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The Drosophila Claudin Kune-kune Is Required for Septate Junction Organization and Tracheal Tube Size Control

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ABSTRACT

The vertebrate tight junction is a critical claudin-based cell–cell junction that functions to prevent free paracellular diffusion between epithelial cells. In Drosophila, this barrier is provided by the septate junction, which, despite being ultrastructurally distinct from the vertebrate tight junction, also contains the claudin-family proteins Megatrachea and Sinuous. Here we identify a third Drosophila claudin, Kune-kune, that localizes to septate junctions and is required for junction organization and paracellular barrier function, but not for apical-basal polarity. In the tracheal system, septate junctions have a barrier-independent function that promotes lumenal secretion of Vermiform and Serpentine, extracellular matrix modifier proteins that are required to restrict tube length. As with Sinuous and Megatrachea, loss of Kune-kune prevents this secretion and results in overly elongated tubes. Embryos lacking all three characterized claudins have tracheal phenotypes similar to any single mutant, indicating that these claudins act in the same pathway controlling tracheal tube length. However, we find that there are distinct requirements for these claudins in epithelial septate junction formation. Megatrachea is predominantly required for correct localization of septate junction components, while Sinuous is predominantly required for maintaining normal levels of septate junction proteins. Kune-kune is required for both localization and levels. Double- and triple-mutant combinations of Sinuous and Megatrachea with Kune-kune resemble the Kune-kune single mutant, suggesting that Kune-kune has a more central role in septate junction formation than either Sinuous or Megatrachea.

EPITHELIA are essential for separating physiologically distinct body compartments and regulating trafficking between them. For proper function, it is imperative that epithelia maintain effective barriers against free paracellular diffusion. To this end, epithelial cells contain occluding junctions, which regulate paracellular permeability. In vertebrates, this is accomplished by tight junctions (TJ), structures that are characterized by regions of close membrane apposition between adjacent cells known as "kissing points" (Tsukita and Furuse 2002). While the TJ is made up of at least 40 different components (Schneeberger and Lynch 2004), the core proteins responsible for the paracellular barrier are the claudins (Angelow *et al.* 2008).

Claudins are four-transmembrane domain proteins that form homo- and heterophilic interactions within the same cell (Furuse *et al.* 1999; Blasig *et al.* 2006) and with claudins in adjacent cells (Furuse *et al.* 1999), thereby establishing the paracellular seal. There are 24 members of the claudin family in mammals, many of which display distinct, tissue-specific expression patterns (Kiuchi-Saishin *et al.* 2002; Angelow *et al.* 2008). Mutations in several claudins can cause significant paracellular permeability defects in mice. For example, mutations in claudin-14 increase TJ permeability in the organ of Corti and cause deafness (Ben-Yosef *et al.* 2003), while loss of claudin-1 compromises epidermal barrier function (Furuse *et al.* 2002).

In Drosophila, primary (ectodermally derived) epithelia lack discernable TJs and instead use pleated septate junctions (SJ) for the paracellular barrier (Baumgartner *et al.* 1996; Lamb *et al.* 1998; Genova and Fehon 2003; Paul *et al.* 2003). However, despite sharing a common barrier function, vertebrate TJs and invertebrate SJs differ in several ways. While vertebrate TJs are positioned apical to adherens junctions (AJ) and contain conserved apical polarity proteins, SJs are basal to AJs and contain conserved basolateral polarity proteins (reviewed in Tepass 2003; Wu and Beitel 2004). In addition, SJs do not contain kissing points, but rather ladder-like septa that span the intermembrane space (Lane and Swales 1982; Tepass and Hartenstein 1994).

Beyond their general epithelial barrier function, SJs are also required for several tissue-specific processes. Glial cells, for example, ensheath nerve fibers and use

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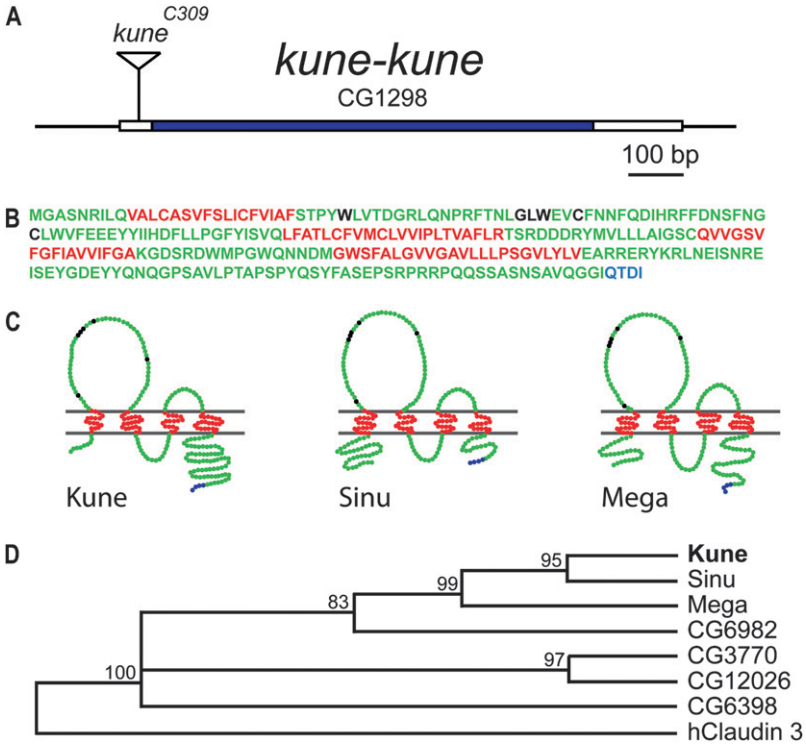

FIGURE 1.—*kune-kune* encodes a Drosophila claudin. (A) A schematic representation of the *kune-kune* (*kune*) locus. The $kune^{C309}$ allele contains a PiggyBac transposable element inserted within the 5′-UTR. (B) The Kune protein is predicted to be 264 amino acids with four transmembrane domains (red) and a C-terminal PDZ binding motif (blue). Residues conserved throughout claudin family members are shown in black. (C) The topology of Kune is similar to other claudins, with intracellular N and C termini and a large extracellular loop followed by two smaller loops. Kune is predicted to have an N terminus that is shorter than the Drosophila claudins Mega and Sinu and a C terminus that is longer. (D) Sequence comparisons show that Kune is more closely related to Sinu and Mega than to other predicted Drosophila claudins. The numbers on branch points in the figure indicate the frequency (%) with which claudins or groups of claudins clustered together in a "bootstrap" analysis where 1000 CLUSTLW alignments were performed on subsets of the dataset using the MacVector program (MacVector, Inc.). This approach more robustly determines relationships of poorly conserved proteins than does single-pass phylogenetic alignment approaches. Distance along the *x*-axis is arbitrary and does not reflect sequence similarity. Human claudin 3 (hClaudin 3) was used as an outgroup.

SJs to maintain the blood–brain barrier (AULD *et al.* 1995; BAUMGARTNER *et al.* 1996; SCHWABE *et al.* 2005). In the embryonic tracheal system, SJs are required for the apical secretion of the lumenal matrix modifying proteins, Vermiform (Verm) and Serpentine (Serp), which act through undefined pathways to restrict tube length (WANG *et al.* 2006). This secretory pathway appears to be specific for Verm and Serp, since other apical proteins are secreted normally in SJ mutants. SJ proteins have also been shown to play a role in morphogenesis of the heart tube, even though this tissue lacks typical SJ septa (YI *et al.* 2008).

Although SJs have clear differences from vertebrate TJs, SJs contain at least two claudins, Megatrachea (Mega) and Sinuous (Sinu), both of which are required for the paracellular barrier (BEHR *et al.* 2003; WU *et al.* 2004; STORK *et al.* 2008). In this article, we identify a third claudin, Kune-kune (Kune), that is an integral SJ protein. Like the other claudins, Kune is required for maintaining epithelial paracellular barrier and tracheal tube size control and is not required for apical-basal polarity. We also find that, of all three characterized claudins, Kune has a more severe SJ phenotype, suggesting that it is a more central player in SJ organization and function than previously characterized Drosophila claudins.

MATERIALS AND METHODS

Fly stocks and genetics: Fly stocks were from the Bloomington Stock Center, the Vienna Stock Center, or from published sources. Alleles used are as follows: $kune^{C309}$, $mega^{G0012}$, $sinu^{nwu7}$, cor^{5}, $Atp\alpha^{DTS1R2}$, and yrt^{75a}. The UAS-*kune*-RNAi^{108224} line was used for all RNAi experiments. The FM7 *dfd*-YFP, CyO *dfd*-YFP, CyO *act*-Z, TM6 *dfd*-YFP, and TM6 *dfd*-Z balancers were used for genotyping single and double mutants (LE *et al.* 2006). *mega*; *kune*; *sinu* triple mutants were identified by the absence of all three proteins, as assessed by immunohistochemistry. All images are from stage 16 embryos unless otherwise indicated. Flies were maintained at 23° for all experiments except for the *kune* rescue. At 23° the UAS-*kune* construct only partially rescued $kune^{C309}$ SJ defects and did not rescue embryonic lethality when driven by *da*-Gal4. Since the Gal4-UAS system is temperature sensitive (DUFFY 2002), we reared *da*-Gal4, $kune^{C309}$/UAS-*kune*, $kune^{C309}$ embryos at 28°. At this temperature, UAS-*kune* rescued the SJ defects and embryonic lethality of $kune^{C309}$. In contrast, *da*-Gal4, $kune^{C309}/kune^{C309}$ embryos reared at 28° were embryonic lethal and phenotypically identical to $kune^{C309}$ embryos reared at 23°.

Immunohistochemistry: The following antibodies were used in this study: mouse (ms) anti-Cor C566.9c and C615.16B 1:500, guinea pig (gp) anti-Cor 1:10000, ms anti-Na, K-ATPase alpha a5 1:25, rabbit (rb) anti-Mega 1:30, rb anti-Sinu 1:250, ms anti-Dlg 1:500, ms anti-Crb cq4 1:20, ms anti-2A12 1:5, gp anti-Verm 1:1000, and rb anti-Serp 1:300. Rb anti-Kune antibody was raised against the C-terminal cytoplasmic region of Kune (aa 193-264) fused with GST and used at 1:1000. Secondary antibodies were used at 1:250 (Molecular Probes and Jackson ImmunoResearch). Lumenal chitin was visualized with rhodamine-conjugated chitin-binding probe (New England Biolabs) (TONNING *et al.* 2005). Embryos were fixed with formaldehyde following standard protocols (SAMAKOVLIS *et al.* 1996), except for Sinu staining, which required heat fixation (WU *et al.* 2004). Measurement of the dorsal trunk (DT) length was performed using confocal sections as described (BEITEL and KRASNOW 2000). Confocal images were acquired on a Leica TCS SP2. Homozygous and heterozygous embryos were imaged on the same slide in the same session using the same laser and

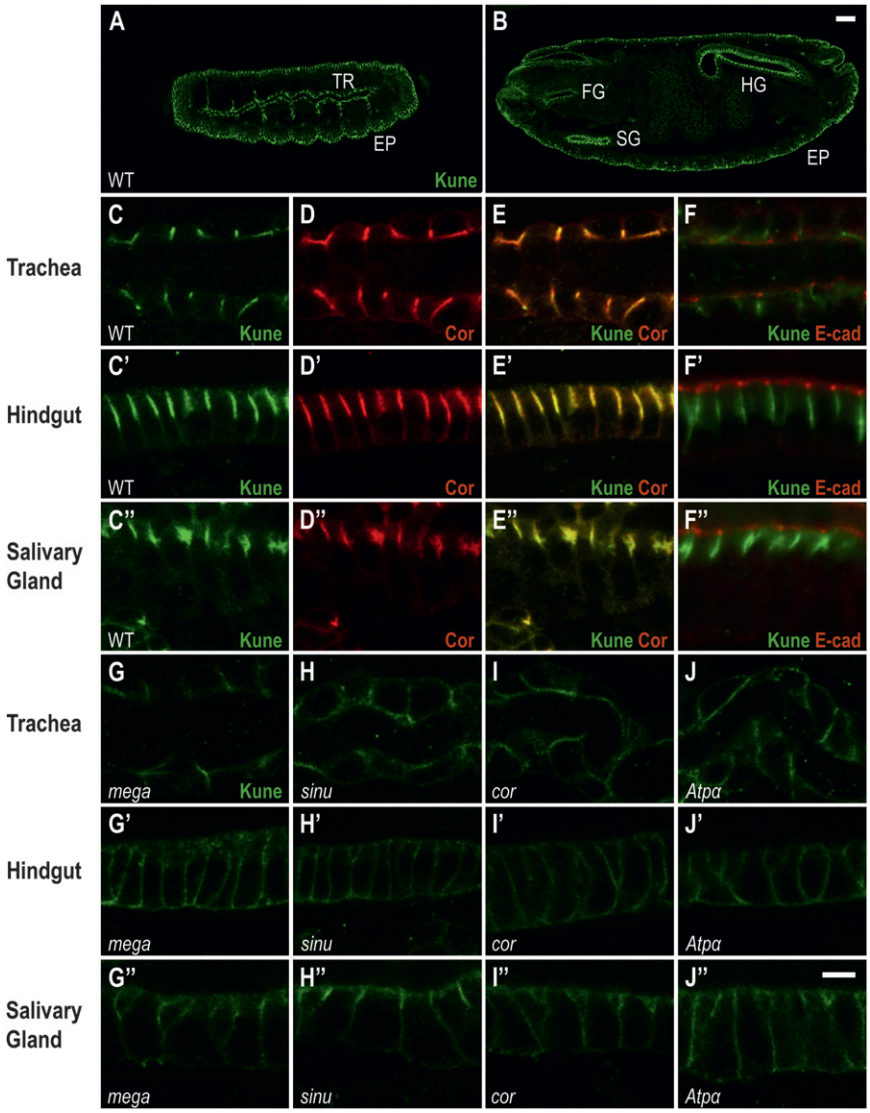

FIGURE 2.—Kune localizes to septate junctions in primary epithelia. (A, B) Kune localizes to primary epithelia, including the trachea (TR), epidermis (EP), foregut (FG), salivary gland (SG), and hindgut (HG). A and B are different focal planes of the same embryo. (C–F″) Kune colocalizes with Cor at septate junctions (SJ) and does not colocalize with DE-cadherin at the adherens junction (F). Images of the trachea (C–F), hindgut (C′–F′), and salivary gland (C″–F″) are shown. (G–J″) Kune localization to the SJ is dependent on the SJ components, Mega, Sinu, Cor, and Atpα, since Kune is reduced and mislocalized to more basal positions in these mutants. Scale bars: 20 μm for A and B in B, 5 μm for C–J″ in J″.

imaging setting so that relative levels of staining could be estimated.

Dye exclusion assay: SJ barrier function of the trachea and salivary gland was assayed by injecting Texas Red-conjugated dextran (Molecular Probes) into the body cavities of stage 16 embryos (LAMB *et al.* 1998). Glial barrier function was assayed by injecting 20- to 21-hr-old embryos. Dye permeability in injected embryos was assayed on a Leica TCS SP2 confocal microscope within 15 min of injection.

Molecular biology: To generate the UAS-*kune* transgene, the *kune* ORF was PCR amplified from genomic DNA, cloned into the pUAS-T attB construct (BISCHOF *et al.* 2007), and sequenced. Transgenic flies were generated using site-directed ϕC31 integration into the attp40 (25C7) site (MARKSTEIN *et al.* 2008) by Genetic Services, Inc. (Cambridge, MA).

RESULTS AND DISCUSSION

***kune-kune* encodes a Drosophila claudin:** The Drosophila genome encodes seven predicted claudin-family molecules (WU *et al.* 2004 and Figure 1D; CG12026 not noted in Wu *et al.*). Two of these, Mega and Sinu, have previously been characterized and were shown to be required for SJ organization and function (BEHR *et al.* 2003; WU *et al.* 2004). Sequence comparisons indicated that, although all seven claudin-like molecules show a large sequence divergence, CG1298 is more closely related to Sinu and Mega than the other Drosophila claudin-family members (WU *et al.* 2004 and Figure 1D). Therefore, although many Drosophila claudin-family members are not required for barrier function (STORK *et al.* 2008), we reasoned that CG1298 may play a role in SJ paracellular barrier formation. Accordingly, we performed a detailed analysis of CG1298, which we have named *kune-kune* (Japanese for "wiggling like a snake," pronounced koon-eh koon-eh and abbreviated *kune*) for its tracheal phenotype (see below).

The *kune* locus contains a single exon that codes for a protein of 264 amino acids (Figure 1, A and B). As is characteristic for claudins, the TMpred transmembrane algorithm (HOFMANN and STOFFEL 1993) predicts Kune to have four transmembrane domains with intracellular

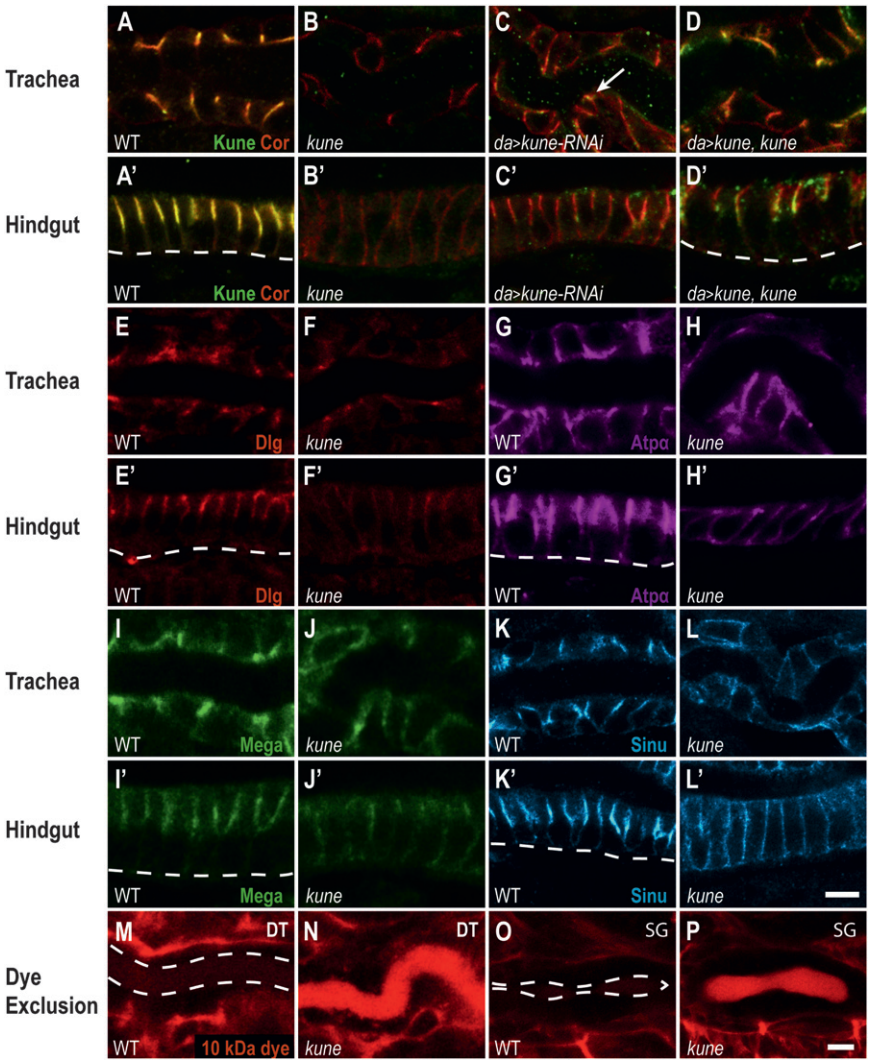

FIGURE 3.—Kune is required for septate junction organization and barrier function. (A, B, A′, B′) *kune*^{C309} embryos show a complete absence of Kune by immunohistochemistry and result in reduced levels and mislocalization of Cor. (C, C′) Expression of *kune*-RNAi also causes Cor mislocalization. Arrow in C indicates the presence of some residual Kune, suggesting that RNAi knockdown is incomplete. (D, D′) Expression of the *kune* ORF using the *da*-Gal4 driver rescues Cor localization. (E–L′) The SJ proteins Dlg (E–F, E′–F′), Atpα (G–H, G′–H′), Mega (I–J, I′–J′), and Sinu (K–L, K′–L′) are reduced and/or mislocalized in *kune* epithelia. Dashed lines indicate the basal surface. (M–P) A fluorescent 10-kDa dye injected into the body cavity of WT embryos is excluded from the lumen of the trachea (M) and salivary gland (O), indicated by dashed lines. The dye readily leaks into the lumens of *kune* embryos (N, P), indicating a loss of the paracellular barrier. Scale bars: 5 μm for A–L′ in L′, 5 μm for M and N in P, 10 μm for O and P in P.

N and C termini, a large initial extracellular loop, and two smaller loops (Figure 1C). Kune also contains a W–GLW–C–C motif in the large extracellular loop (Figure 1, B and C, black residues) and a C-terminal PDZ-binding motif (Figure 1, B and C, blue residues), features that are found in almost all claudin family members (VAN ITALLIE and ANDERSON 2006). Notably, the PDZ-binding motif in Kune is a better match to consensus PDZ-binding motifs than the motif in Sinuous. Furthermore, in contrast to Mega and Sinu, whose N termini are 28 and 38 aa respectively, Kune has a short N terminus of 9 aa that is more typical of vertebrate claudins (ANGELOW *et al.* 2008). Thus, Kune has features that more closely resemble vertebrate claudins than do the so far characterized Drosophila claudins Sinu and Mega.

Kune localizes to septate junctions: To determine the expression pattern of Kune, we generated anti-Kune sera and immunostained wild-type (WT) embryos. As with Mega and Sinu (BEHR *et al.* 2003; WU *et al.* 2004), Kune is highly expressed in ectodermally derived tissues, including the epidermis, salivary gland, trachea, hindgut, and foregut beginning at embryonic stage 13 (Figure 2, A and B). In these tissues, Kune colocalizes with the SJ protein Coracle (Cor) and localizes basal to the adherens junction marker, DE-cadherin (E-cad) (Figure 2, C–F″), suggesting that Kune is a SJ protein. As with many other SJ proteins (AULD *et al.* 1995; BAUMGARTNER *et al.* 1996; BEHR *et al.* 2003; SCHWABE *et al.* 2005), Kune is also expressed in glial cells (Figure 4, A and B).

Since most SJ proteins show interdependence for correct localization and junction function (BEHR *et al.* 2003; GENOVA and FEHON 2003; PAUL *et al.* 2003; FAIVRE-SARRAILH *et al.* 2004; WU *et al.* 2007), we asked whether localization of Kune depends on the presence of other SJ proteins. Indeed, Kune is mislocalized to more basal positions in the primary epithelia of *mega*, *sinu*, *cor*, and *Atp*α null mutants (Figure 2, G–J″), providing strong evidence that Kune is a SJ protein.

Kune is required for septate junction organization and barrier function: To directly assess the function of Kune during development, we identified a PiggyBac insertion, PBac{3HPy}C309, in the 5′-untranslated re-

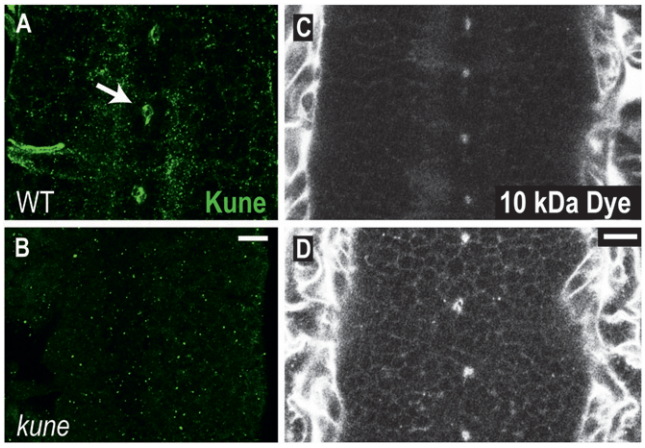

Figure 4.—Kune is required for establishing the blood–brain barrier. (A–B) Kune is expressed in glial cells and is enriched in the glia at the midline (arrow in A). (C–D) A 10-kDa fluorescent dye is excluded from the ventral nerve cord of 20- to 21-hr-old (st 17) WT embryos (C), indicating a functional blood–brain barrier. In contrast, dye penetrates into the nerve cord of *kune* embryos (D). All images are ventral views. Scale bars: 10 μm for A and B in B and 10 μm for C and D in D.

gion (UTR) of *kune* as a putative null mutation (Figure 1A). Embryos homozygous for the $kune^{C309}$ chromosome fail to hatch and completely lack Kune protein as assessed by immunohistochemistry (Figure 3, B and B′). Expression of a UAS-*kune* construct using the ubiquitous *da*-Gal4 driver at 28° (materials and methods) rescued the embryonic lethality of $kune^{C309}$ embryos, demonstrating that lethality was due to loss of Kune. Further, embryos trans-heterozygous for $kune^{C309}$ and Df(2R)BSC696 (which deletes the *kune* locus and also eliminates Kune staining) or homozygous for Df(2R)BSC696 fail to hatch and, as discussed below, display tracheal and septate junction phenotypes that are indistinguishable from $kune^{C309}$ homozygotes (Figure 5C and supporting information, Figure S1, B–C). These results indicate that *kune* is an essential gene and that $kune^{C309}$ is a null or strong loss-of-function allele of *kune*.

To determine if Kune is required for SJ organization and function, we examined the subcellular localization of several SJ proteins in *kune* mutant epithelia. As is seen in other SJ mutants, *kune* epithelia show a reduction and/or mislocalization of the SJ components Cor, Mega, Sinu, Atpα, Discs large (Dlg), and NeurexinIV (Nrx) to more basal locations in all primary epithelia (Figure 3, B, B′ and E–L′ Figure 6, F and I; and Figure S1, D–E″′). This phenotype is also seen in animals that express *kune*-RNAi using the ubiquitous *da*-Gal4 driver, although the phenotype is less severe, presumably due to incomplete knockdown (Figure 3, C and C′). Consistent with the immunohistological evidence of SJ defects, a 10-kDa fluorescent dye injected into the body cavity of *kune* animals readily diffused into the lumen of the trachea and salivary gland, indicating a loss of the paracellular barrier (Figure 3, M–P). Expression of the UAS-*kune* construct with *da*-Gal4 rescued Cor localization and improved the barrier function of *kune* mutants (Figure 3, D and D′, and Figure S1, I and J). Thus, Kune is an essential component of SJs in primary epithelia.

In addition to their roles in epithelial tissues, SJs are also required to establish the blood–brain barrier in flies. In the central nervous system (CNS), surface glial cells completely ensheath the ventral nerve cord and form SJs at glia–glia contacts. This generates a tight paracellular seal that separates the K^+-rich hemolymph

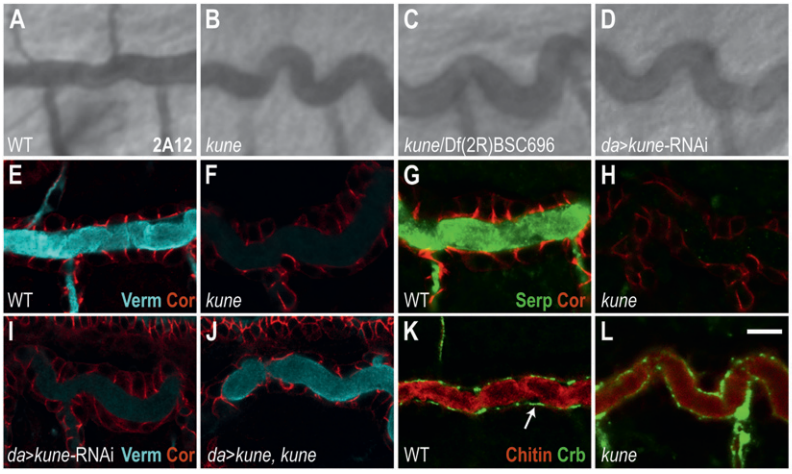

Figure 5.—Kune is required for tracheal tube size control. (A–B) The dorsal trunk of *kune* homozygous embryos (B) is overly elongated, resulting in tubes that follow a tortuous path. (C) This phenotype is identical in *kune*/Df(2R)BSC696 embryos. (D) Embryos expressing *kune*-RNAi using the *da*-Gal4 driver also display overly elongated trachea. (E–H) Lumenal Verm (E and F) and Serp (G and H) are absent in *kune* tracheal tubes. (I) Lumenal Verm is also absent in embryos expressing *kune*-RNAi using the *da*-Gal4 driver. (J) Expression of UAS-*kune* with *da*-Gal4 largely rescues Verm secretion of *kune* mutants. (K and L) WT trachea contain a lumenal, fibrilar chitin cable that is separated from the apical surface of tracheal cells, marked by Crb (arrow in K). In *kune* embryos, the chitin matrix is disorganized and the gap between the cable and the apical surface is missing (L). (M) Measurement of the posterior dorsal trunk (DT) between transverse connectives (TC) 5 and 10 indicate that the length of *kune* DTs is longer than WT. The posterior DT was measured because tracheal elongation of SJ mutants is most prominent at the posterior end. (**) $P < 0.0005$. Scale bar: 10 μm for A–L in L.

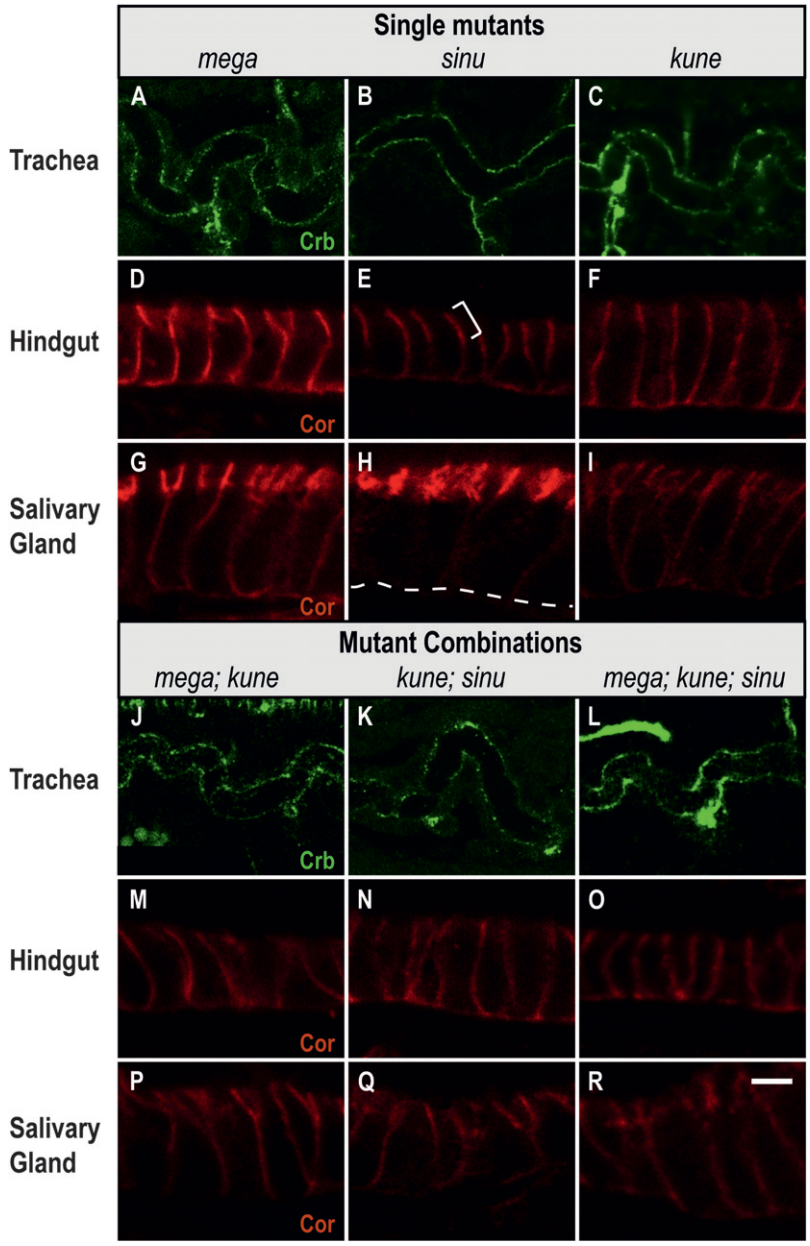

FIGURE 6.—Kune-related claudins function in the same pathway of tracheal tube size control, but have unique roles in SJ organization. (A–C) Mutation to the Kune-related claudins, *mega* (A), *sinu* (B), and *kune* (C) all cause similar tracheal tube elongation defects. (D–F) Staining for Cor in the hindgut reveals that SJ organization is different in each of the three Kune-related claudins. *mega* embryos show complete mislocalization of Cor, but appear to have WT levels (D). Cor is reduced in *sinu* embryos, but retains an enrichment of Cor at the SJ (E, bracket). Cor is reduced and completely mislocalized in *kune* hindguts (F). (G–I) In contrast to *mega* (G) and *sinu* (H) salivary glands, *kune* salivary glands display a dramatic disruption in Cor levels and localization (I). (J–L) The trachea of *mega; kune* (J), *kune; sinu* (K), and *mega; kune; sinu* (L) embryos do not appear to be any worse than the single mutants (A–C). (M–R) Cor levels and localization in the double and triple mutants appear similar to *kune* single mutants (F and I), suggesting that the *kune* phenotype is the most severe. See Figure 2, D′ and D″, for WT comparison. Dashed lines indicate basal surface. Scale bar: 10 μm for A–C and J–L in R, 5 μm for D–I and M–R in R.

from neural cells, which is essential for generation of action potentials (SCHWABE *et al.* 2005). We found that Kune is expressed in glial cells, which is most clearly seen at the central midline (Figure 4A). Dye injections revealed that Kune, like Sinu and Mega (STORK *et al.* 2008), is required for the CNS glial barrier, since the dye penetrated into the nerve cord of *kune* but not WT embryos (Figure 4, C and D). Taken together, the above results all identify Kune as a critical SJ component in multiple tissue types.

Kune is required for tracheal tube size control: Since almost all characterized SJ proteins are required for tracheal tube size control (BEHR *et al.* 2003; PAUL *et al.* 2003; FAIVRE-SARRAILH *et al.* 2004; LLIMARGAS *et al.* 2004; WU *et al.* 2004; HIJAZI *et al.* 2009; WU *et al.* 2007), we examined the tracheal system of *kune* embryos. Staining with the 2A12 lumenal marker demonstrated that the length of the DT of stage 16 *kune* embryos was significantly increased over WT controls and appeared tortuous (thus the name *kune-kune*) (Figure 5, B and M). This phenotype was identical in both *kune*/Df(2R)BSC696 embryos and embryos expressing *kune*-RNAi using the *da*-Gal4 driver (Figure 5, C and D).

It has been established that Sinu, Mega, and other SJ proteins are required for apical secretion of the putative chitin deacetylases Verm and Serp, which restrict tracheal tube length (LUSCHNIG *et al.* 2006; WANG *et al.* 2006). We therefore examined lumenal accumulation of Verm and Serp and the organization of the chitin-based lumenal matrix at embryonic stage 16. As is typical for a SJ component, *kune* mutant embryos and embryos expressing *kune*-RNAi do not accumulate Verm

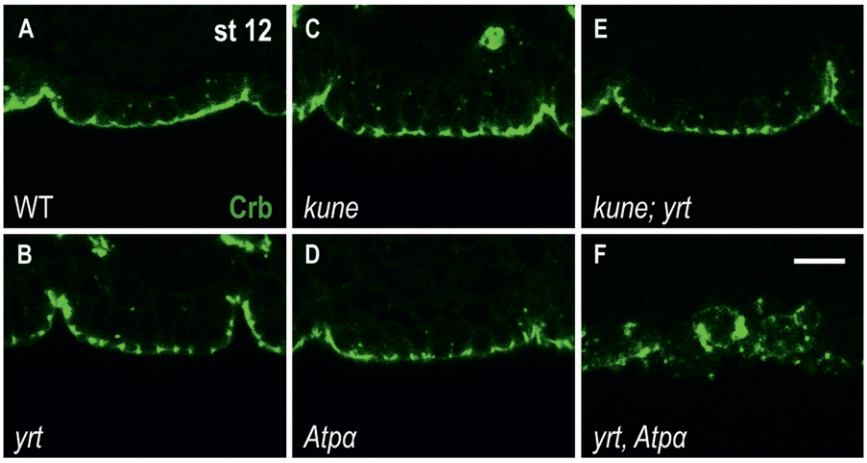

Figure 7.—Kune is not required for epithelial apical-basal polarity. (A–D) Apical localization of Crb is normal in the ventral epidermis of WT (A), *yrt(z)* (B), *kune* (C), and *Atp*α (D) animals at stage 12. (E and F) Embryos containing mutations in *kune* and *yrt* show normal localization of Crb (E). In contrast, Crb is completely mislocalized in *yrt, Atp*α double mutants (F), indicating a loss of apical-basal polarity. Scale bar: 10 μm for A–F in F.

or Serp in the tracheal lumen (Figure 5, E–I). This secretion defect could be largely rescued by expression of UAS-*kune* with the *da*-Gal4 driver (Figure 5J), although not to WT levels. Additionally, staining with a fluorescent chitin binding probe showed that, while the lumen of WT trachea contains a dense, fibrilar chitin cable, *kune* trachea have a diffuse, disorganized lumenal matrix (Figure 5, K and L). *kune* trachea also lack the gap between the chitin cable and the apical surface of the cells that is found in WT trachea (arrow in Figure 5K).

Kune has a more central role in SJ organization than the Drosophila claudins Sinu and Mega: Since Kune is closely related to both Mega and Sinu and all three localize to the SJ, we asked if these claudins are partially redundant in junction organization or tube size control. To test this, we examined the trachea of *mega*, *kune*, and *sinu* single, double, and triple mutants. If the claudins have redundant functions in tube size control, the phenotypes should be worse when multiple claudins are missing. However, the tracheal length defects of *mega; kune* and *kune; sinu* embryos appear no more severe than in any of the single mutants (Figure 6, A–C, J, and K). Strikingly, even embryos lacking all three Kune-related claudins do not appear to have more severe tracheal length defects than single mutants (Figure 6L). In contrast, our previous work has shown that embryos containing mutations in both *sinu* and the SJ gene *varicose* (*vari*) have trachea that are more tortuous than either single mutant (Wu *et al.* 2004). These results suggest that, although some SJ proteins have redundant functions in restricting tracheal tube length, the Drosophila claudins Kune, Sinu, and Mega all function in the same pathway of tracheal tube size control.

Given that tracheal tube length is only a limited readout of SJ function, we compared the effects of single-, double-, and triple-mutant combinations of *kune*, *sinu*, and *mega* on SJ organization using the subcellular localization of Cor as an assay. We focused on the hindgut and salivary gland, since SJ organization is clearest in these columnar cells. Interestingly, the levels and localization of Cor are strikingly different between the three claudin mutations, suggesting that different claudins have unique functions. For example, Cor is completely mislocalized to basal positions in the hindgut of *mega* embryos, but the levels are not dramatically lower than in WT (Figure 6D). On the other hand, the hindguts of *sinu* embryos show lower overall levels of Cor, but retain significant apicolateral enrichment where the SJ is normally found (bracket in Figure 6E). In *kune* mutants, Cor is both reduced and completely mislocalized (Figure 6F). Similar, more pronounced effects are seen in the salivary glands where loss of *mega* causes only slight basolateral mislocalization of Cor, loss of *sinu* causes almost no Cor mislocalization, and loss of *kune* strongly mislocalizes and reduces Cor staining (Figure 6, G–I). The localization and levels of the SJ markers Dlg and Atpα were also more severely disrupted in *kune* mutants than in *sinu* or *mega* mutants (Figure S2), indicating that the effects were not specific to Cor. Interestingly, the levels and localization of Cor are not obviously different between the *kune* single mutant and the double and triple mutants, indicating that the *kune* phenotype is the most severe (Figure 6, M–R). Together, these results suggest that Kune has a more central role in SJ organization than either Sinu or Mega. This possibility is particularly intriguing in light of the greater similarity of Kune to vertebrate claudins than either Sinu or Mega (see above). Perhaps a more central role for Kune in barrier junction formation has constrained its evolution and thus Kune more closely resembles ancestral claudins than do Sinu and Mega, which may have evolved more specialized functions.

It is curious that multiple nonredundant claudins are required for SJ organization and barrier function. The exact reason for this is unclear, but perhaps each claudin interacts independently with specific junctional molecules to establish a SJ scaffold. This would be consistent with their divergent protein sequences and the differences in their N and C termini. Importantly, vertebrate claudins also have nonredundant roles in TJ

function. For example, paracellular barrier function is compromised in the epidermis of mice lacking claudin-1 despite the presence of claudin-4 at the TJ (Furuse *et al.* 2002).

Kune is not required for epithelial polarity: Like other SJ components, Kune does not appear to be required for establishment of apical-basal polarity since the levels and localization of the apical marker, Crumbs (Crb), and the adherens junction marker, E-cad, were normal in *kune* embryos (Figure 5L and Figure S1, F–H). However, it was recently shown that some SJ components have a role in a newly identified phase of Drosophila epithelial polarity that occurs between stages 11 and 13 (Laprise *et al.* 2009). Because of redundancy between SJ components involved in this polarity phase and the SJ component *yrt*, SJ proteins required for polarity can be identified only in a *yrt* zygotic mutant background (Laprise *et al.* 2009). For example, single zygotic mutations in either the SJ gene *Atp*α or *yrt* show normal apical localization of Crb at stage 12 (Figure 7, D and E). *Atp*α, *yrt* double mutants on the other hand show severe mislocalization of Crb, indicative of a loss of polarity (Figure 7F). In contrast, neither *kune* single mutants nor *kune; yrt* double mutants display any obvious polarity defects (Figure 7, C and E), demonstrating that *kune* is not required for either establishment or maintenance of epithelial polarity.

Previous work has shown that neither *mega* nor *sinu* are required for establishment of apical-basal polarity (Behr *et al.* 2003; Wu *et al.* 2004) or for maintenance of epithelial polarity at mid-embryogenesis (Laprise *et al.* 2009). Together with the findings in this article, the available evidence suggests that Drosophila claudins are not required for epithelial polarity. This parallels the situation in *Caenorhabditis elegans* where mutations in the claudin-like proteins CLC1–4 disrupt barrier function, but not epithelial polarity (Asano *et al.* 2003). Similarly, claudins do not appear to be required for epithelial polarity in mammalian epithelial cells, since Eph4 cells can establish normal polarity even when lacking claudin complexes and tight junction strands due to elimination of ZO-1 and ZO-2 (Umeda *et al.* 2006). The absence of a role for claudins in polarity in any characterized species is consistent with the proposal that barrier junctions arose after polarity during the evolution of metazoans (Krupinski and Beitel 2009).

CONCLUSION

Our results show that Kune is an essential claudin that is required in all examined tissues for the organization and function of SJs. Kune expression and localization overlaps with the Drosophila claudins Mega and Sinu, but we find that all three claudins play unique roles in SJ organization. Importantly, Kune more closely resembles vertebrate claudins than either Mega or Sinu and appears to play a more central role in SJ organization. Further work is needed to establish the complete molecular organization of SJs, but such work will be facilitated by the presented characterization of Kune and its interaction with other SJ components.

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LITERATURE CITED

Angelow, S., R. Ahlstrom and A. S. Yu, 2008 Biology of claudins. Am. J. Physiol. Renal Physiol. **295:** F867–F876.

Asano, A., K. Asano, H. Sasaki, M. Furuse and S. Tsukita, 2003 Claudins in *Caenorhabditis elegans*: their distribution and barrier function in the epithelium. Curr. Biol. **13:** 1042–1046.

Auld, V. J., R. D. Fetter, K. Broadie and C. S. Goodman, 1995 Gliotactin, a novel transmembrane protein on peripheral glia, is required to form the blood-nerve barrier in Drosophila. Cell **81:** 757–767.

Baumgartner, S., J. T. Littleton, K. Broadie, M. A. Bhat, R. Harbecke *et al.*, 1996 A Drosophila neurexin is required for septate junction and blood-nerve barrier formation and function. Cell **87:** 1059–1068.

Behr, M., D. Riedel and R. Schuh, 2003 The claudin-like megatrachea is essential in septate junctions for the epithelial barrier function in Drosophila. Dev. Cell **5:** 611–620.

Beitel, G. J., and M. A. Krasnow, 2000 Genetic control of epithelial tube size in the Drosophila tracheal system. Development **127:** 3271–3282.

Ben-Yosef, T., I. A. Belyantseva, T. L. Saunders, E. D. Hughes, K. Kawamoto *et al.*, 2003 Claudin 14 knockout mice, a model for autosomal recessive deafness DFNB29, are deaf due to cochlear hair cell degeneration. Hum. Mol. Genet. **12:** 2049–2061.

Bischof, J., R. K. Maeda, M. Hediger, F. Karch and K. Basler, 2007 An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. Proc. Natl. Acad. Sci. USA **104:** 3312–3317.

Blasig, I. E., L. Winkler, B. Lassowski, S. L. Mueller, N. Zuleger *et al.*, 2006 On the self-association potential of transmembrane tight junction proteins. Cell. Mol. Life Sci. **63:** 505–514.

Duffy, J. B., 2002 GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis **34:** 1–15.

Faivre-Sarrailh, C., S. Banerjee, J. Li, M. Hortsch, M. Laval *et al.*, 2004 Drosophila contactin, a homolog of vertebrate contactin, is required for septate junction organization and paracellular barrier function. Development **131:** 4931–4942.

Furuse, M., H. Sasaki and S. Tsukita, 1999 Manner of interaction of heterogeneous claudin species within and between tight junction strands. J. Cell. Biol. **147:** 891–903.

Furuse, M., M. Hata, K. Furuse, Y. Yoshida, A. Haratake *et al.*, 2002 Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J. Cell Biol. **156:** 1099–1111.

Genova, J. L., and R. G. Fehon, 2003 Neuroglian, Gliotactin, and the Na+/K+ ATPase are essential for septate junction function in Drosophila. J. Cell Biol. **161:** 979–989.

Hijazi, A., W. Masson, B. Auge, L. Waltzer, M. Haenlin *et al.*, 2009 boudin is required for septate junction organisation in Drosophila and codes for a diffusible protein of the Ly6 superfamily. Development **136:** 2199–2209.

Hofmann, K., and W. Stoffel, 1993 TMbase: a database of membrane spanning protein segments. Biol. Chem. Hoppe-Seyler **374:** 166.

Kiuchi-Saishin, Y., S. Gotoh, M. Furuse, A. Takasuga, Y. Tano *et al.*, 2002 Differential expression patterns of claudins, tight junction membrane proteins, in mouse nephron segments. J. Am. Soc. Nephrol. **13:** 875–886.

Krupinski, T., and G. J. Beitel, 2009 Unexpected roles of the Na-K-ATPase and other ion transporters in cell junctions and tubulogenesis. Physiology **24:** 192–201.

Lamb, R. S., R. E. Ward, L. Schweizer and R. G. Fehon, 1998 Drosophila coracle, a member of the protein 4.1 superfamily, has essential structural functions in the septate junctions and developmental functions in embryonic and adult epithelial cells. Mol. Biol. Cell **9:** 3505–3519.

Lane, N. J., and L. S. Swales, 1982 Stages in the assembly of pleated and smooth septate junctions in developing insect embryos. J. Cell Sci. **56:** 245–262.

Laprise, P., K. M. Lau, K. P. Harris, N. F. Silva-Gagliardi, S. M. Paul *et al.*, 2009 Yurt, Coracle, Neurexin IV and the Na(+),K(+)-ATPase form a novel group of epithelial polarity proteins. Nature **459:** 1141–1145.

Le, T., Z. Liang, H. Patel, M. H. Yu, G. Sivasubramaniam *et al.*, 2006 A new family of Drosophila balancer chromosomes with a w- dfd-GMR yellow fluorescent protein marker. Genetics **174:** 2255–2257.

Llimargas, M., M. Strigini, M. Katidou, D. Karagogeos and J. Casanova, 2004 Lachesin is a component of a septate junction-based mechanism that controls tube size and epithelial integrity in the Drosophila tracheal system. Development **131:** 181–190.

Luschnig, S., T. Batz, K. Armbruster and M. A. Krasnow, 2006 serpentine and vermiform encode matrix proteins with chitin binding and deacetylation domains that limit tracheal tube length in Drosophila. Curr. Biol. **16:** 186–194.

Markstein, M., C. Pitsouli, C. Villalta, S. E. Celniker and N. Perrimon, 2008 Exploiting position effects and the gypsy retrovirus insulator to engineer precisely expressed transgenes. Nat. Genet. **40:** 476–483.

Paul, S. M., M. Ternet, P. M. Salvaterra and G. J. Beitel, 2003 The Na+/K+ ATPase is required for septate junction function and epithelial tube-size control in the Drosophila tracheal system. Development **130:** 4963–4974.

Samakovlis, C., N. Hacohen, G. Manning, D. C. Sutherland, K. Guillemin *et al.*, 1996 Development of the Drosophila tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. Development **122:** 1395–1407.

Schneeberger, E. E., and R. D. Lynch, 2004 The tight junction: a multifunctional complex. Am. J. Physiol. Cell Physiol. **286:** C1213–C1228.

Schwabe, T., R. J. Bainton, R. D. Fetter, U. Heberlein and U. Gaul, 2005 GPCR signaling is required for blood–brain barrier formation in drosophila. Cell **123:** 133–144.

Stork, T., D. Engelen, A. Krudewig, M. Silies, R. J. Bainton *et al.*, 2008 Organization and function of the blood-brain barrier in Drosophila. J. Neurosci. **28:** 587–597.

Tepass, U., 2003 Claudin complexities at the apical junctional complex. Nat. Cell Biol. **5:** 595–597.

Tepass, U., and V. Hartenstein, 1994 The development of cellular junctions in the Drosophila embryo. Dev. Biol. **161:** 563–596.

Tonning, A., J. Hemphala, E. Tang, U. Nannmark, C. Samakovlis *et al.*, 2005 A transient luminal chitinous matrix is required to model epithelial tube diameter in the Drosophila trachea. Dev. Cell **9:** 423–430.

Tsukita, S., and M. Furuse, 2002 Claudin-based barrier in simple and stratified cellular sheets. Curr. Opin. Cell Biol. **14:** 531–536.

Umeda, K., J. Ikenouchi, S. Katahira-Tayama, K. Furuse, H. Sasaki *et al.*, 2006 ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. Cell **126:** 741–754.

Van Itallie, C. M., and J. M. Anderson, 2006 Claudins and epithelial paracellular transport. Annu. Rev. Physiol. **68:** 403–429.

Wang, S., S. A. Jayaram, J. Hemphala, K. A. Senti, V. Tsarouhas *et al.*, 2006 Septate-junction-dependent luminal deposition of chitin deacetylases restricts tube elongation in the Drosophila trachea. Curr. Biol. **16:** 180–185.

Wu, V. M., and G. J. Beitel, 2004 A junctional problem of apical proportions: epithelial tube-size control by septate junctions in the Drosophila tracheal system. Curr. Opin. Cell Biol. **16:** 493–499.

Wu, V. M., J. Schulte, A. Hirschi, U. Tepass and G. J. Beitel, 2004 Sinuous is a Drosophila claudin required for septate junction organization and epithelial tube size control. J. Cell Biol. **164:** 313–323.

Wu, V. M., M. H. Yu, R. Paik, S. Banerjee, Z. Liang *et al.*, 2007 Drosophila Varicose, a member of a new subgroup of basolateral MAGUKs, is required for septate junctions and tracheal morphogenesis. Development **134:** 999–1009.

Yi, P., A. N. Johnson, Z. Han, J. Wu and E. N. Olson, 2008 Heterotrimeric G proteins regulate a noncanonical function of septate junction proteins to maintain cardiac integrity in Drosophila. Dev. Cell **15:** 704–713.

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