, nonautonomous effects caused by *sano* overexpression have no directionality. Some WT hairs near the clones point toward the clones (arrows), whereas others point away from the clones (arrowheads). (D) Hair formation delay is shown inside the clones (asterisks), whereas the polarity defects are observed outside of the clones of *sano* overexpressing cells (arrows). Scale bars: 5 μm in (B), 10 μm in (C, D).

**Figure S8** *sano* misexpression disrupts the asymmetric distribution of all PCP proteins analyzed. (A) Stbm (red) loses asymmetric localization in *sano*-overexpressing region (green). The adjacent wild-type cells in B look smaller because they are in the vein (brackets). Scale bars: 5 μm. (B) Dsh (red) is distributed throughout the apical margin in *sano*-overexpressing clones (green).

**Figure S9** *sano* LOF clones in the wings. (A, B) Examples of *sano* loss-of-function (LOF) clones (absence of GFP) showing normal hair cell polarity in the pupal wings. (C, D) Some *sano* clones induced early show only twin spots (bright GFP signal, arrows). Scale bars: 10 μm in (A, B); 50 μm in (C, D).

**Figure S10** Reduction of dsh dosage suppresses gain-of-function phenotype of *Sano*. (A, B) Examples of *MS1096-Gal4/+; UAS-Sano/+* thorax. (C, D) Examples of *MS1096-Gal4/dsh1; UAS-Sano/+* thorax. Reducing the PCP function of dsh suppresses *sano*-overexpressing PCP phenotypes. All flies shown are female and the crosses were done at 18°C.

**Figure S11** Dsh and Fmi localization in the WT trachea. (A, B) Dsh localization in the WT tracheal cells. At early stages, Dsh localizes in the cytoplasm (A), and at later stages, it shows enrichment at the apical membrane (B). Green, Dsh; Red, SAS. (C–E) Fmi localization in the WT tracheal cells. During tracheal morphogenesis, Fmi is detected at the adherens junction in the trachea showing colocalization with E-Cad. Red, Fmi; Blue, E-Cad; Green, SAS.

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**Author Contributions**

Conceived and designed the experiments: SC DJA. Performed the experiments: SC MSV PLB CCC. Analyzed the data: SC DJA. Contributed reagents/materials/analysis tools: KAWJ. Wrote the paper: SC DJA.

**References**

1. Affolter M, Caussinus E (2008) Tracheal branching morphogenesis in Drosophila: new insights into cell behaviour and organ architecture. Development 135: 2055–2064.

2. Casanova J (2007) The emergence of shape: notions from the study of the Drosophila tracheal system. EMBO Rep 8: 335–339.

3. Kerman BE, Cheshire AM, Andrew DJ (2006) From fate to function: the Drosophila trachea and salivary gland as models for tubulogenesis. Differentiation 74: 326–348.

4. Swanson LE, Beitel GJ (2006) Tubulogenesis: an inside job. Curr Biol 16: R51–53.
5. Glazer L, Shilo BZ (2003) Hedgehog signaling patterns the tracheal branches. Development 128: 1599–1606.
6. Chen CK, Kuhnlein RP, Eisenberg KG, Vincent S, Affolter M, et al. (1998) The transcription factors KIRRP and KIRNR RELATED control cell migration and branch morphogenesis during Drosophila tracheal development. Development 125: 4959–4968.
7. Chihara T, Hayashi S (2000) Control of tracheal tubulogenesis by Wingless signaling. Development 127: 4433–4442.
8. Kato S, Chihara T, Hayashi S (2004) Hedgehog and Decapentaplegic instructive growth of cell extensions in the Drosophila trachea. Development 131: 5253–5261.
9. Lilinargas M (2000) Wingless and its signalling pathway have common and separable functions during tracheal development. Development 127: 4407–4417.
10. Vincent S, Ruberte E, Grieder NC, Chen CK, Haerry T, et al. (1997) DPP controls tracheal cell migration along the dorsoventral body axis of the Drosophila embryo. Development 124: 2741–2750.
11. Beitel GJ, Krasonow MA (2000) Genetic control of epithelial tube size in the Drosophila tracheal system. Development 127: 3211–3223.
12. Bachmann A, Draga M, Grawe F, Knust E (2000) On the role of the MAGUK protein encoded by Drosophila van Gogh during embryonic and postembryonic development. BMC Dev Biol 8: 55.
13. Behr M, Riedel D, Schuh R (2003) The claudin-like megalatrachesa is essential in septate junctions for the epithelial barrier function in Drosophila. Dev Cell 5: 611–620.
14. Lilinargas M, Strigini M, Kaitou M, Karagogeos D, Casanova J (2004) Lachesis is a component of a septate junctional function that controls tube size and epithelial integrity in the Drosophila tracheal system. Development 131: 5221–5232.
15. Paul SM, Beitel GJ (2003) Developmental biology. Tubulogenesis CLICs into place. Science 302: 2077–2078.
16. Vu WM, Beitel GJ (2004) A junctional function of apical proportions: epithelial tube-size control by septate junctions in the Drosophila tracheal system. Curc Opin Cell Biol 16: 493–499.
17. Vu WM, Schulte J, Hirschi A, Terpau U, Beitel GJ (2004) Sinuous is a Drosophila claudin required for septate junction function and epithelial tube size control. J Cell Biol 164: 531–542.
18. Laschinger S, Batz T, Armbruster K, Krasonow MA (2006) serpentine and vermisform encode matrix proteins with chitin binding and deacetylation domains that limit tracheal tube length in Drosophila. Cell Biol 16: 186–194.
19. Ambros SJ, Sloan H, Rieser G, Casanova J (2000) mumps/cytox: an enzyme required for chitin and giancy synthesis, involved in trachea, embryochnic cuticle and CNS development-analysis of its role in Drosophila tracheal morphogenesis. Dev Bio 280: 179–193.
20. Devine WP, Lubarsky B, Shaw K, Laschinger S, Messiina L., et al. (2005) Requirement for chitin biosynthesis in epithelial tube morphogenesis. Proc Natl Acad Sci U S A 102: 17014–17019.
21. Tonning A, Hemphila J, Tang E, Nonnmark U, Samakovlis C, et al. (2005) A transient luminal chitin matrix is required to model epithelial tube diameter in the Drosophila trachea. Dev Cell 9: 423–430.
22. Wang S, Jayaram SA, Hemphila J, Senti KA, Tsarouhas V, et al. (2006) Septate-junction-dependent luminal deposition of chitin deacetylase restricts tube growth in the Drosophila trachea. Cell Biol 16: 180–185.
23. Wang Y, Nathans J (2007) Tissue/planar cell polarity in vertebrates: new insights and new questions. Development 134: 647–658.
24. Klein TJ, Mlodzik M (2005) Planar cell polarization: an emerging model point in the right direction. Annu Rev Cell Dev Biol 21: 153–176.
25. Adler PN (2002) Planar signaling and morphogenesis in Drosophila. Dev Cell 2: 525–533.
26. Bastock R, Strutt D (2007) The planar polarity pathway promotes coordinated cell migration during Drosophila oogenesis. Development 134: 3055–3064.
27. Seifert JR, Mlodzik M (2007) Frizzled/PCP signalling: a conserved mechanism regulating cell polarity and directed motility. Nat Rev Genet 8: 126–138.
28. Vinson CR, Conover S, Adler PN (1989) A Drosophila tissue polarity locus encodes a protein containing seven potential transmembrane domains. Nature 338: 263–264.
29. Theisen H, Purcell J, Bennett M, Kansagar D, Yared V, et al. (1994) Dishevelled is identified during wingless signaling to establish both cell polarity and cell identity. Development 120: 547–560.
30. Klingensmith J, Nusse R, Perrimon N, Strutt DI (1997) The Drosophila SEGMENT POLARITY gene starry night encodes a member of the protocadherin family. Development 124: 401–404.
31. Chae J, Kim MJ, Goo JH, Collery S, Gubbi D, et al. (1999) Dishevelled is required during wingless signaling to establish both cell polarity and cell identity. Development 126: 5421–5429.
32. Usui T, Shima Y, Shimada Y, Hirota S, Borgers RW, et al. (1999) Frangipani, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled. Cell 98: 505–505.
33. Taylor J, Alland N, Chantlon J, Adler PN (1998) Van Gogh: a new Drosophila tissue polarity gene. Genetics 150: 199–210.
34. Wollf T, Rubin GM (1998) Strabismus, a novel gene that regulates tissue polarity and cell late decisions in Drosophila. Development 125: 1149–1159.
67. Kakihara K, Shinmyozu K, Kato K, Wada H, Hayashi S (2008) Conversion of plasma membrane topology during epithelial tube connection requires Arf-like 3 small GTPase in Drosophila. Mech Dev 125: 325–336.
68. Uv A, Cantera R, Samakovlis C (2003) Drosophila tracheal morphogenesis: intricate cellular solutions to basic plumbing problems. Trends Cell Biol 13: 301–309.
69. Axelrod JD, Miller JR, Shulman JM, Moon RT, Perrimon N (1998) Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. Gene Dev 12: 2610–2622.
70. Boutros M, Paricio N, Strutt D, Mlodzik M (1998) Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. Cell 94: 109–118.
71. Krasnow RE, Adler PN (1994) A single frizzled protein has a dual function in tissue polarity. Development 120: 1883–1893.
72. Gubb D, Green C, Hurn D, Coulson D, Johnson G, et al. (1999) The balance between isoforms of the prickle LIM domain protein is critical for planar polarity in Drosophila imaginal discs. Genes Dev 13: 2315–2327.
73. Zheng L, Zhang J, Carthew RW (1995) frizzled regulates mirror-symmetric pattern formation in the Drosophila eye. Development 121: 3045–3055.
74. Strutt D, Strutt H (2007) Differential activities of the core planar polarity proteins during Drosophila wing patterning. Dev Biol 302: 181–194.
75. Vinson CR, Adler PN (1997) Directional non-cell autonomy and the transmission of polarity information by the frizzled gene of Drosophila. Nature 397: 526–530.
76. Higashijima S, Kojima T, Michine T, Ishimaru S, Emori Y, et al. (1992) Dual Bar homeo box genes of Drosophila required in two photoreceptor cells, R1 and R6, and primary pigment cells for normal eye development. Genes Dev 6: 50–60.
77. Cooper MT, Bray SJ (1999) Frizzled regulation of Notch signalling polarizes cell fate in the Drosophila eye. Nature 397: 526–530.
78. Lee S, Kolodziej PA (2002) The plakin Short Stop and the RhoA GTPase are required for E-cadherin-dependent apical surface remodeling during tracheal tube fusion. Development 129: 1509–1520.
79. Classen AK, Anderson KI, Marois E, Eaton S (2005) Hexagonal packing of Drosophila wing epithelial cells by the planar cell polarity pathway. Dev Cell 9: 805–817.
80. Capdevila J, Guerrero I (1994) Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in Drosophila wings. EMBO J 13: 4459–4460.