

Report

serpentine and *vermiform* Encode Matrix Proteins with Chitin Binding and Deacetylation Domains that Limit Tracheal Tube Length in *Drosophila*

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Summary

Many organs contain epithelial tubes that transport gases or liquids [1]. Proper tube size and shape is crucial for organ function, but the mechanisms controlling tube diameter and length are poorly understood. Recent studies of tracheal (respiratory) tube morphogenesis in *Drosophila* show that chitin synthesis genes produce an expanding chitin cylinder in the apical (luminal) extracellular matrix (ECM) that coordinates the dilation of the surrounding epithelium [2, 3]. Here, we describe two genes involved in chitin modification, *serpentine* (*serp*) and *vermiform* (*verm*), mutations in which cause excessively long and tortuous tracheal tubes. The genes encode similar proteins with an LDL-receptor ligand binding motif and chitin binding and deacetylation domains. Both proteins are expressed and secreted during tube expansion and localize throughout the lumen in a chitin-dependent manner. Unlike previously characterized chitin pathway genes, *serp* and *verm* are not required for chitin synthesis or secretion but rather for its normal fibrillar structure. The mutations also affect structural properties of another chitinous matrix, epidermal cuticle. Our work demonstrates that chitin and the matrix proteins *Serp* and *Verm* limit tube elongation, and it suggests that tube length is controlled independently of diameter by modulating physical properties of the chitin ECM, presumably by N-deacetylation of chitin and conversion to chitosan.

Results and Discussion

Genetic pathways controlling branching morphogenesis and cell-type diversification of the *Drosophila* tracheal system have been characterized [4]. However, it is not known how tracheal cells measure, regulate, and maintain distinct sizes and shapes of epithelial tubes. Genetic screens have identified genes that influence the diameter, length, and shape of tracheal tubes [5]. Many of these encode components of septate junctions, the

insect cognate of vertebrate tight junctions ([6–9]; reviewed in [10]). Recently, genes involved in the synthesis of a cylindrical chitin matrix secreted by tracheal cells prior to cuticle formation were identified and shown to play an essential role in controlling tracheal tube diameter [2, 3]. Here, we describe the identification and characterization of two genes that encode apical extracellular matrix (ECM) proteins that modify the structure of the chitin matrix and regulate tracheal tube length.

Identification and Genetic Characterization of *serpentine* and *vermiform*, Related Genes that Restrict Tracheal Tube Elongation

In a genomic search for genes regulated by the putative transcription factor Ribbon (S.L. and M.A.K., unpublished data), a nuclear BTB/POZ domain protein that promotes movement and morphogenesis of the apical surface of the tracheal epithelium [11, 12], we identified two adjacent genes at cytological position 76C1-2 (CG32209 and CG8756) that encode structurally related tracheal matrix proteins (see below). The genes are expressed in indistinguishable patterns. mRNAs of both genes were detected in the developing tracheal system beginning at embryonic stage 12 (Figures 1A–1H), just after Ribbon protein is detected, and expression of both genes was reduced in *ribbon* null mutants as determined by in situ hybridization (Figures 1I–1L) and DNA microarray analysis (6-fold reduction of CG32209 and 5-fold reduction of CG8756). Both genes are also expressed in the developing stomodeum beginning at stage 14 and in the epidermis at stage 16. Tracheal expression of both genes starts to fade at stage 15 and is no longer detected at stage 16 (Figures 1C, 1D, 1G, and 1H). The genes were named *serpentine* (*serp*; CG32209) and *vermiform* (*verm*; CG8756) on the basis of their elongated and convoluted tracheal phenotypes described below.

To analyze the developmental functions of the genes, we identified putative null mutations in each gene. A P element insertion in CG8756 (*verm*^{KG}) is embryonic lethal when homozygous (Figure 1R). Excision of the transposon restored viability of the parental chromosome in 9 of 13 excision events, indicating that the lethality is due to the P element. One imprecise excision, *verm*^{ex7}, removed the transposon and 556 bp of flanking genomic DNA, including the first coding exon of CG8756, which is common to all known splice variants and includes the start codon and signal peptide, suggesting that *verm*^{ex7} is a null allele. The parental P element insertion also appears to be a null allele because *Verm* protein expression was not detected by immunostaining (Figures 1P and 2K) and its phenotype was indistinguishable from *verm*^{ex7} (see below). *serp*^{RB} is an insertion in CG32209 of a PiggyBac transposon designed to disrupt mRNA splicing (Figure 1R). It eliminated expression of *Serp* protein (Figures 1N and 1S) and behaved as a null allele in genetic tests (not shown).

Tracheal development in *serp* and *verm* mutants was analyzed with specific markers for tracheal cells and the

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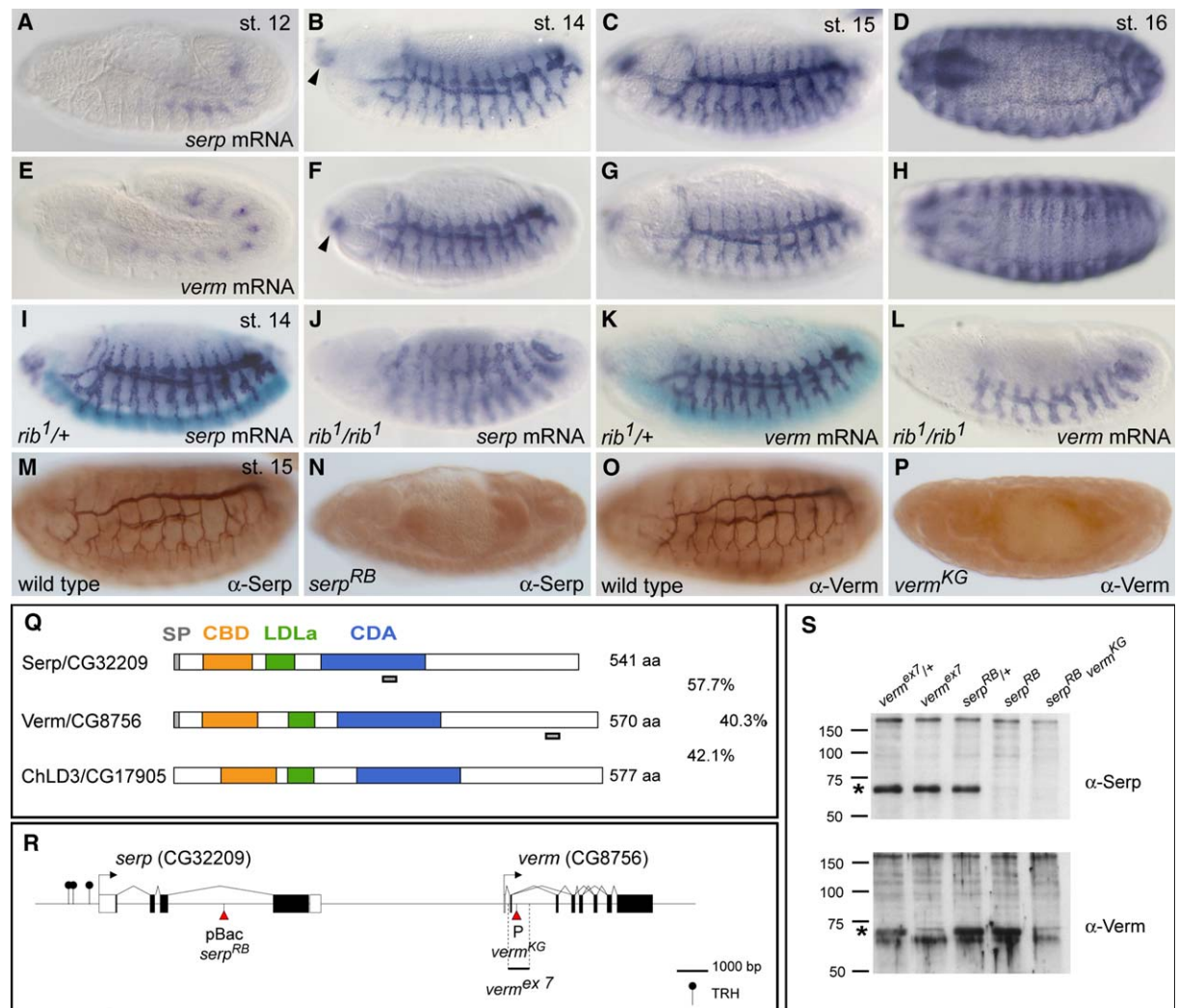


Figure 1. Structure and Expression of *serpentine* and *vermiform* Genes and Their Encoded Proteins

(A–H) Embryonic expression pattern of *serp* and *verm* mRNAs. In situ hybridization of wild-type embryos at the stages indicated is shown. Dorsal is up and anterior is left except as noted. Transcripts of both genes are first detected at stage 12 in the tracheal system. Expression is also detected in the stomodeum beginning at stage 14 (arrowhead in [B] and [F]) and in the epidermis at stage 16 ([D]: dorsal view, [H]: ventral view). Tracheal expression declines at stage 15 and is no longer detected at stage 16. Dorsal-trunk-lumen staining in [D] is nonspecific trapping of probe. (I–L) Expression of *serp* and *verm* mRNAs in *ribbon* mutant embryos. Note reduced expression in homozygous *rib*¹ embryos (J and L) compared to heterozygous *rib*^{1/+} siblings (I and K). X-Gal staining (turquoise in [I] and [K]) was used to distinguish β -galactosidase-expressing heterozygous embryos (*rib*^{1/CyO ftz-lacZ) from homozygotes (*rib*^{1/rib}¹). Microarray experiments (not shown) indicated a 5-fold reduction in mRNA levels for *serp* and a 6-fold reduction for *verm* in stage 13 *rib*¹ homozygotes relative to wild-type embryos. (M–P) Immunostaining of Serp and Verm proteins in stage-15 wild-type and homozygous *serp*^{RB} and *verm*^{KG} embryos. No specific staining with anti-Serp antiserum is detected in the *serp*^{RB} mutant (N), and likewise for anti-Verm antiserum in the *verm*^{KG} mutant (P). (Q) Domain organization of Serp, Verm, and a related *Drosophila* protein (ChLD3). Size of each protein and pairwise amino acid identity among them are indicated at right. The following abbreviations are used: SP (gray), N-terminal signal peptide; CBD (orange), chitin binding domain; LDLa (green) LDL receptor type A ligand binding motif; and CDA, chitin deacetylase domain. Small gray boxes indicate positions of peptides (Serp residues 253–270; Verm residues 504–523) used to generate anti-Serp and anti-Verm antisera. (R) Organization of the *serp* and *verm* loci. Exons are shown by boxes with coding regions indicated by black fill. Four alternative splice forms (corresponding to 52 ESTs) are annotated for *verm*/CG8756. A single splice form (10 ESTs) is annotated for *serp*/CG32209 (FlyBase). Positions of transposon insertions in *serp* and *verm* are indicated below the line by red triangles. The *verm*^{ex7} allele contains a 556 bp deletion generated by imprecise excision of the P[KG] element. TRH indicates positions of TACGTG consensus binding motifs [40] for the Trachealess transcription factor that could contribute to tracheal expression. (S) Immunoblot analysis of Serp and Verm proteins. Immunoblots of extracts of embryos of the indicated genotypes were probed with α -Serp (top) or α -Verm (bottom) antisera. α -Serp detects a 65 kDa band (asterisk) present in wild-type and *verm*^{ex7} deletion mutants, but absent in *serp*^{RB} mutants. α -Verm detects a ~70 kDa band (asterisk) present in wild-type and *serp*^{RB} mutants, but absent in *verm*^{ex7} deletion mutants and in *serp*^{RB} *verm*^{KG} double mutants. Other bands are cross-reacting species that are also detected in extracts of *Df(3L)Exel6135* embryos, which lack the entire *serp* and *verm* coding regions (not shown). Positions and molecular weights (kDa) of protein molecular weight standards are shown at left.}

tracheal lumen (Figure 2). No defects were detected early in tracheal development in any of the homozygous mutants analyzed. Dorsal trunk (DT) branches budded,

fused, and dilated normally. However, during stage 15 (~13 hr after egg lay at 25°C) in both *serp* and *verm* homozygous embryos, the DT began to elongate

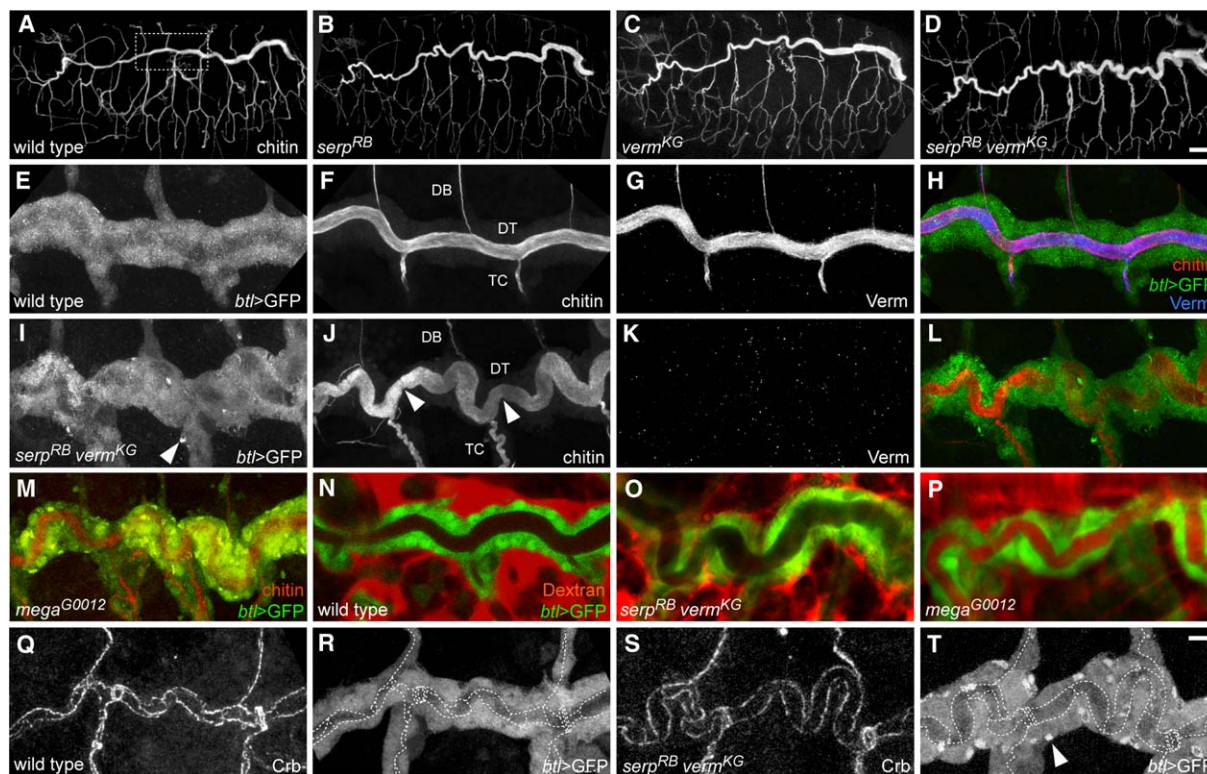


Figure 2. Effects of *serp* and *verm* Mutations on Tracheal Tube Structure

(A–D) Stage-16 embryos of the indicated homozygous genotypes immunostained with a fluorescent chitin binding probe to show the tracheal lumen. Note that tracheal tubes in *serp* (B) and *verm* (C) single mutants are slightly elongated and convoluted compared to wild-type (A), and they are severely elongated and tortuous in *serp verm* double mutants (D). The diameter of the tubes and characteristic taper of the dorsal trunk (major longitudinal branch) is not affected in the mutants.

(E–L) Close-ups of dorsal trunk (corresponding to the boxed region in [A]) of wild-type (E–H) and *serp verm* double mutant (I–L). Tracheal cells are labeled with GFP (E and I), chitin is labeled with a fluorescent chitin binding probe (F and J), and Verm protein is stained with anti-Verm (G and K). In the merged image (H and L), GFP is shown in green, chitin in red, and Verm in blue. Note that the tracheal lumen is elongated and tortuous in *serp verm* mutants (I–L) compared to wild-type (E–H). However, luminal diameter is grossly normal (Figure 2J; compare to *kkv*¹ embryos in Figure 3M, which have highly irregular tube diameters). Arrowheads in (J) indicate slight constrictions in luminal diameter. *serp verm* mutants contain normal levels of luminal chitin (compare [J] to [F]), but tracheal cells have curious bright punctae of GFP staining (arrowheads in [I] and [T]). Tracheal branches are labeled as follows in [F] and [J]: DB, dorsal branch; DT, dorsal trunk; and TC, transverse connective.

(M) Dorsal trunk of a *mega*^{G0012} homozygote labeled as in (L). *mega* encodes a claudin-like septate-junction protein [6]. Note elongated and convoluted lumen similar to *serp verm* double mutant (L).

(N–P) Test of tracheal pericellular-diffusion barrier function in *serp verm* mutants. Rhodamine-labeled dextran was injected into the body cavity of stage-16 embryos of indicated genotypes. Tracheal cells are marked by GFP expressed under control of *btl*-GAL4. The dextran (red) is excluded from the lumen of wild-type control embryo (N) and the *serp verm* mutant (O), but enters the tracheal lumen in the *mega* mutant (P).

(Q–T) Effect of *serp* and *verm* mutations on apical epithelial surface in the tracheal system. Wild-type (Q and R) and *serp verm* mutant (S and T) embryos were immunostained for Crumbs (Crb), an apical junction protein (Q and S), and tracheal cells were marked by cytoplasmic GFP (R and T). Note that apical (inner) cell surfaces are elongated in *serp verm* mutants (compare [S] to [Q]), but the basal (outer) side of tracheal cells does not appear to be similarly elongated and does not follow the tortuous path of the lumen (compare [T] to [R]). Crb staining in (Q) and (S) was traced and is shown by dotted lines in (R) and (T).

Panels (A) through (M) and (Q) through (T) are projections of serial confocal sections. Panels (N) through (P) are single confocal sections of live embryos. Scale bars: (A–D), 25 μm; (E–T), 5 μm.

inappropriately and became convoluted (Figures 2B and 2C). The effects were more dramatic in homozygous *serp*^{RB} *verm*^{KG} double mutants (Figure 2D): The DT began to elongate excessively at stage 15 and by stage 16 (15 hr AEL) was 40% longer than normal and highly convoluted (Figures 2I–2L; Figure S1 in the Supplemental Data available online). Similar effects were observed in other branches including the transverse connective (TC in Figure 2J), although the effects were not as pronounced in smaller-caliber branches, such as the dorsal branch (DB in Figure 2J). The phenotype of hemizygous *serp*^{RB} *verm*^{KG} embryos (in trans to *Df(3L)Exel6135* [13] that removes *serp* and *verm*) was indistinguishable

from homozygous *serp*^{RB} *verm*^{KG} embryos (data not shown). In contrast to the dramatic effects of the mutations on tracheal tube length and shape, there was little or no effect of the mutations on the diameter of the tubes. The DT showed its characteristic posterior to anterior taper (compare Figures 2A and 2D), and was of normal caliber except for slight constrictions that were occasionally observed near DT fusion joints in *serp*^{RB} *verm*^{KG} double mutants (arrowheads in Figure 2J). We conclude that *serp* and *verm* are required to restrict tracheal tube length. This distinguishes them from a second class of tracheal-tube morphogenesis genes that are required to establish and maintain correct tube diameter

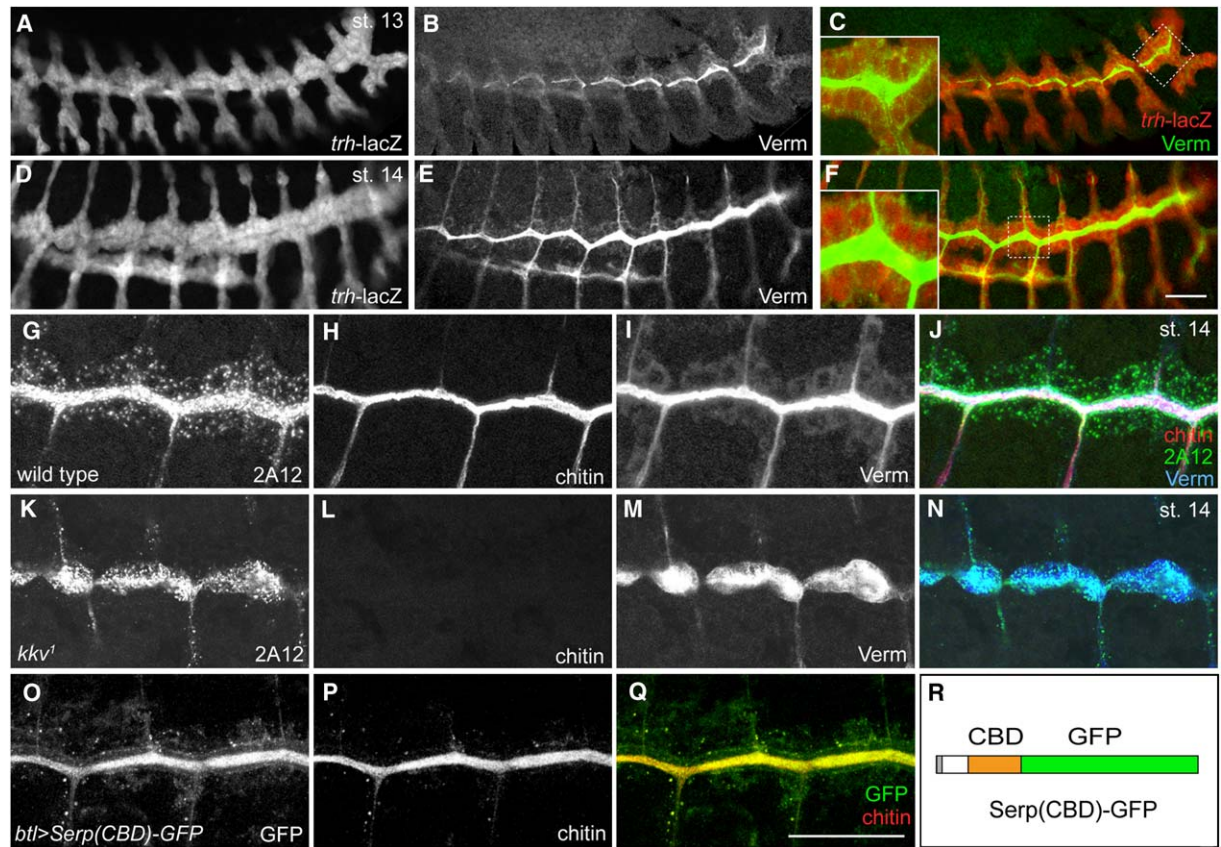


Figure 3. Serp and Verm Are Apical Extracellular Matrix Proteins that Colocalize with Intraluminal Chitin

(A–F) Stage-13 (A–C) and stage-14 (D–F) embryos carrying *trh-lacZ* that were double stained for β -galactosidase (to show tracheal cells; [A and D]) and for Verm protein (B and E). Merged images are shown in (C) and (F). Verm expression in tracheal cells is first detected in the posterior tracheal system at early stage 13 and is gradually expressed more anteriorly. Verm protein is detected in a perinuclear pattern within tracheal cells until early stage 15 (insets in [C] and [F] show higher-magnification images of boxed areas). Luminal Verm protein is first detected in the posterior DT at stage 13 (B) and gradually accumulates more anteriorly (E). By stage 15, Verm protein is present in the lumen of all tracheal branches (not shown). Serp protein shows a temporal and spatial distribution pattern similar to that of Verm (Figures 1M and 1O and Figure S2). (G–J) Close-up of dorsal trunk in stage-14 wild-type embryo triple stained for the anonymous tracheal luminal antigen 2A12 [19], chitin, and Verm. Merged image is shown in (J). Verm protein is concentrated in a cylindrical structure that is slightly smaller in diameter than the luminal cross-section, in the region that contains chitin (H–J). 2A12 antigen is distributed throughout the lumen; it shows a slightly broader intraluminal distribution than Verm and is not concentrated in the chitin cylinder (compare [G] and [H] and note green marginal zone of 2A12 staining in lumen in [J]). (K–N) Close-up of dorsal trunk in a stage-14 *kkv*¹ chitin synthase mutant embryo triple stained as above. In *kkv*¹ mutant embryos, which lack chitin (L) and show irregular tube diameter (K), Verm protein shows an inhomogeneous luminal distribution (M and N). (O–R) A transgene expressing the N terminus of Serp including the signal peptide and chitin binding domain (CBD) fused to GFP (panel [R]) was expressed in the tracheal system under the control of *btl*-GAL4. A close-up of the dorsal trunk in an embryo expressing the transgene and double stained for GFP and chitin is shown (O–Q). The N terminus of Serp including the CBD is sufficient for localization of the fusion protein to the chitinous matrix.

Panels (A) through (Q) are projections of serial confocal sections. Scale bars in (A)–(F) and (G)–(Q): 25 μ m.

and are involved in chitin synthesis (e.g., *kkv*; see Figures 3K–3N; [2, 3]).

The Apical Surface of the Tracheal Epithelium Expands in *serp verm* Double Mutants, but Epithelial-Barrier Function Is Maintained

In *serp*^{RB} *verm*^{KG} double mutants, the tracheal lumen was excessively long and formed dramatic corkscrew-like twists. Immunostaining for Crumbs (Crb) protein, which localizes to the apical marginal zone of epithelial cells (reviewed in [14]), showed that the apical tracheal surface was similarly elongated and convoluted in the mutants (compare Figure 2Q to Figure 2S). However, the basal (outer) surface of the tracheal epithelium did not appear to follow the convoluted path of the lumen

and apical surface (Figures 2I, 2J, 2L, and 2T; see also Figure 4F). This suggests that *serp* and *verm* act to selectively restrict expansion of the lumen and apical surface of the tracheal epithelium. A similar mutant phenotype has been described for *grainyhead* (*grh*), which encodes a transcription factor proposed to restrict tracheal tube elongation through transcriptional regulation of apical matrix genes [15]. Serp and Verm proteins are still expressed in *grh*^{IM} mutant embryos (Figure S2), suggesting that *serp* and *verm* are not critical targets of GRH.

The selective effect of *serp* and *verm* mutations on the apical surface and the length and convolution of tracheal tubes also resembles the tube-morphogenesis defect of mutants in *megatrachea* (compare Figures 2L and 2M; [6]) and other genes that encode components of septate

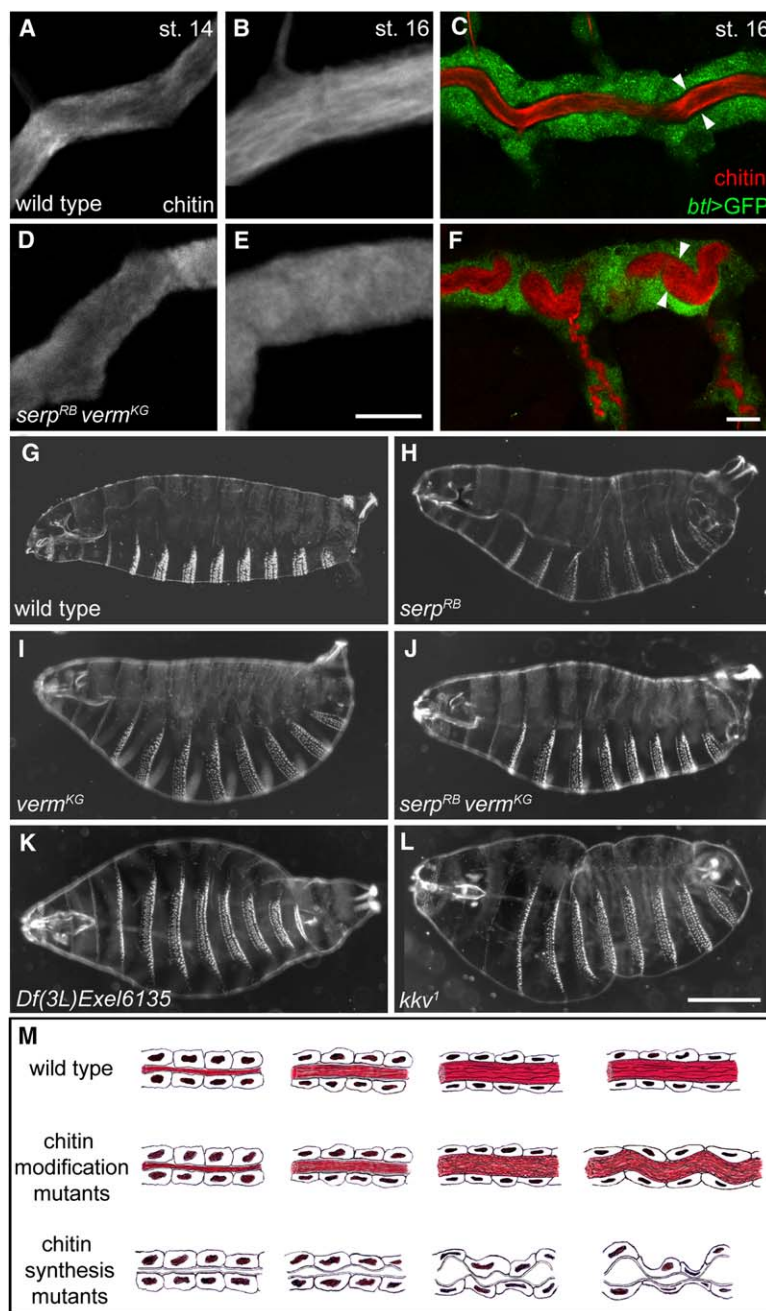


Figure 4. *serp* and *verm* Are Required for the Normal Structure and Function of Chitin Extracellular Matrices

(A–F) Effect of *serp* and *verm* on the structure of the luminal chitin cylinder in the tracheal system. High-resolution confocal images of wild-type (A–C) and *serp verm* double-mutant (D–F) embryos expressing GFP in the tracheal system that were immunostained for chitin and GFP. Embryo stages are indicated. In wild-type (A and B), the chitin cylinder has a smooth surface and fibrous texture, and there is a narrow gap between the luminal chitin cylinder and the apical cell surface (arrowheads in [C]). In the *serp verm* mutant (D and E), the surface of the chitin cylinder is irregular and chitin texture appears cloudy and unstructured, and there is no gap (arrowheads in [F]) between chitin and the apical surface. Note that the lumen winds independently of the basal epithelial surface in the *serp verm* mutant in a single confocal section (F). Panels (A), (B), (D), and (E) are projections of serial confocal sections. Panels (C) and (F) are single confocal sections. Scale bars: 5 μ m. (G–L) Effect of *serp* and *verm* mutations on epidermal cuticle. Dark-field photomicrographs of devitelinized embryonic cuticle preparations of embryos of the indicated genotypes. Note that *serp* and *verm* single (H and I) and double (J) mutants show expanded and deformed cuticles, associated with a lack of cuticle rigidity, as do *Df(3L)Exel6135* homozygotes (K) that lack both *serp* and *verm* as well as neighboring genes. Cuticle phenotypes of the single, double, and deficiency mutants were similar in strength and penetrance. *kkv¹* chitin synthase mutants show a qualitatively similar, but more severe and fully penetrant phenotype (L). Note that *Chld3* is expressed in the epidermis and could act in a partially redundant fashion with *serp* and *verm*. The scale bar in (G)–(L): 100 μ m. (M) Model summarizing the genetically separable roles of chitin synthesis and modification in controlling tracheal tube length versus diameter. Four time points in the dorsal-trunk-tube expansion process are schematized for wild-type embryos (top panels), *serp* and *verm* chitin modification mutants (middle panels), and *cystic*, *knk/gnarled*, and *kkv* chitin synthesis mutants (lower panels). Chitin is shown in red. In chitin modification mutants, chitin cylinder and lumen lengthen excessively and become convoluted. In chitin synthesis mutants, the chitin cylinder is absent and tubes do not dilate properly.

junctions (SJs; reviewed in [10]). Although the mechanism by which SJs influence tube length is not understood, all of the SJ mutants that have been tested affect the pericellular-diffusion barrier function of tracheae and other epithelia [8, 16]. To determine whether tracheal barrier function is compromised in *serp verm* double mutants, we injected rhodamine-labeled dextran (MW ~10 kDa) into the body cavity of mutant and control embryos and analyzed its distribution 25 min later [16]. In *mega^{G0012}* and other SJ mutants, dextran passes through the tracheal epithelium and into the lumen (Figure 2P). By contrast, in *serp verm* double mutants, dextran was excluded from the tracheal lumen, as it

was in the wild-type control (Figures 2N and 2O). We conclude that epithelial barrier function is grossly intact in the *serp verm* double mutant and that the tube-morphogenesis defect does not result from disruption of SJ barrier function. Below, we present evidence that the defect arises from alterations in chitin structure.

***serp* and *verm* Define a Family of Proteins with Chitin Binding and Deacetylation Domains and an LDL-Receptor Ligand Binding Domain**

serp and *verm* encode similar proteins. Both have a predicted N-terminal signal peptide, a peritrophin-A-like chitin binding domain (CBD), a single type-A

LDL-receptor ligand binding domain repeat (LDLa), and a polysaccharide deacetylase domain (Figure 1Q). The deacetylase domains show similarity to the NodB domain, which is shared by a group of bacterial and fungal enzymes with chitin deacetylase (CDA) activity (see alignment in Figure S3; [17]). CDAs modulate the physical and chemical properties of chitin by deacetylation of the β ,1-4 N-acetyl-D-glucosamine polymer, which converts chitin into chitosan (reviewed in [18]). Biological functions of CDAs and chitosan (quantitatively deacetylated chitin) have been characterized in bacteria and fungi but have not been previously described in animals. The Serp and Verm protein family was called ChLD, on the basis of the predicted domain structure (chitin binding, LDL receptor ligand binding, chitin deacetylase). The same domain organization, except for a predicted signal peptide, is shared by the product of one other *D. melanogaster* gene, which we call *Chld3* (CG17905, located at cytological position 36A13-14; Figure 1Q). *Chld3* mRNA was not detected in the embryonic tracheal system, but was detected in the epidermis at stage 16 (data not shown). Related proteins are found in other insects and in *C. elegans* (Figure S3).

Serp and Verm Are Apical Matrix Proteins that Associate with the Luminal Chitin Cylinder

Antisera were generated against short synthetic peptides derived from the *serp* and *verm* coding sequences, respectively (Figure 1Q). Immunostaining and immunoblot analysis demonstrated that the anti-Serp and anti-Verm antisera do not cross-react with the other protein (Figures 1M–1P and 1S). Serp and Verm proteins are first detected in early stage-13 embryos in a punctate perinuclear distribution in tracheal cells (Figures 3A–3C, inset in [C]; data not shown for Serp). About 1 hr later, just before DT branches fuse, the two proteins begin to accumulate in the lumen of the DT. They appear first in the lumen of the most posterior DT segments and slightly later in more anterior DT segments (Figures 3B and 3E). During stages 14 and 15, the proteins appear in the lumen of all of the other tracheal branches (Figures 3D–3F). Intracellular staining of Serp and Verm proteins persists through stage 15, but is no longer detected at stage 16.

Chitin forms a cylinder inside the tracheal lumen, and this cylinder is first detected in the DT just before DT dilation and then expands as the lumen dilates (Figure 3H [2, 3]). Luminal chitin is secreted hours before the chitinous tracheal procuticle forms at the end of embryogenesis [2, 3], and it is degraded or expelled from the tracheal lumen when the tubes are mature and just before they fill with gas (data not shown). The chitin cylinder has a slightly smaller diameter than that of the lumen, which is bounded by the apical surface of the tracheal epithelium (Figures 3G, 3H, 3P, and 3Q). Strong staining of Serp and Verm protein colocalized with the chitin cylinder, whereas there was only weak staining in the small gap between the cylinder and the apical surface of DT cells (Figures 3I and 3J; Figures S2A–S2C, S2G–S2I). This contrasts with the distribution of another luminal antigen, 2A12 [19], which was distributed throughout the entire luminal space including the gap (Figures 3G and 3J).

The colocalization of Serp and Verm with the chitin cylinder, and the presence in each protein of a chitin binding domain (CBD), suggest that the proteins directly

associate with the chitin cylinder. Two experiments support this. First, the organized and regular distribution of Serp and Verm proteins in the lumen required chitin. In embryos homozygous for *kkv*¹ (a mutation in the chitin synthase I gene), which lack tracheal chitin [2, 3, 20, 21], Serp and Verm proteins were still expressed and secreted into the lumen. However, the distribution of the proteins was altered—they formed irregular, amorphous masses in the lumen (Figure 3M and data not shown for Serp), as did 2A12 antigen (Figure 3K). Second, the CBD is apparently sufficient for localization to the chitin cylinder. A transgene expressing just an N-terminal fragment of Serp including the CBD fused to GFP [Serp(CBD)-GFP; Figures 3O–3R] showed the same colocalization with the chitin cylinder as endogenous Serp protein (Figures S2A–S2C) and full-length Serp-GFP (not shown). We conclude that Serp and Verm proteins associate with the luminal chitinous matrix and that this association is likely to be mediated at least in part by the CBD.

serp and *verm* Influence the Structure of Chitin but Not Its Luminal Accumulation

We investigated whether *serp* and *verm* mutations affect the synthesis or structure of chitin. There was no detectable effect in *serp verm* double-mutant embryos on the level of luminal chitin staining (Figures 2F and 2J), demonstrating that *serp* and *verm* function is not required for the synthesis, secretion, or luminal accumulation of chitin. Likewise, the secretion and luminal accumulation of 2A12 antigen and the zona pellucida protein PioPio [22, 23] were not disrupted in *serp verm* mutants (not shown). However, the morphology and structure of the luminal chitin cylinder was altered in *serp verm* mutant embryos. High-resolution confocal imaging of luminal chitin stained with a fluorescently labeled chitin binding protein revealed that the chitin cylinder in wild-type embryos is fibrous and has a smooth surface (Figures 4A–4B). By contrast, in *serp verm* mutants, the fibrous structure of the chitin cylinder is abolished and the surface of the cylinder is irregular (Figures 4D and 4E). Also, the small gap between the chitin cylinder and the apical epithelial surface is absent (compare Figures 4C and 4F). Morphological defects in chitin structure are apparent in *serp verm* mutants by stage 14 (Figure 4D), several hours before the elongated-tube phenotype begins to manifest. This implies that the defects in chitin structure are not a secondary consequence of the disruption in tube morphology, and support an alternative model in which a *serp*- and *verm*-dependent alteration in chitin structure influences tube length. *serp* and *verm* are also expressed in epidermal cells, and the mutations affect body shape (Figures 4G–4L), presumably by altering the structure and rigidity of epidermal cuticle, another chitinous matrix.

Summary and Model of Serp and Verm Function in Tube Length Control

We have described two new tracheal-tube morphogenesis genes that are expressed in similar patterns, encode similar proteins, and function partially redundantly to regulate tube length and curvature. The genes define a new molecular class of tube morphogenesis genes encoding apical extracellular matrix proteins that modify

the chitin matrix. Both proteins contain chitin binding and deacetylase domains, and both are secreted into the apical tracheal matrix, where they associate with, and modify the structure of, the chitin cylinder that fills most of the luminal space. In *serp verm* double mutants, the chitin cylinder still forms, but it lacks its normal fibrillar appearance. The chitin cylinder, along with the surrounding lumen and apical tracheal surface, becomes excessively long and convoluted, a process that normally occurs gradually over the next hours and days of development as the tubes expand to their mature sizes and acquire their characteristic shapes.

Our results, along with the recent identification of chitin biosynthesis genes in tracheal-tube morphogenesis, demonstrate the dual and genetically separable functions of chitin in tracheal tube diameter and length control (Figure 4M). The chitin synthesis genes *cystic*, *knk/gnarled*, and *kkv* chitin synthase are required to synthesize the expanding chitin cylinder in the tracheal lumen, which is proposed to promote and coordinate the dilation of the surrounding epithelium so that tubes reach their proper diameter [2, 3]. By contrast, the chitin modification genes *serp* and *verm* are not required for synthesis of chitin and have little effect on dilation and tube diameter. Rather, they influence the structure of the chitin cylinder and the length and curvature of the tubes. The chitin cylinder may therefore function as an internal template that plays a critical role in defining the diameter, length, and shape of the tube that surrounds it. Below, we propose a molecular model for the role of Serp and Verm proteins and the chitin cylinder in tube lengthening and suggest how this mechanism could be regulated to control the longitudinal growth of tracheal tubes during development.

There are four postulates of the model. First, we propose that the Serp and Verm proteins bind and modify chitin. Second, this modification alters the physical properties of the chitin cylinder, keeping it rigid and short. Third, these changes in the chitin cylinder are sensed by the surrounding tracheal cells, perhaps through a direct link between an apical-membrane component and a constituent of the chitin cylinder. Fourth, this signal restricts apical-membrane biogenesis, in a manner that limits polarized growth of the cell membrane specifically along the longitudinal axis of the tube.

Much data supports the first two postulates. Serp and Verm proteins are secreted into the tracheal lumen, where they associate with the chitin cylinder, an association that is likely mediated by the chitin binding domain (Figures 3H–3J and 3O–3Q). The chitin modification is most probably deacetylation of N-acetylglucosamine residues by the chitin deacetylase domain of the proteins. This is a well-known structural modification of chitin in yeast and fungi, and the enzymatic catalysis of this reaction has received much attention because of the commercial use of deacetylated chitin (chitosan) in water treatment, in the food industry, and in medical applications such as fabrication of artificial skin [18]. Deacetylation increases the solubility and decreases the density of chitin fibrils in vitro [24, 25], and it may influence the structure and orientation of chitin fibrils in arthropod cuticle [26]. This apparently increases the rigidity of the chitin matrix, as implied by the defect in cell wall rigidity in a yeast mutant lacking chitin deacetylase activity [27,

28] and by the lax epidermal cuticle in *serp verm* double mutants (Figures 4G–4L).

The last two postulates of the model are more speculative. We do not know if or how the chitin cylinder is attached to the apical cell surface. There is a characteristic $\sim 0.5\ \mu\text{m}$ gap that is between the cell surface and the chitin cylinder and is visible in fixed specimens and could contain an anchoring complex (Figure 4C). The gap is eliminated in *serp verm* double mutants (Figure 4F). How the proposed link between chitin and the apical cell surface, or a signal generated by this complex, limits apical-membrane biogenesis along the longitudinal axis of the tube is even more obscure. Perhaps it exerts a mechanical effect on the apical cell surface, physically constraining apical membrane elongation, or maybe it influences the distribution or activity of apical-basal cell-polarity regulators such as Crumbs [14]. Whatever the mechanism, the genetic results make clear that absence of Serp and Verm proteins results in a dramatic expansion of the lumen and apical tracheal surface—but only along the longitudinal axis of the tube.

Longitudinal growth of tracheal tubes normally occurs gradually and continuously during development, beginning soon after the tubes form during embryogenesis and continuing throughout larval life [5]. This allows the size and transport capability of the tracheal network to keep pace with the increasing oxygen demand of the growing larva. Because this growth occurs in the absence of tracheal cell division and is only periodically interrupted by a burst of radial growth, it must involve the gradual and continuous expansion of tracheal cells specifically in the longitudinal axis of the tube. This is reminiscent of the effects of *serp* and *verm* mutations in the embryo, except that in the mutants, tube elongation occurs more precipitately than normal. Because the *serp* and *verm* genes are expressed early and broadly in the developing tracheal system, under control of Ribbon and possibly Trachealess (Figures 1I–1L and 1R), they could act as governors on tube growth from the onset, keeping tube elongation in check. Controlled downregulation of Serp and Verm expression or activity during development could gradually release this constraint and give rise to the controlled longitudinal tube growth that is normally observed. Septate-junction mutants [10] have a similar tube-elongation phenotype to that of *serp* and *verm* mutants, so septate junctions could function to promote Serp and Verm expression or activity or to antagonize the negative regulatory pathway.

Our findings in the *Drosophila* tracheal system could have implications for the mechanisms of tube size and shape regulation in other tubular epithelial organs, including those of vertebrates. For example, blood vessels grown in vitro from human endothelial cells contain a fibrous luminal matrix that is of unknown composition and function and has been postulated to play a role in tube morphogenesis [29]. Although there are many molecular differences among the luminal ECMs of blood vessels, tracheal tubes, and other tubular organs in animals, they could act similarly to regulate and maintain the diameter, length, and shape of the surrounding tubes. Indeed, synthetic mandrils are used in this way in blood-vessel engineering [30]. It will be important to identify and characterize the components of the luminal matrices of blood vessels and other types of tubes, and

to determine whether dynamic and specific changes in the structure and physical properties of these matrices are used to regulate tube size and shape in vivo, as proposed for *Drosophila* tracheal tubes.

Experimental Procedures

Fly Stocks and Genetics

Fly stocks were obtained from the Bloomington stock center. The following mutant alleles and enhancer trap strains are described in FlyBase (<http://flybase.bio.indiana.edu/>): *grh*^{IM}, *kkv*¹, *mega*^{G0012}, *rib*¹, *rib*^{P7}, and *trh-lacZ* (*1-eve-1*). *btl-GAL4* [31] was used to drive expression of UAS transgenes in the tracheal system. PBac[RB]e02821 [32] is a PiggyBac insertion referred to here as *serp*^{RB}. P[*SUPor*-P]KG07819 [33] is a P element insertion referred to here as *verm*^{KG}. Df(3L)Exel6135 [13] deletes ten predicted genes, including *serp* (CG32209) and *verm* (CG8756). *verm*^{ex7} was generated by imprecise excision of P[y⁺, w⁺ *SUPor*-P]KG07819. The region encompassing the deletion was amplified from genomic DNA of homozygous embryos with oligonucleotides KG07819-F (5'-CCGTTCACCTCCATT CATT) and KG07819-R (5'-TCTTACGGTAGCGTTGGTC), cloned into pCR2 (Invitrogen), and sequenced with M13 forward and reverse primers. The *serp*^{RB} *verm*^{KG} double mutant was constructed by P transposase-mediated recombination in the male germ line [34]. First, the chromosome bearing the P[y⁺, w⁺ *SUPor*-P]KG07819 insertion in the *verm* gene was mutagenized with ethyl methanesulfonate (25 mM; [35]) to mutate the w⁺ minigene carried by the P element, resulting in a P[y⁺ w⁻] P element. Several y⁺ w⁻ lines were recovered, one of which was crossed in *trans* to PBac[w⁺ RB]e02821 in a background expressing P transposase (Δ 2-3). Males of the genotype y w Δ 2-3/Y; P[y⁺, w⁻ *SUPor*-P]KG07819 / PBac[w⁺ RB]e02821 were crossed en masse to y w females, and male F₁ progeny were screened for y⁺ w⁺ recombinants. Several independent recombinant lines were tested for the presence of Serp and Verm proteins on immunoblots of extracts of homozygous embryos. One recombinant line, which lacked both Serp and Verm proteins, is referred to as *serp*^{RB} *verm*^{KG} and was used for further experiments. Homozygous *serp*^{RB} *verm*^{KG} embryos showed tracheal and epidermal phenotypes indistinguishable from those in Df(3L)Exel6135 homozygotes or *serp*^{RB} *verm*^{KG} / Df(3L)Exel6135 double hemizygotes.

Molecular Biology

The UAS-Serp-GFP transgene was constructed as follows. The *serp* cDNA was excised from cDNA clone RE22242 in pFic1 (*Drosophila* Genomics Resource Center, <http://dgrc.cgb.indiana.edu/>) with NotI and KpnI and inserted into pUAST [36] digested with NotI and KpnI to generate pUAST-Serp. A C-terminal AgeI-XbaI fragment of *serp* in pUAST-Serp was replaced by a PCR fragment containing an in-frame BamHI site at the 3' end and a PCR fragment containing the coding sequence of eGFP (Invitrogen) with an in-frame BamHI site at the 5' end to make pUAST-Serp-GFP. UAS-Serp(CBD)-GFP was constructed by amplifying the N-terminal coding sequence of Serp (residues 1–125 including the signal peptide and the CBD) from RE22242 with oligonucleotides EcoRI-*serp*F (5'-TACAGAATTCAA CAGGTCCACCTAAAGATG) and *serp*-cbd-NotIR (5'-TACAGCGGC CGCACAGGGGCTCATCCGATG) and digesting the PCR product with EcoRI and NotI. This fragment was ligated along with the eGFP sequence (containing an in-frame NotI site at the 5' end and an XbaI site at the 3' end) into pUAST digested with EcoRI and XbaI. The sequences of PCR-amplified portions of the resulting plasmids were confirmed by DNA sequencing. Transgenic lines were established by P element-mediated germline transformation.

Immunostaining and Immunoblotting

The following antibodies were used: mAb 2A12 (used at 1:5 dilution; [19]); mAb Cq4 directed against Crb protein (1:10; [37]); rabbit anti-Pio (1:20; [23]); mouse-anti-GFP (1:300; Clontech); and mouse anti- β -galactosidase (1:1000; Sigma). Rabbit antisera were raised against synthetic peptides derived from the CG32209 and CG8756 coding sequences, respectively (Bio-Synthesis, Lewisville, Texas). Peptide sequences were selected to show high immunogenicity and likelihood for surface exposure, minimal conservation between

Serp and Verm proteins, and presence in all annotated protein isoforms (Figure 1Q):

Serp-C: TARKGHEIAVHSITHNDE (aa 253–270/541)

Verm-C: TELNSLRDFQEWKEKCDVKG (aa 504–523/570)

Antibodies were affinity-purified on columns containing the peptide used for immunization and used at 1:1000 dilution. Primary antibodies were detected by using secondary antibodies conjugated with Biotin, HRP, Cy3, Cy5 (Jackson ImmunoResearch), or Alexa488 (Molecular Probes). Embryos were fixed in 4% formaldehyde in PBS/heptane for 20 min and devitellinized by shaking in methanol/heptane. A chitin binding domain protein labeled with either rhodamine or FITC (New England Biolabs) was used at a dilution of 1:500 to detect chitin [2].

For immunoblotting, embryos were homogenized in sample loading buffer, and the equivalent of ten embryos was loaded per lane on SDS-PAGE gels (10% polyacrylamide). Immunoblotting and detection of HRP-conjugated anti-rabbit IgG secondary antibodies by enhanced chemiluminescence (Amersham) were done according to manufacturer's instructions.

In Situ Hybridization

In situ hybridization was carried out according to standard procedures [38]. Digoxigenin-labeled antisense RNA probes were generated by in vitro transcription in the presence of digoxigenin-labeled UTP (Roche) with PCR products as DNA templates. Oligonucleotides were designed to amplify an exon fragment (~1 kb) from genomic DNA. Reverse primers contained the T7 RNA polymerase promoter (underlined):

CG32209-F: 5'-GATGATCACCATCACGTTTCG

CG32209-R: 5'-TAATACGACTCACTATAGGGTCTGCAGTTGATG GTCT

CG8756-F: 5'-AACGTGGACAACATCGACCT

CG8756-R: 5'-TAATACGACTCACTATAGGGTTGGGCAGAGAGCAG TAGG

Digoxigenin-labeled RNA probes were detected by using alkaline phosphatase-conjugated anti-digoxigenin-Fab fragments (1:2000; Roche). For in situ hybridization analysis of *rib* target genes, embryos were collected from flies carrying *rib*¹ or *rib*^{P7} balanced over CyO, *ftz-lacZ*. Embryos were first stained with X-Gal and then subjected to in situ hybridization to allow direct comparison of homozygous and heterozygous embryos.

Cuticle Preparations

Embryos were collected on apple juice agar plates and aged for 24 hr. Unhatched embryos were dechorionated in 5% sodium hypochlorite (Klorix bleach), washed in water, devitellinized by shaking in methanol/heptane, washed in methanol, and mounted in Hoyer's medium [39].

Rhodamine-Dextran Injections

Embryos from flies carrying a *btl-GAL4* UAS-GFP chromosome (to mark tracheal cells) were injected with rhodamine-labeled 10 kDa-dextran (10 mg/ml; Sigma) as described [16] and examined on a Leica TCS SP confocal microscope.

Supplemental Data

Supplemental Data include three figures and are available with this article online at: <http://www.current-biology.com/cgi/content/full/16/2/186/DC1/>.

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