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Drosophila *convoluted/dALS* Is an Essential Gene Required for Tracheal Tube Morphogenesis and Apical Matrix Organization

Lianna E. Swanson,* Marcus Yu,* Kevin S. Nelson,* Patrick Laprise,†,[1] Ulrich Tepass† and Greg J. Beitel*,[2]

**Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208 and* †*Department of Cell and Systems Biology, University of Toronto, Toronto, Ontario M5S 3G5, Canada*

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ABSTRACT

Insulin-like growth factors (IGFs) control cell and organism growth through evolutionarily conserved signaling pathways. The mammalian acid-labile subunit (ALS) is a secreted protein that complexes with IGFs to modulate their activity. Recent work has shown that a Drosophila homolog of ALS, dALS, can also complex with and modulate the activity of a Drosophila IGF. Here we report the first mutations in the gene encoding dALS. Unexpectedly, we find that these mutations are allelic to a previously described mutation in *convoluted* (*conv*), a gene required for epithelial morphogenesis. In *conv* mutants, the tubes of the Drosophila tracheal system become abnormally elongated without altering tracheal cell number. *conv* null mutations cause larval lethality, but do not disrupt several processes required for tracheal tube size control, including septate junction formation, deposition of a lumenal/apical extracellular matrix, and lumenal secretion of Vermiform and Serpentine, two putative matrix-modifying proteins. Clearance of lumenal matrix and subcellular localization of clathrin also appear normal in *conv* mutants. However, we show that Conv/dALS is required for the dynamic organization of the transient lumenal matrix and normal structure of the cuticle that lines the tracheal lumen. These and other data suggest that the Conv/dALS-dependent tube size control mechanism is distinct from other known processes involved in tracheal tube size regulation. Moreover, we present evidence indicating that Conv/dALS has a novel, IGF-signaling independent function in tracheal morphogenesis.

INSULIN and insulin-like growth factors (IGFs) control energy homeostasis and growth through evolutionarily conserved signaling pathways (reviewed by Pavelic *et al.* 2007). Key regulators of these pathways are IGF binding proteins (IGFBPs) that modulate IGF activity, transport, and stability. The mammalian acid-labile subunit (ALS) forms ternary complexes with IGFs and IGFBP-3 or IGFBP-5 (reviewed by Boisclair *et al.* 2001). It has recently been shown that a Drosophila homolog of ALS, dALS, forms a ternary complex with Drosophila insulin-like peptide-2 (Dilp2) and the binding protein IMP-L2 (Arquier *et al.* 2008). Surprisingly, we find that the gene encoding dALS is allelic to *convoluted* (*conv*), a gene previously shown to be required for regulating the length of epithelial tubes in the Drosophila tracheal system (Beitel and Krasnow 2000).

The Drosophila tracheal system is a ramifying network of epithelial tubes that delivers oxygen directly to target tissues (reviewed by Kerman *et al.* 2006). The tracheal system is among the best characterized systems for investigating branching morphogenesis and for control of epithelial tube size, an essential feature of many vital organs such as lung and kidney. The dimensions of tracheal tubes are regulated by at least two distinct mechanisms, one of which involves a transient lumenal extracellular matrix (ECM) (Tonning *et al.* 2005) and the putative matrix-modifying proteins Vermiform (Verm) and Serpentine (Serp) (Luschnig *et al.* 2006; Wang *et al.* 2006). Importantly, lumenal secretion of Verm requires the septate junction (SJ), which restricts paracellular diffusion similar to the vertebrate tight junction, but is located in the basolateral membrane and contains polarity proteins that promote basal membrane identity (Wu and Beitel 2004; reviewed in Swanson and Beitel 2006). Mutations that disrupt the matrix or Verm secretion cause individual tracheal cells, and thus the overall tubes, to become too long (Araujo *et al.* 2005; Moussian *et al.* 2005, 2006; Luschnig *et al.* 2006; Tonning *et al.* 2006; Wang *et al.* 2006). It has not been established whether the ECM provides a signal to the epithelial cells that causes them to adjust their dimensions or whether the ECM serves as a mandrel that shapes the tubes by physical forces. However, in addition to the matrix-based size control mechanism, there is evidence that growth factor signaling constitutes a second mechanism that controls tracheal tube size

[1]*Present address:* Laval University Cancer Research Center, CRCHUQ-Hôtel-Dieu de Québec, Québec, QC G1R 2J6, Canada.

[2]*Corresponding author:* Hogan Hall, Room 2-100, Northwestern University, Evanston, IL 60208. E-mail: beitel@northwestern.edu

because mutation of *chico*, which encodes the Drosophila homolog of the mammalian insulin receptor substrates 1–4, shortens tracheal tube length to match the reduced body size of the *chico* mutant larva (Beitel and Krasnow 2000).

Our analysis of the *conv/dALS* locus reveals that Conv activity defines a new step in the matrix-based size control process. Further, although Conv/dALS could potentially act through the insulin growth factor pathway to regulate tube size, our results suggest that the tracheal matrix organization function of Conv/dALS represents a distinct function from the IGF pathway function.

MATERIALS AND METHODS

Fly strains and genetics: Fly strains were obtained from the Bloomington or Szeged stock centers except for $conv^{R278}$, $conv^{Y58}$, and the *ppl*-Gal4 and *cg*-Gal4 fat body drivers. $conv^{R278}$ was created by EMS mutagenesis of al^1 dp^{ov1} b^1 pr^1 c^1 px^1 sp^1 followed by backcrossing to remove the markers. The $conv^{R278}$ genomic sequence is CACCATCGATGTCTTGCACAATAA CATCTCC (C → T change is underlined). $conv^{Y58}$ was created by mobilization of transposable element insertion $SelD^{SH1599}$. The primers 5′-GCCTAGTAGTCCGAATCCAGTA and 5′-TGGCCCATATAGACGAACTAG were used to identify $conv^{Y58}$, which templates a 103-bp band, while the wild-type (WT) chromosome produces a 2.3-kb band. The sequence across the $conv^{Y58}$ deletion endpoints is 5′-CCAGTAATATCGGTATGCC CAT. Fat body drivers *ppl*-Gal4 and *cg*-Gal4 were a gift from P. Léopold.

Molecular biology: RNAi was performed as previously described (Kennerdell and Carthew 1998), using primers 5′-AAACGGGAAATTCTTTTTTCAG and 5′-AATTTTATAT TAGCAATTGTAC for *CG8561*. A 6-kb fragment for *CG8561* genomic rescue construct was amplified from pBACe3.6 (BACPAC resources), using primers 5′-CTGGATCCGAGCGA GATGTCACAACAGGGTTGTATTA and 5′-ATGCGGCCGCT GAATGCTGATTTATGCCATTCGCCAC, and cloned into pCasper5 (Le *et al.* 2007). The genomic rescue construct with the $conv^{R278}$ mutation was created by Quickchange (Stratagene, La Jolla, CA) of the WT construct and subcloning into pUASTattB (Bischof *et al.* 2007). The WT *conv* cDNA was amplified by RT–PCR from OregonR RNA, using primers 5′-GCGAACTTGGTTTTGGGCCATGGGATAA and 5′-TCTTGGT GCACAGTTTTCCCATGC, and cloned into pBluescriptC5 (Le *et al.* 2007) and then into pUASTattB. The 3.3-kb fragment was moved into the *Sac*II site of the pAC5-YFP plasmid in frame with the C-terminal YFP tag and the tagged construct was cloned into the pUASTattB vector. Transgenic flies were made either using P transposase (Rubin and Spradling 1982; Spradling and Rubin 1982) or the ϕC31 integration systems (Bischof *et al.* 2007). The complete sequence of all constructs was determined using BigDye sequencing (Applied Biosystems, Foster City, CA). The accession number for *conv* cDNA is EU814872.

Immunohistochemistry and imaging: Embryos were fixed and stained as previously described (Samakovlis *et al.* 1996). For clathrin stains, embryos were fixed in 4% paraformaldehyde and devitellinized in 80% ethanol to avoid the use of methanol. For assessment of lumenal clearance, WT and $conv^{R278}$ mutant embryos were collected in 1-hr lots and aged at 24°. Stage 16 embryos were predominantly found in the lot aged for 15 hr after collection, stage early 17 embryos were in the 16-hr lot, and stage mid-17 embryos were in the 17-hr lot. Primary antibodies used were anti-lumenal 2A12 1:5 (DSHB), anti-Coracle 1:500 (Fehon *et al.* 1994), anti-GFP 1:1000 (Abcam), rat anti-DE-cadherin DECAD2 1:20 (Oda *et al.* 1994), anti-Verm and Serp 1:300 (Luschnig *et al.* 2006), FITC-conjugated anti-Chitin binding probe 1:500 (New England Biolabs, Beverly, MA), and anti-clathrin 1:100 (Sigma, St. Louis). Secondary antibodies were used at 1:200 (Jackson Laboratories; Molecular Probes, Eugene, OR). To assess protein levels, heterozygous and homozygous embryos were imaged on the same slide in the same session. Electron micrograph (EM) images were obtained as in Wu *et al.* (2004). Paracellular barrier function was assessed using a 10-kDa dextran dye as described in Paul *et al.* (2003). None of nine $conv^{Y58}/Df(2R)7131$ embryos showed dye leakage.

Sequence analysis and biochemistry: Sequence alignment was done using ClustalW (Figure 2). Protein domains were predicted using SMART (Letunic *et al.* 2004). Western blotting used *btl*::Gal4 UAS-Conv-YFP 0- to 20-hr embryos or *Escherichia coli* expressing GFP homogenized in lysis buffer (Genova and Fehon 2003) and probed with rabbit polyclonal anti-GFP (Abcam) at 1:10,000. WT is OregonR.

RESULTS AND DISCUSSION

***CG8561* encodes *convoluted*:** Although the original $conv^{K6507b}$ allele was isolated from a screen of transposon-induced mutations, it is not associated with a transposable element (Beitel and Krasnow 2000). To obtain additional alleles, we performed a noncomplementation screen and isolated $conv^{R278}$, which fails to complement $conv^{K6507b}$ and causes the same overly long tracheal phenotype as $conv^{K6507b}$ (Figure 1D).

To clone *conv*, we used recombination mapping and deficiency complementation to define a region of ~20 genes containing both $conv^{K6507b}$ and $conv^{R278}$ (Figure 1I, supplemental Table S1). Sequencing and dsRNAi knockdown of all predicted genes in this interval revealed that *CG8561* was the only gene that had a codon-altering base change in the $conv^{R278}$ strain and whose knockdown by dsRNAi injection caused a "long trachea" phenotype (Figure 1, E and K). To confirm that *CG8561* encodes *conv*, we created transgenic flies bearing a 6-kb genomic fragment that entirely contained *CG8561* but overlapped the RNAs of adjacent genes by <29 bp. This genomic fragment rescued the tracheal defects (24/24) and lethality caused by the $conv^{R278}$ and $conv^{k6507b}$ mutations, as well as the tracheal defects caused by the small deficiency *Df(2R)7131* that deletes *CG8561* (Figure 1G and data not shown). Importantly, the rescue ability of this genomic fragment was eliminated by introduction of base changes that either correspond to the $conv^{R278}$ mutation or create a premature stop codon in the middle of the third exon (Figure 1H and data not shown). Together with the ability of a *CG8561* cDNA to rescue tracheal defects (see below), these results demonstrate that *CG8561* encodes *conv*.

Convoluted is a leucine-rich repeat protein with similarity to mammalian ALS and Drosophila adhesion and signaling proteins: *conv* has very low embryonic expression levels, and no *conv* cDNAs were present in

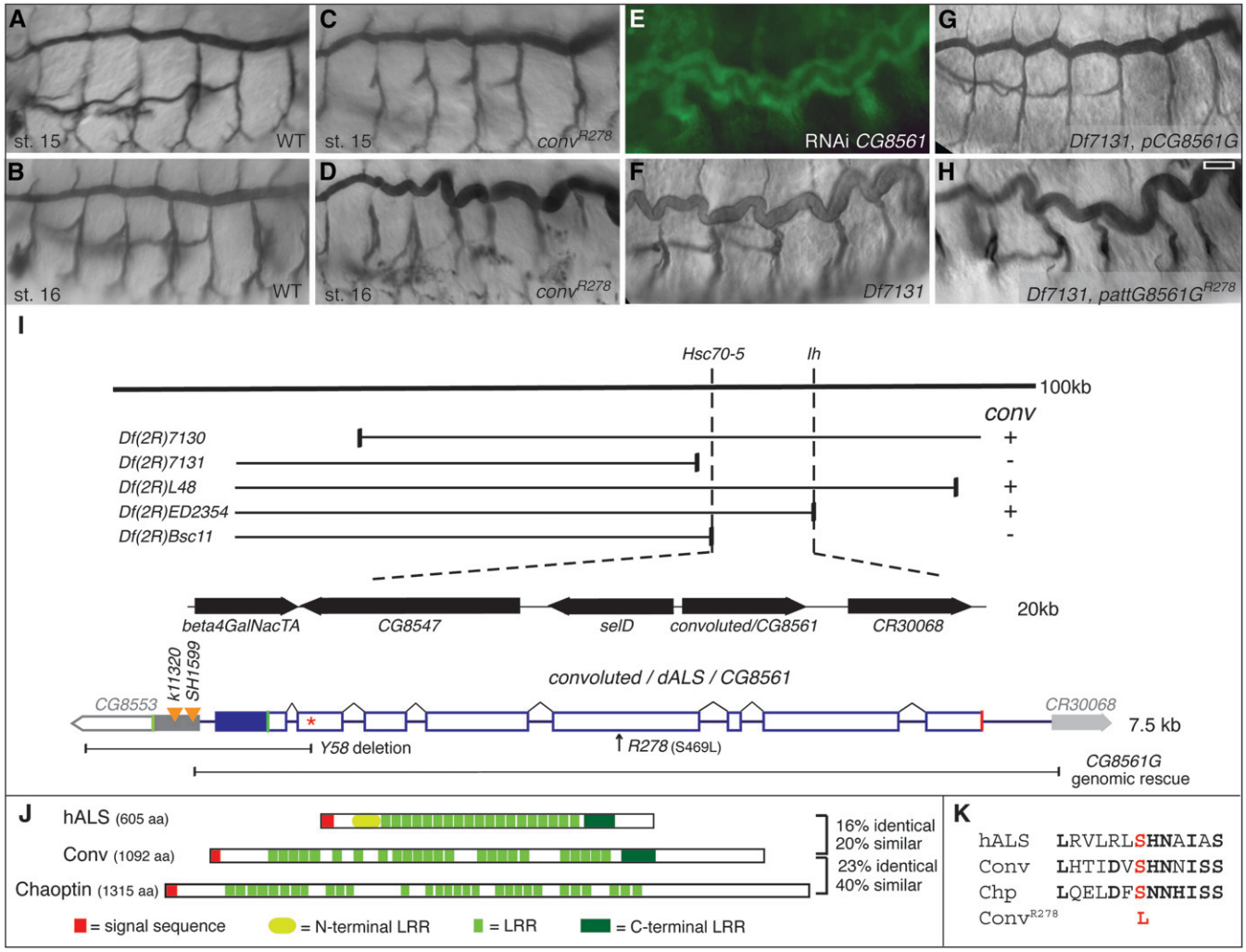

Figure 1.—Convoluted/dALS is an LRR protein required for tracheal tube morphogenesis. (A–D) The trachea in *conv* mutant embryos appear normal through stage 15 (C), but by stage 16 become overly long and "convoluted" (D). (E) RNAi knockdown of *CG8561* causes the *conv* phenotype. (F–H) Embryos homozygous for *Df(2R)7131* show the *conv* phenotype (F), which can be rescued by a genomic construct containing *CG8561* (G) but not by the same construct bearing the $conv^{R278}$ point mutation (H). (I) Schematics of the *CG8561* genomic region and transcript organization. Complementation tests between genetic deficiencies and $conv^{K6507b}$ and $conv^{R278}$ located *conv* between *Hsc70-5* and *Ih*. Sequence analysis and RNAi (E) identified *CG8561* as *conv*. The mutations for $conv^{R278}$ and $conv^{Y58}$ are indicated, as is the fragment used for the genomic rescue construct and the location of the stop codon that eliminates rescue ability of the construct (red asterisk). No base changes in the 6-kb rescue fragment were found in the $conv^{K6507b}$ allele. Green boxes, start codons; red box, stop codon; open bars, open reading frame; solid bars, noncoding transcribed regions; orange triangles, *P*-element insertions in SelD. (J) The domain organization of Conv and its similarity to human ALS (hALS) and the Drosophila adhesion protein Chaoptin are shown. The percentage of similarity and identity is relative to Conv. (K) ClustalW alignments of Conv, hALS, and Chaoptin in the region of the $conv^{R278}$ mutation. Residues in common with Conv are in boldface type, and the $conv^{R278}$ mutation is in red. Trachea were visualized with *btl*::Gal4 UAS-GFP in E and with mAb 2A12 in A–D and F–H. Bar in H (for A–H), 10 μm.

the publicly available cDNA libraries. We therefore used RT–PCR to assemble a complete cDNA and to confirm the predicted gene structure. The full-length cDNA is 3.3 kb long and has an open reading frame (ORF) of 1092 amino acids (aa) preceded by in-frame stop codons (Figure 1I and Materials and Methods). No evidence for alternative splicing was observed.

The Conv protein has a strongly predicted signal sequence at the N terminus and multiple leucine-rich repeats (LRR) that are commonly involved in protein–protein interactions. A BLASTP search revealed that the closest human homolog of Conv is ALS of the insulin growth factor binding complex (Boisclair *et al.* 2001). In strong support of Conv having functional as well as sequence similarity to human ALS, recent work by Arquier *et al.* (2008) has demonstrated that Conv/dALS can bind and antagonize Dilp2 and that altering Conv/dALS levels can affect metabolism. As we have previously shown that larval tracheal length is reduced in *chico* mutants, which have reduced insulin-like growth factor signaling (Beitel and Krasnow 2000), the increased length of trachea in *conv* mutants is consistent

with *conv* functioning to regulate tracheal tube length through insulin-like peptide signaling. Furthermore, the *R278* mutation is located in a region of significant similarity between ALS and Conv/dALS and could potentially disrupt Conv/dALS insulin binding functions (Figure 1K).

Alternatively, although ALS is the closest human homolog of Conv/dALS, there are substantial differences between the two proteins that have not previously been noted (Figure 1J). ALS is 605 aa long, has 19 tightly packed typical LRR domains, and has both N- and C-terminal class LRR domains. In contrast, Conv is 1092 aa long, has 23 somewhat dispersed LRR domains, and has a C- but not N-terminal class LRR domain. BLASTP searches of the Drosophila proteome using Conv/dALS reveal that Conv/dALS and human ALS (BLASTP score 181) have similarity that is less than or comparable to Conv/dALS and other Drosophila LRR proteins, including the cell adhesion protein Chaoptin (Reinke *et al.* 1988; Krantz and Zipursky 1990) (score 199), the Toll-7 and Toll-6 receptors (scores 181 and 179), and the 18-wheeler transmembrane protein (score 171) that controls salivary gland cell shape (Tauszig *et al.* 2000; Kolesnikov and Beckendorf 2007). Similarly, ClustalW alignment and Bootstrap tree analysis indicates that Conv/dALS is as related to Chaoptin and other LRR proteins as it is to ALS (Figure 2). Thus, while strong evidence supports Conv/dALS being the functional homolog of human ALS in IGF signaling, the divergence of human ALS and Conv/dALS is also consistent with Conv/dALS having an unanticipated IGF-independent function in tracheal morphogenesis.

***convoluted* is an essential gene:** Because Conv/dALS might be a multifunctional protein and it was unclear whether $conv^{K6507b}$ and $conv^{R278}$ were null alleles, we created a *conv* null allele with which to definitively characterize Conv/dALS functions. Imprecise excision of the *P*-element $SelD^{SH1599}$ created the small deficiency $Df(2R)conv^{Y58}$ that deletes the entire intergenic region 5′ of *conv* as well as the first exon and a half of *conv* that include the transcriptional start site (Figure 1I, materials and methods). No *conv* transcript is detected by RT–PCR in $conv^{Y58}$ homozygotes. Although $conv^{Y58}$ also disrupts the adjacent gene *SelD*, *SelD* does not have a role in tracheal development because no tracheal defects are apparent in $SelD^{K11320}$, $SelD^{SH1599}$, or nine new excision alleles that disrupt *SelD* but complement $conv^{R278}$ (data not shown). More definitively, the 6-kb *conv* genomic fragment that does not include any *SelD* ORF completely rescues the tracheal defects of $conv^{Y58}$ and *Df(2R)7131* homozygous embryos (Figure 1, G and I). We therefore consider $conv^{Y58}$ a molecular null allele of *conv*.

Embryonic morphogenesis appears normal in both $conv^{R278}$ and $conv^{Y58}$, with the notable exception of tracheal tube size-control defects (see below). Conv/dALS function is largely or entirely dispensable for neural and muscle function as larvae homozygous for $conv^{R278}$ or $conv^{Y58}$ hatch and begin crawling. However, neither these homozygous larvae nor larvae *trans*-heterozygous for $conv^{R278}/conv^{Y58}$ survive past the second larval stage, which is presumably due to the 100% penetrant failure of *conv* mutant trachea to fill with air and thereby provide oxygen to target tissues (Figure 3, B and D). Thus *conv/dALS* is an essential gene under normal conditions, which is in contrast to the viable null phenotype of Imp-L2, the insulin-binding protein required for Conv/dALS to bind Dilp2-containing complexes, and in contrast to the viable null phenotype of *chico*, the fly homolog of human insulin receptor substrates 1–4 (Bohni *et al.* 1999; Honegger *et al.* 2008).

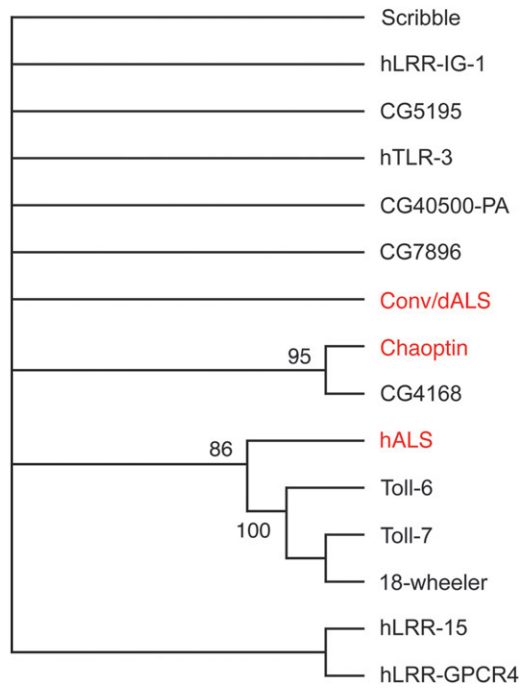

Figure 2.—Convoluted is not closely related to other LRR proteins. A phylogenetic tree shows the relationship between Convoluted/dALS and other human and Drosophila LRR proteins as calculated using a "bootstrap" procedure that more robustly determines relationships of divergent proteins than do typical single-iteration approaches. When tested with the set of closest human (proteins starting with "h") and Drosophila homologs of Convoluted identified by BLAST searches, Convoluted/dALS did not reproducibly show close relationships to any of the LRR proteins in 1000 samplings of the data set. Drosophila Scribbled was used as an outgroup for rooting the trees. Proteins were aligned using the ClustalW and bootstrap algorithms implemented by the MacVector 7 program (MacVector, Cary, NC).

Convoluted is required for tracheal tube-size control, but not for SJ assembly, chitin matrix deposition, or chitin deacetylase secretion: The trachea of embryos homozygous or *trans*-heterozygous for *conv* mutations becomes abnormally long during stage 16 (Figure 1D). The onset and severity of the phenotypes caused by $conv^{R278}$, $conv^{Y58}$, *Df(2R)7131*, or $conv^{R278}$ in *trans* to $conv^{Y58}$ or *Df(2R)7131* are indistinguishable, suggesting that $conv^{R278}$ is null for the tracheal functions of *conv*.

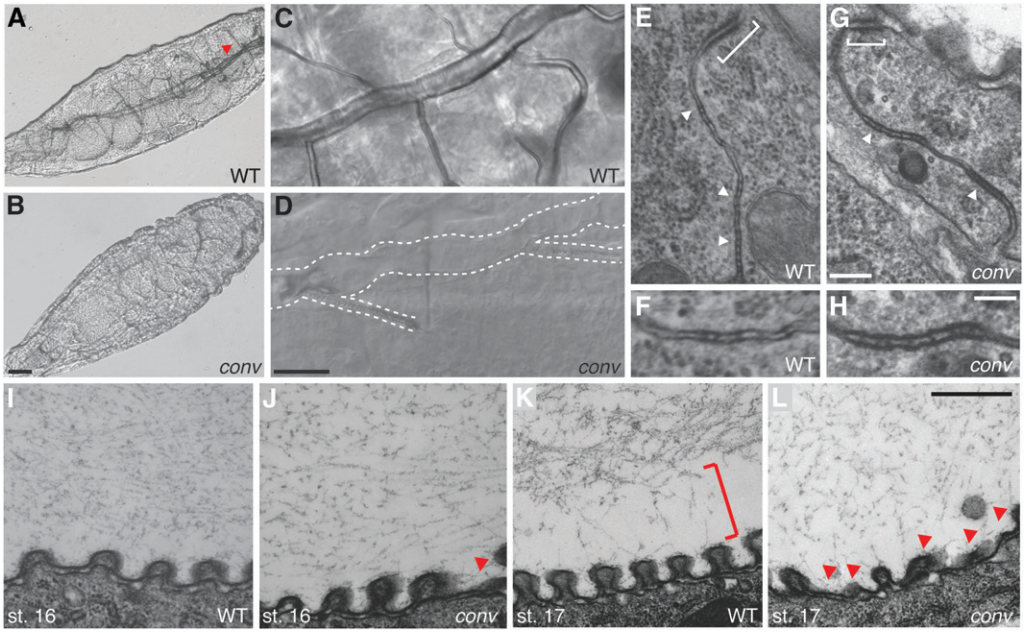

FIGURE 3.—Convoluted is required for extracellular matrix organization and for gas filling, but not for septate junction ultrastructure. (A–D) Both WT (A and C) and *conv* (B and D) larvae hatch and move, but most regions of the trachea in *conv* mutants are not filled with air and are poorly visualized (trachea in A indicated with red arrowhead and in D with dotted lines). (E–H) Adherens junctions (brackets) and septate junctions (arrowheads in E and G; higher magnification in F and H) appear normal in EM images of stage 17 embryos. (I–L) EM images show that at stage 16 the lumenal matrix is more dense in WT (I) than in *conv* embryos (J) and that some taenidial ridges (red arrowheads) are abnormal in *conv* mutants. By stage 17, *conv* mutants lack the normal gap (red bracket in K) between the matrix and the lumen wall and the taenidial defects are more pronounced. Genotypes: *conv* is $conv^{R278}/conv^{Y58}$ in B and D and $conv^{R278}/Df(2R)7131$ in G, H, J, and L. Bars: B (for A and B), 25 μm; D (for C and D), 10 μm; G (for E and G), 200 nm; H (for F and H), 100 nm; and L (for I–L), 500 nm.

To understand the role of Conv/dALS in tracheal tube-size control, we asked if *conv* mutations affect known mechanisms of tube-size control (reviewed in SWANSON and BEITEL 2006). Localization of apical polarity and SJ markers appears normal in *conv* null mutants, as is the ultrastructure of intercellular septa that form paracellular diffusion barriers (Figures 3, G and H, and 4E and data not shown). Consistent with this, *conv* mutants have no paracellular barrier defects as evidenced by wild-type-like impermeability of trachea and salivary glands in $conv^{Y58}/Df(2R)7131$ embryos to a 10-kDa dextran dye (data not shown). Chitin biosynthesis is not affected, as chitin accumulates normally in the tracheal lumen (Figure 4F), as do the putative chitin deacetylases Verm and Serp (Figure 4, G and H). Together, these results indicate that Conv/dALS is not required for the known tracheal tube-size control mechanisms that operate before stage 16.

Convoluted is required for tracheal lumenal and apical extracellular matrix organization: The defect common to most currently identified mutations affecting tracheal tube-size control is that they disrupt organization of the tracheal apical and/or lumenal extracellular matrix (reviewed in SWANSON and BEITEL 2006). In *conv* mutants, EMs show that at stage 16, the lumenal matrix appears to be less dense and somewhat grainier than in WT (Figure 3, I and J). By stage 17 the lumenal matrix in *conv* mutants appears even more sparse (Figure 3, K and L), and while WT embryos create a gap between the lumenal matrix and the tracheal tube surface (Figure 3K, bracket), in *conv* mutants the matrix still extends to the tracheal surface (Figure 3L). The failure of *conv* mutants to create a gap is also detectable by immunohistochemical staining of Verm. In stage 17 WT embryos, gaps are visible between the apical surface and the Verm-stained lumenal matrix (Figure 4D, spaces adjacent to arrowheads that mark the apical surface of the tracheal cell), while in *conv* mutants Verm continues to occupy the entire lumenal space and is not organized into fibrils (Figure 4H).

In addition to lumenal matrix defects, *conv* mutants have grossly abnormal morphogenesis of the apical matrix (cuticle) that lines the tracheal surface. In *conv* mutants, the taenidia, which normally are stereotyped periodic ridges in the highly organized apical matrix, are frequently misshapen and flattened (Figure 3, I–L). Thus, Conv/dALS is required for normal organization and modulation of apical and lumenal matrices that control tracheal tube size. This result is unexpected because neither human ALS nor components of the Drosophila IGF pathway have been observed to be required for apical extracellular matrix organization (BOHNI *et al.* 1999; HONEGGER *et al.* 2008) and we have not observed aberrant embryonic tracheal morphologies in either *chico* or *Imp-L2* mutants (BEITEL and KRASNOW 2000 and data not shown).

Convoluted defines a new step in the matrix-based tracheal tube-size control process: As mutations in genes encoding SJ proteins and *conv* both cause lumenal matrix defects, we performed additional genetic tests to determine whether there were differences between the effects of *conv* and SJ mutations on the

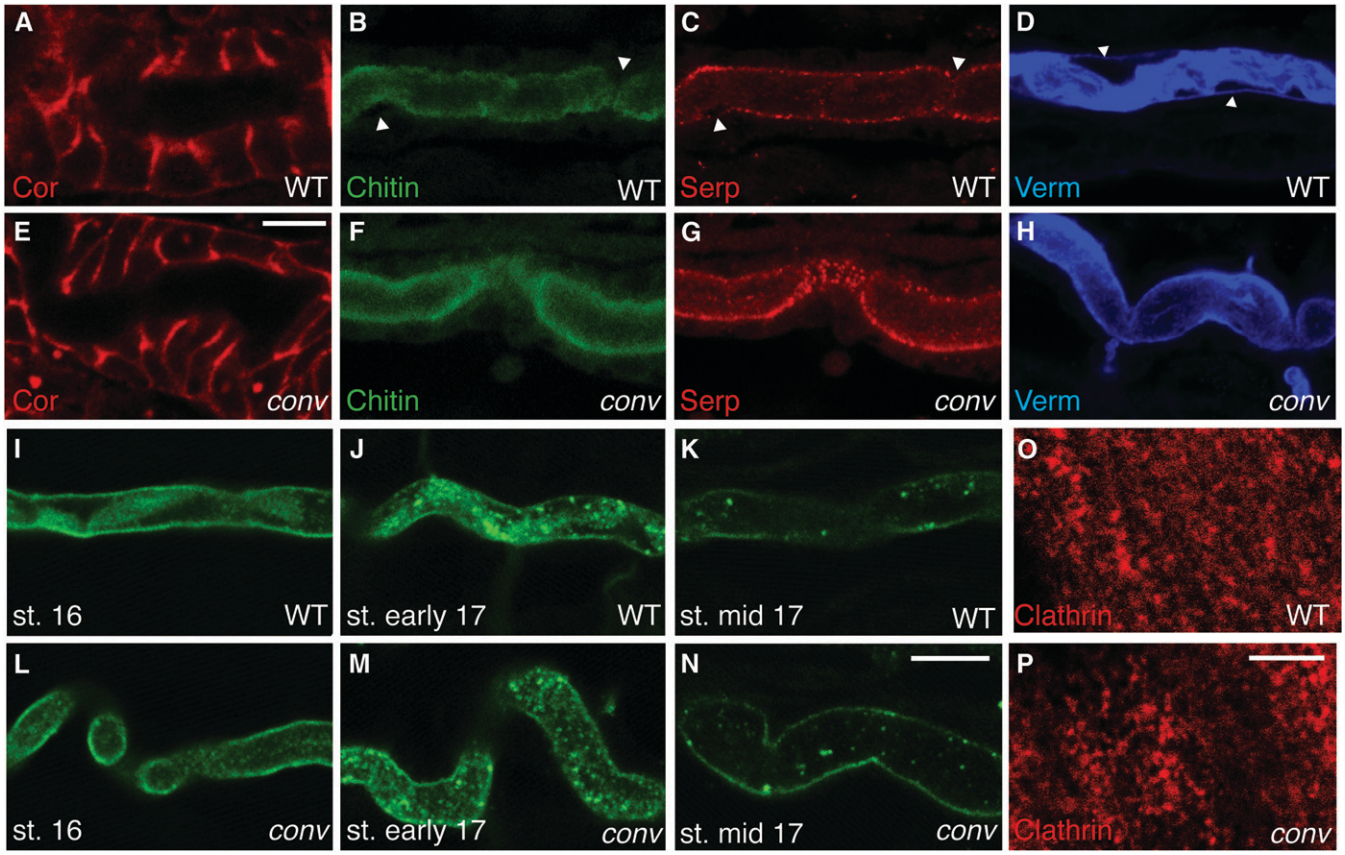

FIGURE 4.—Convoluted is not required for junction formation or secretion. (A and B) Localization of the canonical SJ marker Coracle is normal in *conv* mutants. (B–H) Lumenal matrix components chitin, Serp, and Verm are present at similar levels in stage 17 WT (B–D) and *conv* (F–H) embryos, but the gaps that normally appear between the lumenal walls and the matrix (white arrowheads) are missing in *conv* mutants. (I–N) Clearance of the lumenal antigen 2A12 proceeds equivalently in WT (I–K) and *conv* (L–N) embryos. (O and P) Clathrin shows diffuse cytoplasmic staining in the epidermis of both WT (O) and *conv* (P) embryos. Clathrin staining in the trachea was also cytoplasmic in both WT and *conv* embryos (data not shown). Genotypes: *conv* is $conv^{R278}/conv^{Y58}$ in E–H and is $conv^{R278}/conv^{R278}$ in I–P. WT, OregonR; st., stage (see MATERIALS AND METHODS). Bars: E (for A and E), 5 μm; N (for B–N), 10 μm; and P (for O and P), 5 μm.

extracellular matrix. We examined the genetic interactions of *varicose* (*vari*), which encodes an adaptor protein critical for SJ formation (WU *et al.* 2007), and of *conv* with *piopio* (*pio*) and *dumpy* (*dp*). Pio and Dp are extracellular matrix proteins deposited in the tracheal lumen that contain ZP domains (WILKIN *et al.* 2000; JAZWINSKA *et al.* 2003; BOKEL *et al.* 2005). ZP domains are named after proteins that form the zona *p*elucida, a gel-like substance surrounding mammalian oocytes (JOVINE *et al.* 2005). Intriguingly, ZP domains can polymerize to form strands (JOVINE *et al.* 2002), which in the trachea could potentially play a role parallel to that of the chitin-based fibrillar matrix. Alternatively, Pio and Dp are transmembrane proteins and thus could act as mediators of chitin-fibril-based signaling or scaffolding. Pio and Dp are required for the cell intercalation that produces unicellular tracheal branches (JAZWINSKA *et al.* 2003; RIBEIRO *et al.* 2004). In strong *pio* and *dp* mutants intercalation fails to stop and unicellular tracheal branches become disconnected (Figure 5, F and N). As the multicellular tracheal tubes in *pio* and *dp* mutants have normal length and diameter, these ZP proteins were not thought to have a role in tracheal tube-size control. However, we find that both a strong mutation in *pio*, $pio^{2R\text{-}20}$, and a viable mutation in *dp* that does not cause branch breaks, dp^{ov1} (Figure 5D), significantly enhanced the tracheal tube elongation defects of both weak and strong mutations in *vari* (Figure 5, G and H). This enhancement was specific because a lethal mutation affecting DE-cadherin, shg^{G119}, that reduces DE-cadherin levels by >50% (Figure 5L) and causes sporadic tracheal branch breaks, did not enhance the *vari*, *dp*, or *pio* mutant phenotypes (Figure 5, I and J, and data not shown).

In contrast to the enhancement of the *vari* length defects by *dp* and *pio*, double-mutant combinations of a strong *conv* allele and *dp* or *pio* did not have increased tracheal length (Figure 5, O and P), even when the *dp conv* double mutant was constructed with a stronger allele of *dp*, dp^{OVLR}, that causes a fully penetrant branch-break phenotype (Figure 5, N and P). Thus, although the exact role of *pio* and *dp* in tracheal length control

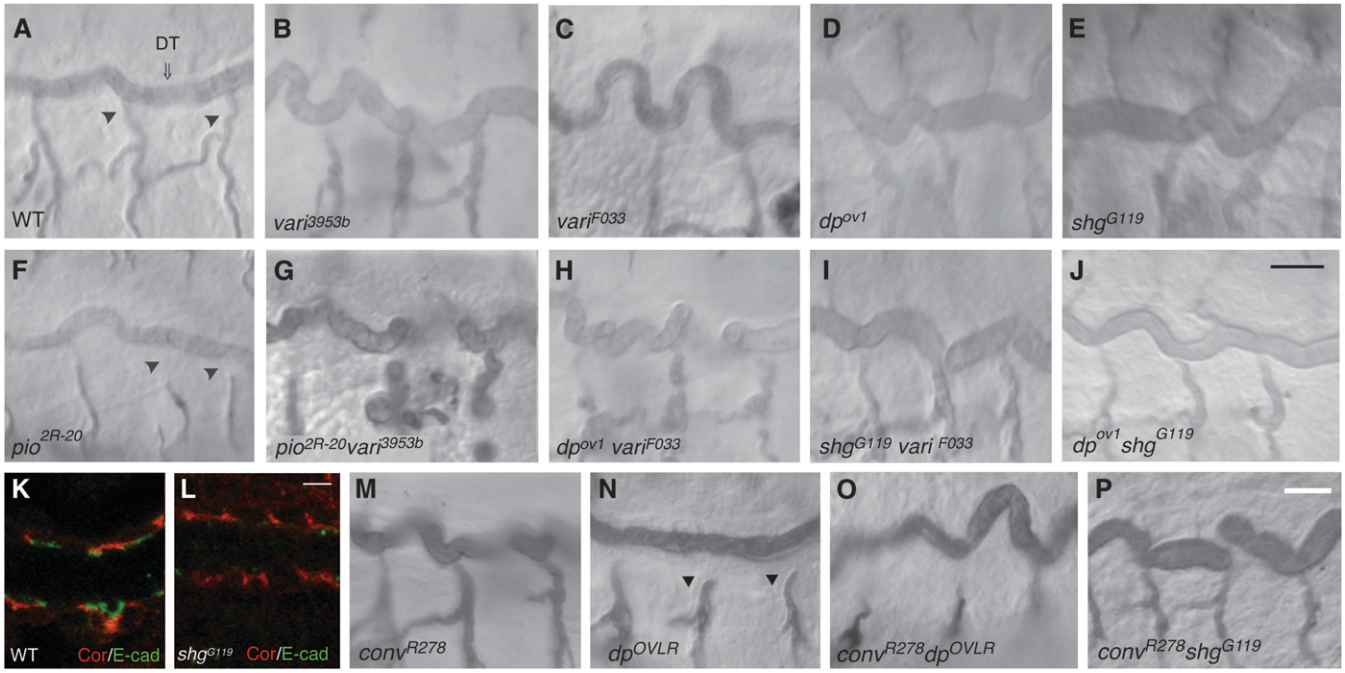

Figure 5.—Mutations in the ZP proteins Pio and Dp enhance Vari but not Conv/dALS mutations. (A–H) Whereas in *vari* (B and C) and *conv* (M) mutant embryos the tracheal dorsal trunk (DT) is long and tortuous compared to wild type (WT) (A), *pio* (F) and *dp* mutants have straight trachea (C and N). Strong *pio* and *dp* mutants have disconnected tracheal branches (arrowheads in F and N). The tracheal length defects caused by a strong *vari* mutation (C) are enhanced (I) by a weak mutation in *dp* that does not by itself cause length increases or branch breaks (D). The trachea length defects caused by weak mutation in *vari* (B) are also enhanced (G) by a *pio* mutation (F). (I–L) The specificity of the interactions of *vari* with *dp* and *pio* is demonstrated by the failure of the viable shg^{G119} allele (E) to enhance the tracheal length defects of a null *vari* allele (I) despite the fact that shg^{G119} homozygotes have significantly reduced tracheal E-cadherin staining (K and L) and have sporadic tracheal branch breaks (not shown). (M–P) Neither a strong mutation in *dp* (N) nor the weak DE-cadherin mutation shg^{G119} (E) enhance the tracheal length defects caused by a strong *conv* mutant (O and P). Bars: J (for A–J), 10 μm; L (for K and L), 2.5 μm; and P (for M–P), 10 μm.

remains to be determined, these results provide genetic evidence that *conv* has distinct effects from a SJ mutant on lumen matrix and size control. This possibility is further supported by double-mutant combinations of *conv* and *vari* and of *conv* and *coracle* showing enhanced tracheal length defects (data not shown). Thus, the interactions of *conv* mutations with mutations in ZP and SJ genes indicate that Conv has a distinct role from SJ proteins controlling tracheal tube size.

Mutations in *conv* cause tracheal tube-length and matrix defects similar to those caused by mutations in the *wurst* locus, which encodes a J-domain transmembrane protein that is required for clathrin-mediated endocytosis of lumenal material (Behr *et al.* 2007). However, in contrast to *wurst* mutants, lumenal clearance of the 2A12 marker (Figure 4, I–N) and Verm (data not shown) in the *conv* mutant was the same as in wild type. Similarly, in *conv* mutants, clathrin had a dispersed cytoplasmic localization in epidermal and tracheal cells that was indistinguishable from that of wild-type embryos (Figure 4, O and P, and data not shown) and markedly different from the striking membrane localization observed in *wurst* mutant epidermis (Behr *et al.* 2007). Therefore, Conv/dALS is not required for lumenal clearance and does not appear to regulate endocytosis. Taken together, our data suggest that in the temporal sequence of events, Conv/dALS acts after SJ proteins but before Wurst in controlling tracheal tube size and defines a new step in this process.

Expression of Convoluted in the trachea but not the fat body rescues epithelial morphogenesis defects: If Conv/dALS functions as a matrix-organizing or cell-adhesion protein, one would expect it to be localized to the tracheal apical cell surface or lumen. Unfortunately, our attempts to raise antibodies to Conv/dALS protein have been unsuccessful. We therefore attempted to determine the subcellular localization of Conv/dALS by expressing a YFP-tagged protein in the tracheal system using the ubiquitous *da*-Gal4 that efficiently expresses and rescues many tracheal genes (Wodarz *et al.* 1995; Wu *et al.* 2004, 2007; Paul *et al.* 2007). When expressed with *da*-Gal4, Conv::YFP showed little accumulation in the tracheal system and its subcellular localization could not be reliably determined in tracheal cells by YFP fluorescence or by anti-YFP immunofluorescent staining (data not shown). Interestingly, although little Conv::YFP was evident in the trachea, *da*-Gal4 driving Conv::YFP almost completely rescued embryonic tracheal morphological defects (90%, $n = 20$; no rescue of adult viability, $n > 100$), suggesting

either that very little Conv/dALS is required in the embryonic tracheal system or that Conv/dALS does not act in the embryonic tracheal system.

To more directly address whether Conv/dALS acts in the tracheal system, we expressed Conv/dALS and Conv::YFP using the *btl*-Gal4 driver that expresses only in the tracheal system and in some glia in the central nervous system (Shiga *et al.* 1996). With this driver, Conv::YFP localized to the tracheal cytoplasm, suggesting that it was inefficiently trafficked to the cell surface (Figure 6B). Similar results were obtained by Arquier *et al.* (2008) with a myc::dALS construct expressed in the fat body and in cultured S2 cells. For the Conv::YFP fusion, the cytoplasmic localization was not an artifact of cleavage of the C-terminal YFP tag as Western blots showed that almost all detectable GFP immunoreactivity was in a high molecular weight band that corresponds to the correct size of the Conv::YFP fusion protein (Figure 6C). There was no obvious accumulation of tagged protein in the tracheal lumen, apical surfaces, or basal surfaces.

Despite poor trafficking of Conv::YFP in the tracheal system, both tagged- and untagged-expression constructs fairly efficiently rescued embryonic tracheal defects in *conv* mutants (66%, $n = 18$, and 80%, $n = 25$, respectively; Figure 6, B and E), but did not rescue the viability defects. As this rescue was achieved using the *btl*-Gal4 driver, this result is consistent with Conv/dALS acting as a cell-adhesion or matrix-organizing factor in the tracheal lumen. However, this result is also consistent with local rescue of IGF pathway function since Honegger *et al.* (2008) have shown that ectopic expression of Imp-L2 in imaginal eye cell clones can locally reduce ommatidia size. We therefore expressed Conv/dALS in the fat body using the *ppl*-Gal4 driver (Colombani *et al.* 2003). Although this driver was successfully used in combination with Conv/dALS constructs by Arquier *et al.* (2008) to alter IGF signaling pathway functions, no rescue of the tracheal defects was observed using either the untagged or the tagged constructs (Figure 6, F and G). A similar lack of rescue was observed using the *cg*-Gal4 driver (Hennig *et al.* 2006), which expresses in the fat body as well as in hemocytes (Figure 6H). These results suggest that Conv/dALS acts autonomously in the tracheal system.

Concluding remarks: Drosophila Conv/dALS has been characterized as an important player in the IGF signaling pathway where it forms a ternary complex containing Imp-L2 (Arquier *et al.* 2008). Here we report the first characterization of mutations in the gene encoding Drosophila ALS and find that in contrast to *Imp-L2*, *conv/dALS* is an essential gene. Surprisingly, Conv/dALS has an important role in tracheal epithelial morphogenesis, where it limits tube elongation. Although these results raise the fascinating possibility that Conv/dALS could act by dampening IGF signaling to prevent abnormal tracheal growth, the observations

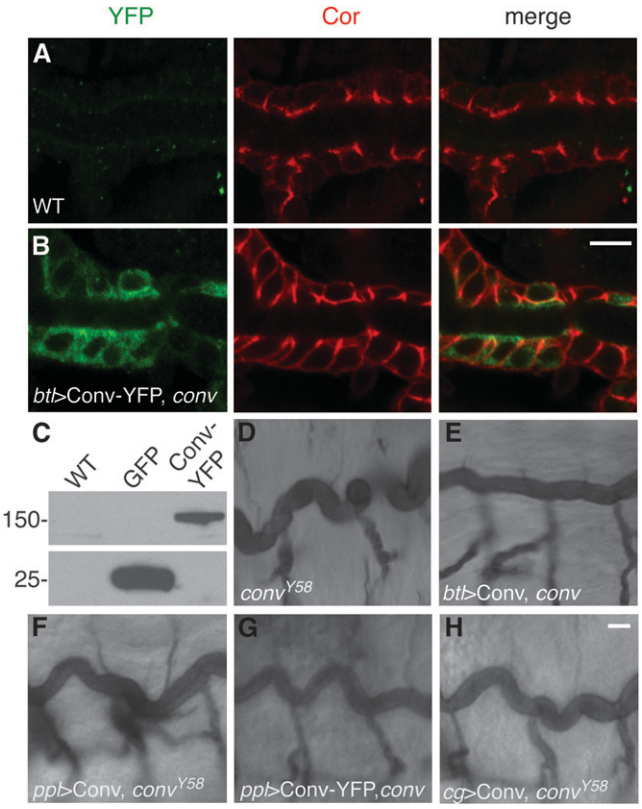

Figure 6.—Expression of Convoluted/dALS in the trachea rescues tracheal defects. (A and B) Specific expression of YFP-tagged Conv in the trachea can rescue *conv* mutants (note straight trachea in B), although anti-GFP staining shows a predominantly cytoplasmic localization (B). (C) Western blotting using anti-GFP shows that the immunoreactivity is Conv-YFP fusion protein and not just cleaved tag. Full-length Conv-YFP and YFP are predicted to be 149 and 29 kDa, respectively. Molecular weight markers are indicated. (D and E) Expression of untagged Conv/dALS in the tracheal system also rescues the tracheal defects of $conv^{Y58}$ homozygotes (E). (F–H) Expression of untagged (F and H) or tagged (G) Conv in the fat body using the *ppl*-Gal4 (F and G) or *cg*-Gal4 drivers does not rescue the tracheal defects of the $conv^{Y58}$ homozygotes. The genotype in B is *btl::*Gal4, $conv^{R278}$/*UAS-Conv-YFP*, $conv^{Y58}$; that in E is *btl::*Gal4, $conv^{R278}$/*UAS-Conv*, $conv^{Y58}$; and that in G is *ppl::*Gal4, $conv^{R278}$/*UAS-Conv-YFP*, $conv^{R278}$. Bars: B (for A and B), 5 μm; and H (for D–H), 10 μm.

that Imp-L2 is not required for tracheal morphogenesis and that Conv/dALS appears to act autonomously in the tracheal system suggest that Conv/dALS has a tracheal-matrix organizing function that is distinct from its IGF-binding function. The exact role of Conv/dALS in matrix organization is unclear, but the apparently low level of embryonic Conv expression suggests that Conv/dALS acts as an important regulator rather than a structural component of the lumenal extracellular matrix.

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