Lachesin is a component of a septate junction-based mechanism that controls tube size and epithelial integrity in the *Drosophila* tracheal system

Marta Llimargas[1,*,†], Maura Strigini[2,*], Markella Katidou[3], Domna Karagogeos[2,4] and Jordi Casanova[1,†]

[1]Institut de Biologia Molecular de Barcelona (IBMB), CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain
[2]IMBB/FORTH, Vassilika Vouton, Iraklio, Crete GR-71110, Greece
[3]Department of Biology, University of Crete, Iraklio, Crete GR-71110, Greece
[4]Medical School, University of Crete, Iraklio, Crete GR-71110, Greece

*These two authors contributed equally to the work
[†]Authors for correspondence (e-mail: mlcbmc@cid.csic.es and jcrbmc@cid.csic.es)

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Summary

Organ morphogenesis requires the coordinated activity of many mechanisms involved in cell rearrangements, size control, cell proliferation and organ integrity. Here we report that Lachesin (Lac), a cell surface protein, is required for the proper morphogenesis of the *Drosophila* tracheal system. Homozygous embryos for *Lac* mutations, which we find fail to complement the previous identified *bulbous (bulb)* mutation, display convoluted tracheal tubes and tube breaks. At the cellular level, we can detect enlarged cells, suggesting that *Lac* regulates organ size by influencing cell length rather than cell number, and cell detachments, indicating a role for *Lac* in cell adhesion. Results from an in vitro assay further support that Lac behaves as a homophilic cell adhesion molecule. Lac co-localizes with Septate Junction (SJ) proteins, and ultrastructural analysis confirms that it accumulates specifically at this type of cellular junction. In *Lac* mutant embryos, previously characterized components of the SJs are mislocalized, indicating that the proper organization of SJs requires Lac function. In addition, mutations in genes encoding other components of the SJs produce a similar tracheal phenotype. These results point out a new role of the SJs in morphogenesis regulating cell adhesion and cell size.

Key words: *Drosophila*, Tracheal system, Septate junctions, Epithelial integrity, Organ size, GPI-linked IgCAM

Introduction

Many essential organs, such as the lungs, kidney or the vascular system, consist of complex branched tubular structures that carry out basic functions. The morphogenesis of these organs (tubulogenesis) requires the coordinated activity of many mechanisms involved in cell rearrangements, cell proliferation, cell shape changes, size control and tissue integrity. Among them, the control of organ and body size has been the subject of many studies that have led to the conclusion that this control often depends on the regulation of cell number (Conlon and Raff, 1999).

The respiratory organ of *Drosophila melanogaster*, the tracheae, has proved to be an excellent model system for the study of organ morphogenesis and tubulogenesis. The available data indicate that the mechanisms of tubulogenesis have been highly conserved during evolution (Affolter et al., 2003; Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003). Therefore, a detailed understanding of tracheal morphogenesis should contribute to our understanding of the morphogenesis of other branched tubular structures.

The tracheal system arises from 20 clusters of ectodermal cells that invaginate inside the embryo. By a process of directed cell migration, cell rearrangements and cell shape changes, the tracheal cells then follow a stereotyped pattern of branching and branch fusion without further cell division, giving rise to the mature tracheae at the end of embryogenesis (Manning and Krasnow, 1993; Samakovlis et al., 1996). Genetic analysis has identified many genes and genetic pathways required for the different steps of tracheal morphogenesis, such as branching and tube fusion (Affolter and Shilo, 2000). The cells forming the tracheal tubes remain attached to one another, and the tissue maintains its epithelial integrity while it completes the whole branching process, making tracheal development an appropriate model to study epithelial integrity. In addition, the size and diameter of the tubes are finely regulated to ensure functionality during animal growth. Although more than 10 genes have been reported to affect the size and integrity of tracheal tubes (Beitel and Krasnow, 2000; Wilk et al., 2000), the cellular and molecular mechanisms involved in this control remain unclear.

The general conclusion from many studies is that the direction of migration of the tracheal cells relies on a set of positional cues provided by nearby cells. Thus, the establishment of interactions between tracheal cells and their substrates is a crucial step in tracheal cell migration, a process ultimately determined by molecules expressed at their surface.

In addition, tracheal cells remain clustered as they migrate, and need to communicate to allow concerted growth of the organ, indicating that they establish specific interactions among themselves that might also require cell surface proteins. To gain further insight into the mechanisms involved in tracheal morphogenesis we have begun a search for cell surface molecules with a pattern of expression consistent with a role in this developmental process. As a result of this analysis, we have found that the gene coding for the Lachesin (Lac) cell surface protein is expressed in the tracheal cells.

Our results indicate that Lac accumulates at the Septate Junctions (SJs), specific invertebrate cell junctions located in the apical part of the lateral membrane of ectoderm-derived cells. They first appear midway through embryogenesis and are characterized by a ladder-like arrangement of septa crossing the intercellular cleft. SJs have been proposed to play a role in the formation of a trans-epithelial diffusion barrier, establishing and/or maintaining cell polarity, cell adhesion and cell-cell interactions (Tepass and Hartenstein, 1994; Tepass et al., 2001; Lane and Skaer, 1980; Noirot-Timothée and Noirot, 1980). Several proteins are known to associate with SJs, and many of these form supramolecular complexes (Knust and Bossinger, 2002; Tepass et al., 2001). Until recently SJs were believed to be unique to invertebrates, where they were assumed to perform some of the roles of tight junctions in vertebrates. However, a new scenario is emerging, as many homologues of the *Drosophila* SJ proteins, as well as similar structures to the SJs, have been identified in mammals (Tepass et al., 2001).

Here we show that Lac behaves as a homophilic cell adhesion molecule required to provide epithelial integrity to the tracheal tubes and to control tubular epithelium length. Moreover, we can detect enlarged cells in the tracheal tubes of *Lac* mutant embryos, suggesting that *Lac* regulates organ size by influencing cell length rather than cell number. In addition, we have found that mutations in genes encoding previously characterized components of the SJs produce a similar tracheal phenotype. Finally, we show that there is an interdependence of Lac and other components of SJs for their proper localization. Thus, this work identifies a new component of the SJs with adhesion properties, and unveils a novel role for the SJs during morphogenesis in the regulation of cell adhesion and cell size.

Materials and methods

Fly strains

The BG1462 line was obtained from the H. Bellen collection; the P-element is inserted at position +46 of the *Lac* transcript (http://flypush.imgen.bcm.tmc.edu/pscreen/). We generated the Lac^2 mutation by an imprecise excision of BG1462. In order to estimate the extent of the deletion, we performed PCR on genomic DNA extracted from homozygous Lac^2 embryos. The PCR results show that the deletion proximal endpoint is found within ~430 bp upstream of position +46 of the *Lac* transcription unit, whereas the distal endpoint is found after position +828 of the transcription unit but before the *Lac* second exon (further details of our strategy are available upon request). Given that no gene is predicted in this intronic sequence of *Lac*, the deletion associated with the Lac^2 mutation removes the *Lac* ATG without affecting nearby genes. $bulb^{k11012b}$ is a non-strict loss-of-function mutation described by Beitel and Krasnow (Beitel and Krasnow, 2000). The following fly lines are described in FlyBase (http://flybase.bio.indiana.edu): $cora^5$, grh^{B37}, Nrx^{4304}, lgl^4, $scrib^2$, Df(2R)CB1, Df(2R)BSC3 and Df(2R)vg135. The *Lac*GFP line is described by Morin (Morin et al., 2001). Tracheal specific expression was achieved with the Gal4 system (Brand and Perrimon, 1993), using a *btlGal4* line (Shiga et al., 1996). *UASLac* transgenic flies were generated by cloning the *Lac* cDNA (Karlstrom et al., 1993) into pUAST as an *Eco*RI fragment and injecting it into *yw* embryos; several independent lines were obtained, including F1.F7/10 carrying the transgene on the second chromosome, which was recombined onto the Lac^1 chromosome and used for the rescue experiments. Rescue of Lac^1 was tested by crossing females of genotype *w*; Lac^1 *UASLac*/CyOGFP to males of genotype *w*; Lac^1/CyOGFP; *btlGal4*/TM2. Other UAS lines used were a *UASnodGFP* line and a *UAStauGFP* line (Bolivar et al., 2001).

Immunostaining, in situ hybridization and permeabilization assays

In situ hybridization was performed according to standard protocols, with *Lac* RNA probes generated using the whole cDNA as template, and produced with the Megascript kit (Ambion).

To generate an anti-Lac antibody, mice were immunized and boosted with a recombinant Lachesin-HIS fusion protein (corresponding to roughly the first 250 amino acids, i.e. the first and second Ig domains). Specificity of the antibody was tested by staining Lac^1 and Df(2R)CB21 homozygous embryos, and by detection of Lac protein overexpressed by the Gal4 system. The sera from all three mice recognize Lac protein, both in immunostaining experiments (1:1000) and western blots (1:5000-10000). In double staining protocols, where we needed to use a mouse antibody directed against a protein other than Lac, we made use of a *LacGFP* line (Morin et al., 2001) that reproduces the same expression pattern as the endogenous Lac protein.

Immunostaining was performed on embryos fixed in 4% formaldehyde for 20-30 minutes according to standard protocols, except for Arm staining, for which we used a heat fixation protocol (Peifer, 1993). The following antibodies were used: anti-GFP (Molecular Probes), mAb2A12 (Developmental Studies Hybridoma Bank; DSHB), anti-Crb (Cq4, DSHB), anti-Dlg (from A. Müller), anti-Arm (N27A1, DSHB), anti-Cora (from R. G. Fehon), anti-Nrx (from S. Baumgartner), anti-FasIII (DA15 from D. Brower), anti-Nrg (1B7 from C. S. Goodman), and anti-βGal (Cappel). Biotinylated or Cy3-, FITC- and Cy5- secondary antibodies (Jackson ImmunoResearch) were used at a dilution of 1/300. For HRP histochemistry, the signal was amplified using the Vectastain-ABC kit (Vector Laboratories). For fluorescent staining, the signal was amplified using TSA (NEN Life Sciences) when required. Confocal images were obtained with a Leica SP1 microscope.

Permeabilization assays were performed by injecting rhodamine-labeled dextran (M_r 10,000; Molecular Probes, Eugene, OR) into the hemocoel of embryos, as described by Lamb et al. (Lamb et al., 1998)

Electron microscopy

Dechorionated *Drosophila* embryos were cryofixed by high pressure freezing using a EMPact (Leica). Freeze-substitution was performed in an 'Automatic Freeze substitution System' (AFS; Leica), using acetone containing 0.5% of uranyl acetate, for 3 days at –90°C. On the fourth day, the temperature was slowly increased, by 5°C/hour, to –50°C. At this temperature, samples were rinsed in acetone, and then infiltrated and embedded in Lowicryl HM20 for 10 days. Ultrathin sections were picked up on Formvar-coated gold grids. For immunogold localization, samples were blocked with 5% BSA in PBS for 20 minutes, and incubated at room temperature for 2 hours with anti-Lac antibody diluted 1/25 in PBS. Washes were performed with 0.25% Tween 20 in PBS, prior to adding goat anti-mouse conjugated to 10 nm colloidal gold for 1 hour at room temperature. Finally, samples were washed and contrasted with 2% uranyl acetate for 20 minutes, then observed in a Jeol 1010 electron microscope with a SIS

Mega View III CCD. Control samples were treated equally but were not incubated with the primary antibody.

Bead aggregation assays

Chimaeric Lac-Fc protein was made by cloning a *Lac* fragment, corresponding to amino acids 1-327, in frame with the Fc fragment of the human IgG in the pcDNA3.1-Fc vector (a gift from Katja Bruckner). The chimaeric protein was recovered from the conditioned medium of transiently transfected HEK293 cells, and was concentrated to approximately 3 μg/ml using Centricon filters (Amicon). Polystyrene beads (Fluospheres; Molecular Probes), 1 μm in diameter, were coated with rabbit anti-human Fc (Jackson) and coupled to Lac-Fc. Control beads were generated by incubating anti-Fc coated beads with conditioned medium from mock-transfected cells. The bead aggregation assay was performed according to Pavlou et al. (Pavlou et al., 2002), with a 1 hour incubation at room temperature. Diluted aliquots were mounted on microscope slides and examined for the formation of bead clusters, using a confocal microscope (Biorad Radiance 2100).

Results

Lachesin, a cell surface protein of the Ig superfamily, is expressed in the trachea

To gain further insight into the mechanisms involved in tracheal morphogenesis we have begun a search for cell surface molecules expressed in the trachea. We have found that the gene coding for the *Drosophila* Lac cell surface protein is expressed in a dynamic pattern including the developing trachea. *Lac* mRNA is first detected in the cellularized blastoderm, where it is excluded from the ventral side. Its expression persists in the early ectoderm, uniformly until completion of gastrulation, and with a peculiar segmental pattern consisting of an alternate strong-weak stripe in each segment from around stage 10, which progressively fades away and disappears by stage 15. Strong expression is detected in specific tissues such as the trachea (from early stage 13), hindgut, foregut and nervous system. In the nervous system *Lac* is detected in subsets of neurons starting from stage 11, and, later, in subsets of glia too, where it has been reported to be a target of *glial cell missing* (Egger et al., 2002; Freeman et al., 2003) (Fig. 1D-G, and data not shown).

Lac was first identified in the grasshopper embryo as a membrane protein specifically expressed in neurogenic cells and subsets of neurons and axons; subsequently, a fly homologue was identified (Karlstrom et al., 1993), but its role during development has not been examined. The Lac protein contains three immunoglobulin (Ig) domains, and a C-terminal hydrophobic domain characteristic of GPI-linked proteins (Fig. 1C). Vertebrate proteins displaying the same domain arrangement are known as IgLONs.

Lachesin is required for tracheal morphogenesis

To assess the role of the Lac protein in tracheal morphogenesis we have analyzed the BG1462 line, which has a P-element inserted into the 5′UTR of the *Lac* gene (Fig. 1B). BG1462 is late embryonic lethal, both when homozygous and over two deficiencies that uncover the *Lac* gene (Fig. 1A). In addition, its lethality can be reverted by precise excision of the P-element. Thus, we have renamed BG1462 as Lac^1.

We have also generated the Lac^2 mutation by imprecise excision of BG1462. Lac^2 is a deletion that removes the *Lac* ATG without affecting nearby genes (see Materials and methods). Comparison of the tracheal phenotypes in homozygous conditions, and over deficiencies that uncover the gene, indicate that both Lac^1 and Lac^2 behave as strong loss-of-function alleles or null alleles. In situ hybridization with a *Lac* probe showed no, or very low, signal in homozygous Lac^1 and Lac^2 embryos (not shown). Immunostaining with a specific anti-Lac antibody that we generated (see Materials and methods, and below) produced a similar result: we did not detect protein expression in homozygous Lac^2 embryos, although residual levels of signal were detected in Lac^1 homozygous embryos in tissues other than the trachea (not

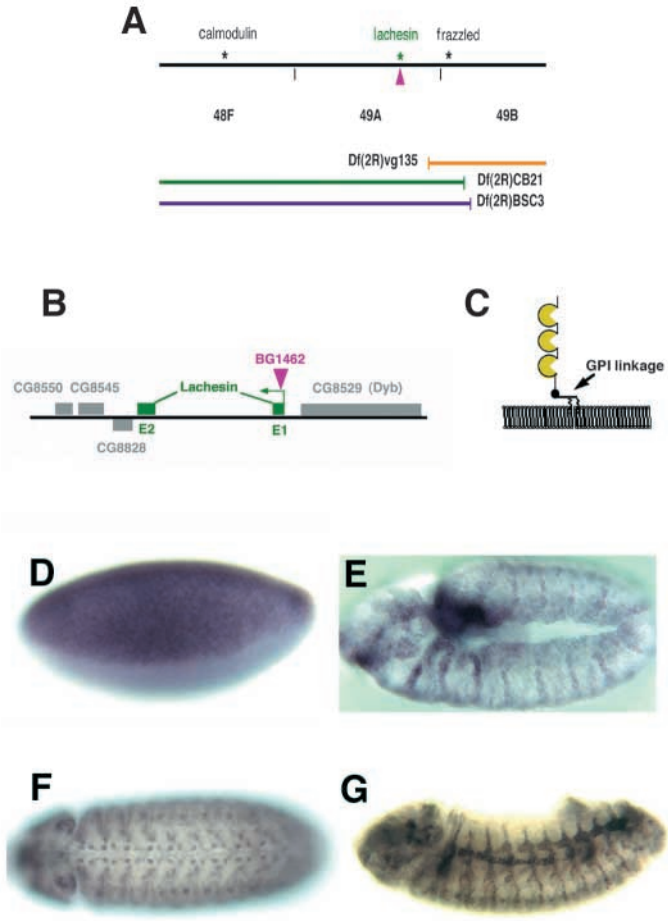

Fig. 1. Organization and expression of the *Lac* gene, and structure of the Lac protein. (A) The *Lac* gene is uncovered by Df(2R)CB1 and Df(2R)BSC3, but not by Df(2R)vg135. (B) The *Lac* gene encompasses around 12 kb and its cDNA is around 2.1 kb. BG1462 is inserted at position 46 of the *Lac* cDNA, 270 bp before the ATG. BG1462 is late embryonic lethal both when homozygous, and over the deficiencies that uncover the *Lac* gene, but not over Df(2R)vg135. Lac^2 is an imprecise excision of BG1462 that removes the ATG. (C) The Lac protein contains three Ig domains and a C-terminal hydrophobic domain, characteristic of GPI-linked proteins. (D-G) In situ hybridization with an antisense *Lac* probe does not detect any signal in oocytes (not shown). (D) At stage 5, the *Lac* transcript is detected in the cellularized blastoderm but it is excluded from the ventral side. (E) At germ band extension, a striped pattern of *Lac* transcript can be detected in the ectoderm. Later on, the *Lac* transcript accumulates in some cells of the nervous system (F) and in the tracheal cells (G).

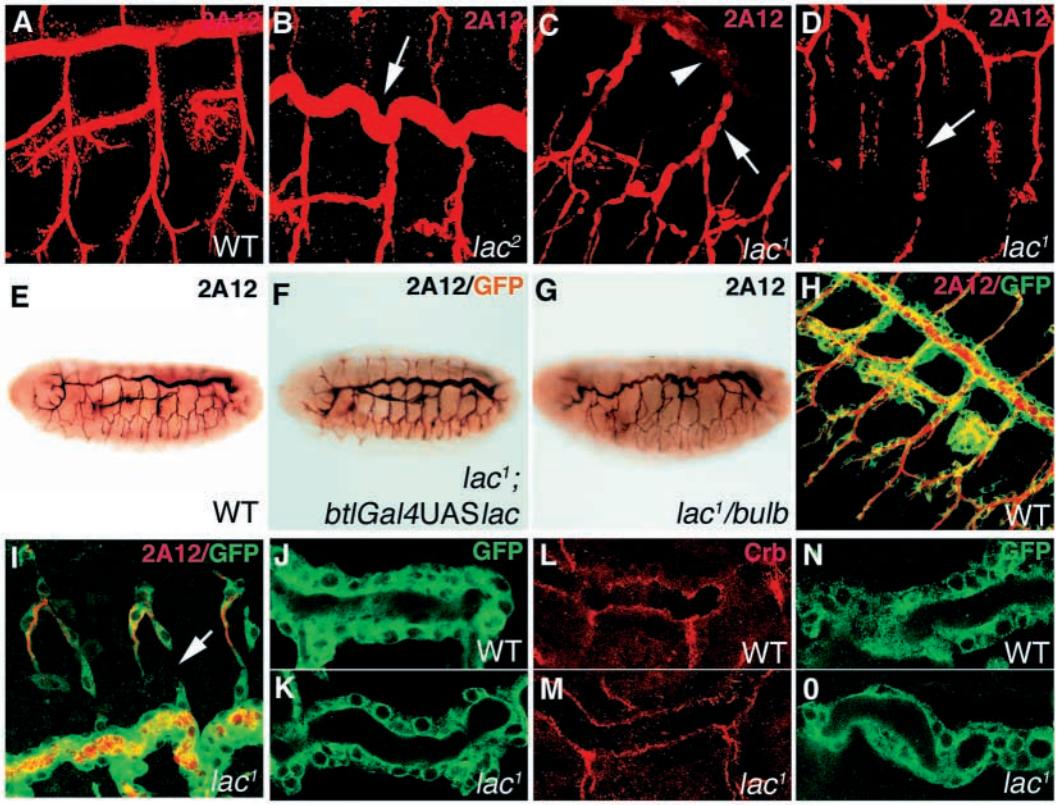

Fig. 2. Characterization of the tracheal phenotype of *Lac* mutants. (A-D,H,I) Projections of several confocal sections showing details of 2-3 tracheal metameres of wild type (A,H), Lac^2 (B) or Lac^1 (C,D,I) mutants at stage 16, stained only with mAb2A12 to show the lumen (red), or with mAb2A12 and an anti-GFP antibody to detect the expression of *tauGFP* in tracheal cells (green), which highlights cell shape. Note the defects in mAb2A12 accumulation (arrowhead in C), branch sinuosity (arrows in B,C), and branch breaks (arrows in D,I). (E) Lateral view of a stage 15 wild-type embryo stained with mAb2A12. (F) Lateral view of a stage 15 Lac^1 embryo, in which *Lac* and *tauGFP* are expressed in the tracheal cells stained with mAb2A12 (black) and anti-GFP (brown). Note the rescue of the tracheal phenotype. (G) Lateral view of a Lac^1/*bulb embryo* at stage 16 stained with mAb2A12 (black), showing the same tracheal phenotype as Lac^1 or Lac^2 mutants. (J-O) Details of the dorsal trunk between tracheal metameres 7 and 8 at stage 16 of wild type (J,L,N) and Lac^1 mutants (K,M,O). Embryos are labelled with anti-GFP (J,K) to show the increased length of the tracheal cells (highlighted by expression of *tauGFP*) and, as a consequence of the dorsal trunk, with a Crb antibody (L,M), and with a GFP antibody (N,O) to detect nodGFP accumulation in tracheal cells.

shown). Consistent with these results, Lac^1 and Lac^2 mutants display the same tracheal phenotype.

The early events of tracheal development, such as guidance and primary branching, occur normally in *Lac* mutant embryos. In addition, branch fusion and extension of terminal branches also show a normal pattern, indicating that *Lac* is not required for these processes. However, from stage 15, Lac^1 and Lac^2 mutants start to display several defects that become more apparent by stage 16. In particular, most branches become more sinuous or convoluted than in the wild-type; this is especially conspicuous in the dorsal trunk. The lumen shows an uneven appearance, with expansions and constrictions along the tubes, and in addition we detect an abnormal accumulation of lumen components when monitoring the lumenal antigen 2A12. We also observe numerous lumenal breaks and discontinuities, mainly in the dorsal and lateral branches (Fig. 2B-D). Moreover, tracheal tubes do not inflate at the end of embryogenesis indicating that they do not become functional (not shown).

We have assessed the specific tracheal requirement of *Lac* activity by inducing *Lac* expression in the tracheal cells of otherwise Lac^1 mutants, and have confirmed that these embryos display a normal tracheal system (Fig. 2F).

A group of mutants, previously reported by Beitel and Krasnow, display a tracheal phenotype similar to that of *Lac* (Beitel and Krasnow, 2000). One of these mutations, *bulb*, maps to the same region as the *Lac* gene. The following observations suggest that *bulb* is a mutation of the *Lac* gene. First, the *bulb* mutation fails to complement both Lac^1 and Lac^2: bulb/Lac^1 embryos (Fig. 2G) show the same kind of tracheal phenotype as *bulb* and Lac^1 homozygotes, and they do not survive. Second, although in *bulb* embryos Lac is expressed in several tissues in a similar way to the wild type, we do not detect Lac expression in tracheal cells (not shown). Thus, *bulb* appears to be a regulatory mutation of *Lac*.

Lachesin controls the size and epithelial integrity of tracheal tubes

To analyze in more detail the nature of the *Lac* mutant defects we have used a *tauGFP* construct to visualize the tracheal cells. We have found that the convoluted shape of the tracheal tubes, mainly the dorsal trunk, is a consequence of them having lengthened too much. However, this extra growth does not appear to derive from an increase in the number of tracheal cells, but rather from an increase in the length of the cells. For example, we have scored the same number of cells in the dorsal trunk between metameres 7 and 8 (18±2 cells on average in this interval in the wild-type, n=14; and 18.3±1.7 in Lac^1, n=16), but the total length of the branch in this interval in *Lac* mutants is 110% of that of wild-type embryos (n=10 intervals measured for Lac^1 and for wild-type embryos) (Fig. 2J,K). Accordingly we do not detect extra tracheal cell proliferation in *Lac* mutants, as assessed by the absence of α-phosphorylated histone H3 staining. Similar observations were

reported for *bulb* (Beitel and Krasnow, 2000). Moreover, we do not detect an enlargement of the nuclei of the mutant cells, as compared with the wild type. These results reveal that *Lac* plays a role in regulating organ size, probably by affecting the shape of the cells.

We have found that many lumenal breaks are the result of an anomalous behaviour of the tracheal cells, which detach from one another in some of the tracheal branches (Fig. 2I). We observed cell detachments that break the tracheal tubes, particularly in those branches where cells elongate more and are thought to be subjected to stronger pulling forces. Indeed, these are the cells where a lessening in cell adhesion should be more readily detected. This phenotype points to a role for the Lac protein in cell adhesion.

On the other hand, the tracheal cells of *Lac* mutants retain a normal epithelial polarity, as judged by the distribution of an apical marker, such as Crumbs (Crb) (Fig. 2L,M) (Wodarz et al., 1995), and a normal polarization of the cytoskeleton, as judged by scoring the minus end of the microtubules with a nodGFP transgene (Bolivar et al., 2001) (Fig. 2N,O).

Lachesin functions as a homophilic adhesion molecule in a bead aggregation assay

Lac shows closest similarity to members of the Ig superfamily belonging to the neuronal Ig cell adhesion molecule (IgCAM) class, which includes vertebrate L1, NCAM, TAG1, Contactin and IgLONs, and *Drosophila* Neuroglian, Wrapper and Klingon (reviewed by Karagogeos, 2003; Tessier-Lavigne and Goodman, 1996). Members of this family have been shown to engage in homophilic and/or heterophilic interactions to mediate cell adhesion, raising the possibility that the molecular mechanism by which Lac contributes to tracheal development could be based on its adhesive properties. In order to establish whether Lac also works as a homophilic cell adhesion molecule, we tested its activity in a bead aggregation assay (Faivre-Sarrailh et al., 1999; Pavlou et al., 2002). In this assay, chimaeric molecules consisting of the protein of interest and the Fc fragment of human IgG are coupled to polystyrene beads, and assayed for their ability to form aggregates (see Materials and methods). Whereas control beads do not aggregate, Lac-Fc coated beads form aggregates (Fig. 3), showing that Lac can work as a homophilic cell adhesion molecule. This in vitro assay confirms the adhesion properties of the Lac protein suggested by the cell detachment phenotype we observed in mutant tracheae.

Lachesin accumulates at the Septate Juntions

We used the antibody raised against Lac to determine the subcellular localization of the protein. Protein distribution essentially recapitulates mRNA expression, although the protein appears to accumulate in the ectodermal derivatives until the end of embryogenesis (Fig. 4A,B), when *Lac* expression has already disappeared from most tissues. Interestingly, the Lac protein is not homogeneously distributed on the cell surface, but rather it accumulates in a lateral region. To precisely determine in which compartment Lac is localized, we carried out double-labelling experiments (Fig. 4).

Drosophila epithelial cells contain an adhesive belt, the ZA (zonula adherens), which encircles the whole cell just below the apical surface, and in which E-Cadherin and its associated proteins are found. Apical to ZA, a subapical region (SAR) is

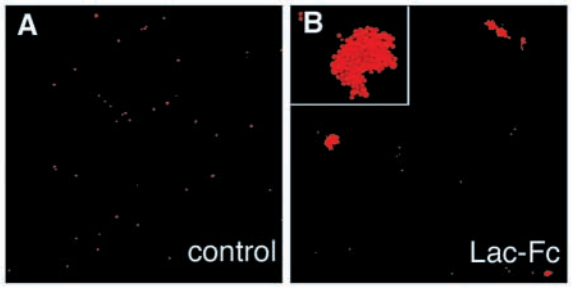

Fig. 3. Lac behaves as a homophilic cell adhesion protein. Confocal microscopy analysis of the bead aggregation assay. (A) Control or (B) Lachesin-Fc-coupled beads were incubated in solution for 1 hour at room temperature and observed under the confocal microscope for formation of bead clusters. Whereas control beads do not form aggregates, Lachesin-Fc beads form large aggregates. Inset (B) shows one aggregate at higher magnification.

observed, where different protein complexes localize, such as the Crb complex. Septate Junctions (SJ) are found basal to the ZA, and are also composed of different protein complexes (Knust and Bossinger, 2002; Tepass et al., 2001). Lac is found adjacent, and lateral, to the SAR protein Crb (Fig. 4C,D). It is also more basally located than the cytoplasmic protein Armadillo (Arm), which accumulates in the ZA via association with E-Cadherin (Peifer, 1993) (Fig. 4E,F). The lack of co-localization between Lac and Crb or Arm is especially conspicuous in the large columnar cells of the hindgut (Fig. 4D,F). By contrast, Lac co-localizes with Coracle (Cor; Cora – FlyBase), Neurexin IV (Nrx), Disclarge (Dlg) and Fasciclin 3 (Fas3), well-known components of the SJs (Baumgartner et al., 1996; Fehon et al., 1994; Genova and Fehon, 2003; Woods and Bryant, 1991). In particular, Lac completely co-localizes with Cora (Fig. 4G,H) and Fas III (not shown), and partially with Nrx (Fig. 4I,J) and Dlg (not shown), whose staining appears to be more enhanced in the most apical region of the apicolateral membrane corresponding to the region of the SJs.

To more finely determine the localization of Lac protein along the apicolateral membrane, we analyzed embryos by immunoelectron microscopy. In order to preserve the structure and antigenicity of the sample, we carried out electron microscopy analysis following cryofixation and freeze-substitution techniques (see Materials and methods). The results show that Lac distribution is restricted to the membrane region, where the septae are observed (Fig. 5), further confirming that Lac is associated with SJs.

Interestingly, *Lac* is expressed in the same tissues in which SJs have been reported. However, SJ formation occurs midway through embryogenesis, which is later than the onset of *Lac* expression. As is the case for Dlg, Scrib and Lgl (Tepass et al., 2001), we propose that Lac is gradually integrated or recruited into the SJs while or once they are formed.

Lachesin is required to maintain the trans-epithelial diffusion barrier

SJs have been proposed to play a prominent role in the formation of trans-epithelial diffusion barriers (Baumgartner et al., 1996; Lamb et al., 1998). As our subcellular localization experiments showed that Lac is a component of the SJs, we tested whether *Lac* is also involved in the formation of such a barrier in the tracheal tubes. We performed a dye permeability

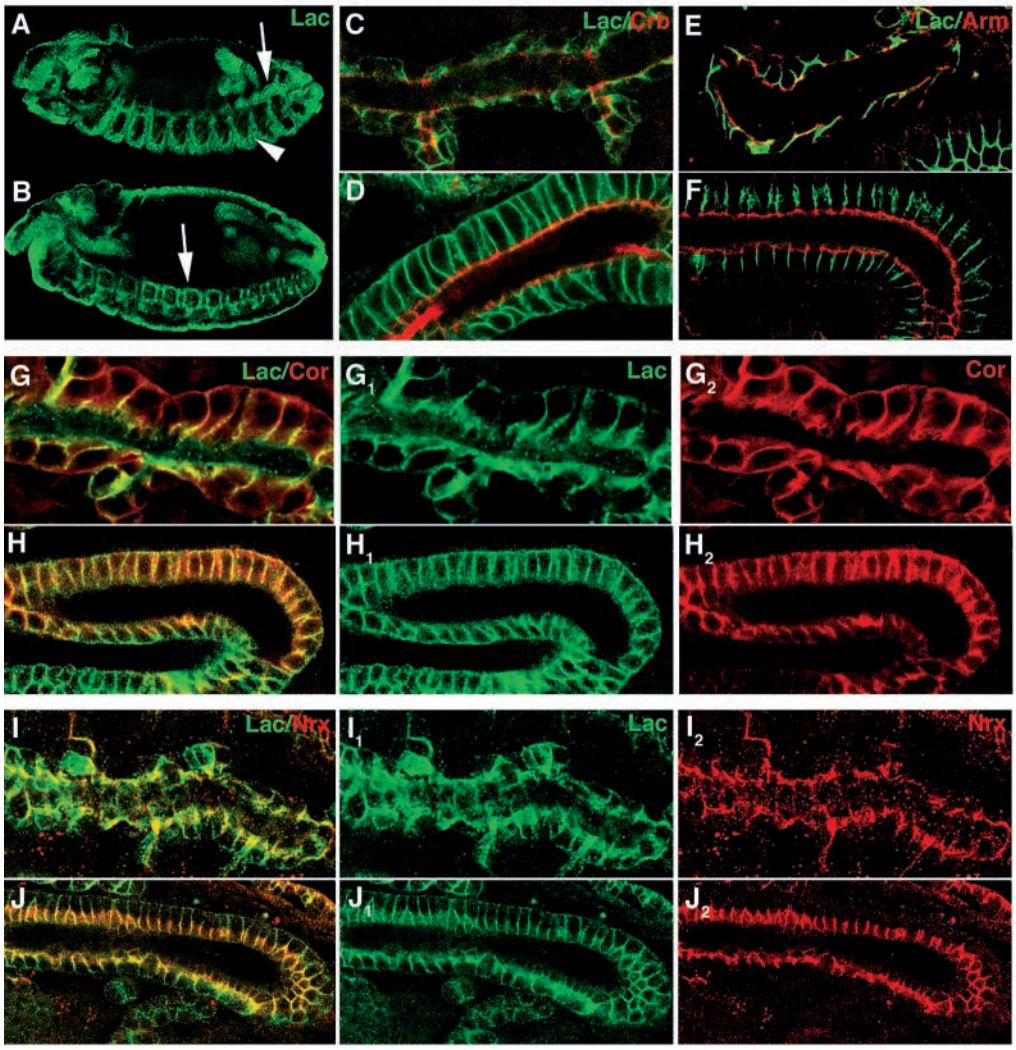

Fig. 4. Lac co-localizes with SJs proteins. (A,B) Stage 14 wild-type embryo labelled with an antibody against Lac; two different focal planes are shown, trachea and epithelium (arrow and arrowhead, respectively, in A) and the CNS (arrow in B). (C-F) Details of the tracheal dorsal trunk (C,E) or the hindgut (D,F) of *LacGFP* stage 16 embryos labelled with anti-GFP to detect Lac accumulation (green), and an anti-Crb (C,D; red) or an anti-Arm antibody (E,F; red). (G,H) Detail of a stage 16 tracheal dorsal trunk (G-G_2) or the hindgut (H-H_2) of wild-type embryos, labelled with anti-Lac (G_1,H_1; green) and anti-Cora (G_2,H_2; red). Note, co-localization of the two proteins. (I,J) Detail of a stage 16 tracheal dorsal trunk (I-I_2) or the hindgut (J-J_2) of wild-type embryos labelled with an anti-Lac antibody (I_1,J_1; green) and a Nrx antibody (I_2,J_2; red).

assay, by injecting a 10 kDa rodhamine-labeled dextran into the hemocoel (Lamb et al., 1998) of *Lac* mutant embryos at the end of embryogenesis. In wild-type embryos, the dextran did not show any diffusion into the tracheal lumen, even more than one hour after injection (Fig. 6A). However, in *Lac* mutant embryos, the dye diffused very quickly and completely filled the tracheal lumen (Fig. 6B), showing that these mutants are unable to establish or maintain the tracheal diffusion barrier. In addition, we found that the dye was internalized in the salivary glands, indicating that they are also affected. In agreement with this, we observed necrotic tissue in the salivary gland region of *Lac* mutant embryos at later stages (data not shown). As we have shown earlier, *Lac* mutants display defects in the accumulation of tracheal lumen components such as 2A12. The fact that *Lac* is necessary to maintain the trans-epithelial diffusion barrier suggests that the improper accumulation of lumen components in *Lac* mutants could be due to leakage of the tracheal tubes rather than to a defect in secretion.

Interdependence of Lac and other SJs proteins for their proper localization

Localization or stabilization of some (but not all) SJs proteins has been found to be dependent upon that of other components. For example, Cora has a very faint staining and a diffuse localization in *Nrx* mutants (Baumgartner et al., 1996), whereas localization of Dlg or Fas3 shows no obvious defect in those same mutants (Genova and Fehon, 2003; Ward et al., 1998). To understand how Lac relates to other SJs proteins we looked at their mutual requirement for localization.

We analyzed the expression of several SJs proteins in Lac^2 mutants. We could not detect differences in the levels of expression of these proteins in the tissues analyzed. However, we detected some changes in the subcellular localization of the SJs proteins we tested, as is the case for Cora, Dlg, Scrib, Nrx, Fas3 and Nrg (Fig. 6C-H and not shown). These proteins were no longer tightly localized to the SJs region, but rather were spread into more basolateral positions, although they were still found in the membrane and an apically concentrated distribution was still observed. These differences were particularly conspicuous in the salivary glands. The salivary glands consist of large columnar cells, where the SJs are restricted to a relatively small region in the lateral membrane, making it easier to observe a mislocalization of the SJs proteins. By contrast, these defects were not so easily detectable in other tissues, including the trachea, because of the wider distribution of the SJs on the lateral cell surface (Fig. 6E,F; and not shown). These results show that *Lac* plays a role in the proper recruitment or accumulation of several SJs proteins into the complexes.

To determine the requirement of known SJs proteins in Lac localization, we assayed Lac distribution in *cora* (Fig. 6I,J) and zygotic *lgl* mutants (not shown). We could not detect defects in the levels of Lac protein, but we detected subtle changes in

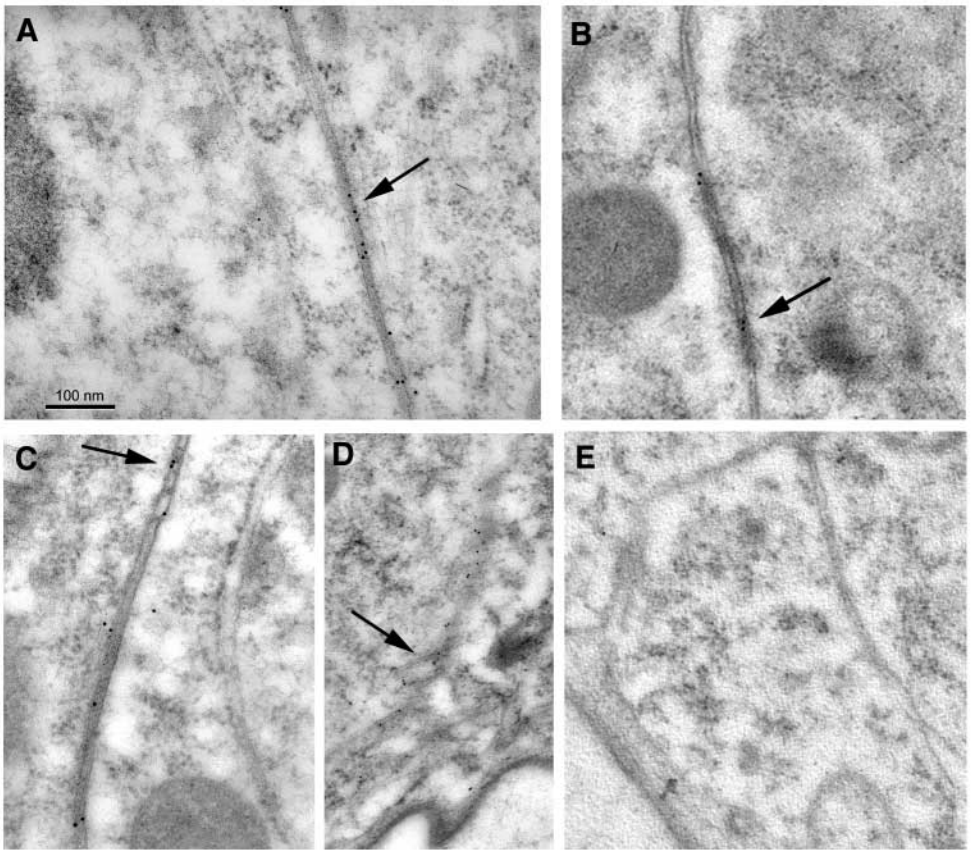

Fig. 5. Lac accumulates at the SJs. Electron micrographs of the ectoderm of wild-type embryos at the end of embryogenesis, labelled with an anti-Lac antibody (A-D). The labelling is visible as gold particles (arrows). (A) Lac specific accumulation along the lateral membrane. (B,C) Lac in the SJs, visible as stretches of electron-dense membrane. (D) Tangentially sectioned SJs with Lac accumulation. (E) Shows a control sample treated equally, but not incubated with the primary antibody (see Materials and methods).

the localization of Lac, which was not so sharply enhanced at the most apical part of the apicolateral membrane. Again, this mild change in Lac localization was more obvious in the salivary glands than in other tissues (Fig. 6I-L). These results indicate that the impairment of SJ integrity affects Lac accumulation.

SJs are required for tracheal morphogenesis

All together, our results suggest Lac as a new component of the SJs. Therefore, we reasoned that SJs could be more generally involved in ensuring proper cell length and cell adhesion during tracheal morphology, and thus we have examined mutants for genes encoding other SJ proteins. In particular, we examined *cora* and *Nrx* mutant embryos, and found that they both display tracheal phenotypes very similar to those of *Lac* mutant embryos (Fig. 6M,N). In particular, both mutants show overgrown tubes with unusual expansions, defects in the accumulation of lumen antigens and lumen breaks. These results demonstrate that there is a general role for SJ in tracheal morphogenesis, and that Lac, as a new component, contributes to this function.

Discussion

A role for SJs in tracheal morphogenesis

The results reported here demonstrate that there is a general role for SJs in tracheal morphogenesis and that Lac contributes to this function. Previous experiments have suggested that the length of the tracheal tubes was controlled at the apical membrane of the tracheal cells (Beitel and Krasnow, 2000), by a mechanism that is dependent on the activity of the Grainy head (Grh) transcription factor in response to Bnl/FGF signalling (Hemphala et al., 2003). The same authors have indicated that an additional Grh-independent mechanism must also function in the lateral cell compartment to control tube length and integrity. Our observation that Lac and other SJs proteins are also required for tube length and integrity leads us to propose that this independent lateral mechanism is based on the function of SJs. In this regard, the localization of none of the SJs proteins analyzed is dependent on *grh* (Hemphala et al., 2003) (M.L., M.S., M.K., D.K. and J.C., unpublished). Very recently, an independent paper has been published on the role of the SJ protein Na^+/K^+ ATPse in tracheal development (Paul et al., 2003). Consistent with our results, these authors also show that SJs play a role in tracheal epithelial tube-size control.

The tracheal tubes seem particularly sensitive to defects in SJs; in particular, there is a loss of epithelial integrity not reported for other ectodermal tissues. In this regard, the specific and strong expression of *Lac* in tracheal cells from stage 13 might hint at a strong requirement for Lac, and possibly SJs, during tracheal morphogenesis. It is important to note that SJs start to form late in embryogenesis, only after most of the morphogenetic events have taken place; however, at these stages, the tracheal tree has not yet completed its morphogenesis and could require fully functional SJs. The integrity of tracheal tubes may be dependent on the ability of their cells to adhere to one another, as they are subjected to stronger pulling forces than most other ectodermal tissues. Interestingly, differences at the level of the cellular junctions have been reported between epidermal and tracheal cells (Tepass and Hartenstein, 1994).

Lac protein and the SJs

Co-localization studies with confocal microscopy, immunoelectron microscopy analysis, functional studies on the permeability of the trans-epithelial barrier, and the mutual requirement of Lac and other SJ proteins for their correct subcellular localization indicate that Lac is a new component

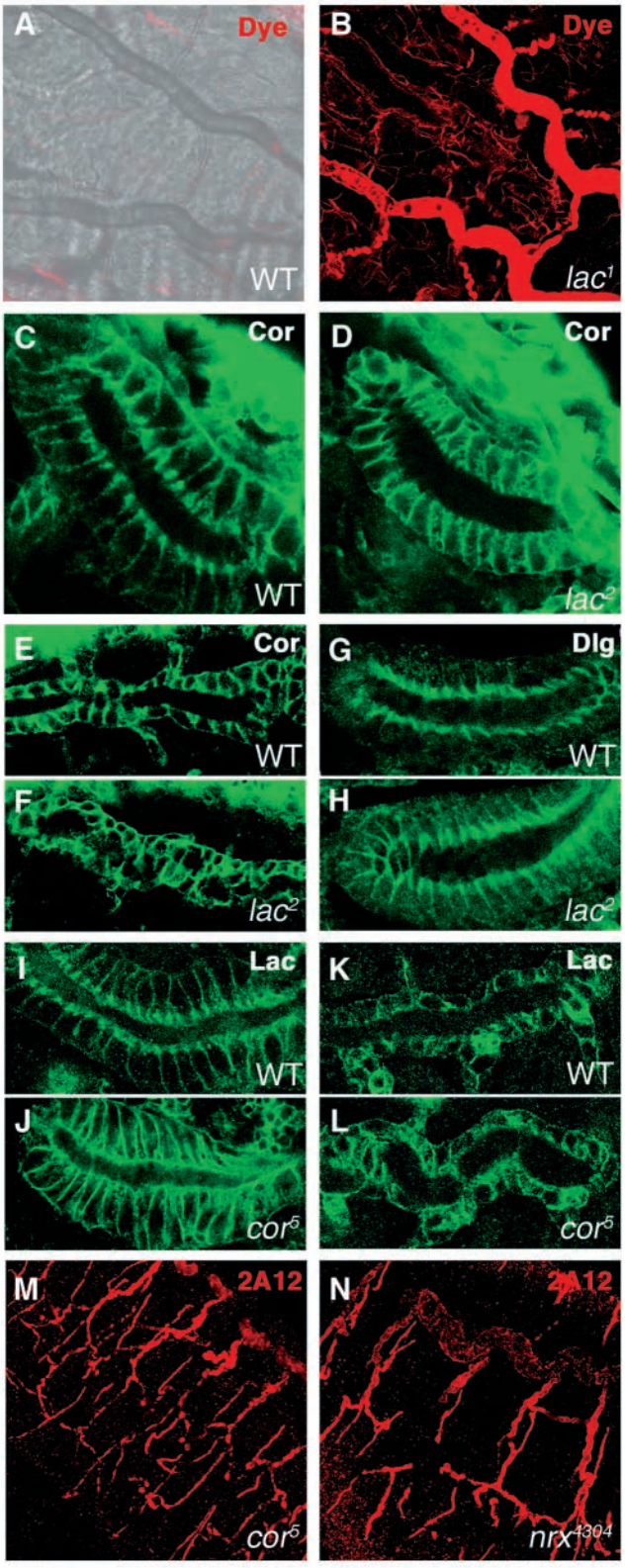

Fig. 6. Lac is required for the distribution of SJs proteins, which are also required for tracheal morphogenesis. (A,B) Detail of the posterior part of stage 16 embryos, in dorsal view, 40 minutes to 1 hour after injecting dye. In the wild type (A), the tracheae (visible with transmission) remains non-permeable, whereas in *Lac*^{1} mutants (B) the tracheae completely fills with dye. (C,D) Salivary glands of wild type (C) and *Lac*^{2} mutants (D) labelled with anti-Cora antibody. (E,F) Detail of the dorsal trunk of wild type (E) or *Lac*^{2} mutants (F) labelled with anti-Cora antibody. (G,H) Salivary glands of wild type (G) and *Lac*^{2} mutants (H) labelled with anti-Dlg antibody. (I,J) Salivary glands of wild type (I) and *cora*^{5} mutants (J) labelled with anti-Lac antibody. (K,L) Detail of the dorsal trunk of wild type (K) or *cora*^{5} mutants (L) labelled with anti-Lac antibody. (M-N) Detail of 3-4 tracheal metameres of *cora*^{5} (M) or *Nrx*^{4304} (N) mutants stained with mAb2A12. Compare with Fig. 2A-D.

of the SJs. As mentioned before, localization or stabilization of some (but not all) SJ proteins has been found to be dependent upon that of other components. For example, Cora has a very faint staining and a diffuse localization in *Nrx* mutants (Baumgartner et al., 1996), whereas localization of Dlg or Fas3 shows no obvious defect in those same mutants (Genova and Fehon, 2003; Ward et al., 1998). SJ components whose proper localization is interdependent are thought to physically interact, and to belong to the same supramolecular complexes in the SJs (Baumgartner et al., 1996; Bilder et al., 2003). These results led to the proposal that SJs are formed by the recruitment of distinct components into different protein complexes. However, it still remains unknown which protein/s would initially be required to target these protein complexes to the SJs (Tepass et al., 2001). Our results indicate that several SJ proteins are mislocalized in *Lac* mutants to a certain degree. One possibility is that Lac interacts with one of the components of the SJs, and that in the absence of this interaction SJs are not properly assembled. Lac is a cell surface protein, associated with the outer leaflet of the membrane bilayer via its GPI-tail, and could potentially interact, in cis and/or in trans, with transmembrane proteins belonging to SJ complexes. It has been shown that Contactin and TAG1, vertebrate GPI-linked proteins of the Ig superfamily, bind to vertebrate Nrx-like proteins in the context of axo-glial interactions in the region of the node of Ranvier (Bhat et al., 2001; Boyle et al., 2001; Traka et al., 2003). We have tested a similar hypothesis for Lac and Nrx with the S2 cell aggregation assay (Bieber, 1994). Although Lac-expressing transfected S2 cells form aggregates, consistent with the results of the bead-aggregation assay (Fig. 3), they do not aggregate with untransfected S2 cells that endogenously express Nrx (Baumgartner et al., 1996) (M.L., M.S., M.K., D.K. and J.C., unpublished). This negative result suggests that Lac does not interact, at least in trans, with Nrx. Ig superfamily members often show heterophilic interactions with other family members (reviewed by Karagogeos, 2003), raising the possibility that Lac interacts with components of SJs carrying Ig domains.

However, our results also indicate that the mislocalization of the analyzed SJ proteins in *Lac* mutants is not as severe as the one described for proteins that are thought to physically interact, as the levels of the proteins analyzed seemed normal. Therefore, our results do not necessarily point to a direct interaction of Lac with those proteins, and could suggest, instead, that SJs are not perfectly assembled in *Lac* mutants and, as a consequence, their components are less well localized or stabilized. In that scenario, Lac could recognize a pre-formed structure of the SJs in order to be properly localized; Lac localization appears to require functional SJs because it is indeed abnormal in mutants that affect SJs structure, such as

cora (Lamb et al., 1998). It has been suggested that SJ multiprotein complexes present in adjacent cells have to interact with each other to ensure proper assembly of the junction (Genova and Fehon, 2003). The nature of Lac as a homophilic cell adhesion protein suggests that it could mediate such intercellular interactions, and could have a role as a component of SJs specifically ensuring or reinforcing the SJs-mediated adhesion between neighbouring cells.

Cell adhesion, cell shape and organ size

Our results suggest a new role for SJs in morphogenesis, by the control of cell length and adhesion. Organ and body size often depend on control of cell number (Conlon and Raff, 1999), and SJs have been previously suggested to control cell proliferation (Lamb et al., 1998; Tepass et al., 2001). Our results suggest that, in the case of the tracheal system, SJs regulate organ size by influencing cell length rather than cell number. We want to point out that these cells do not need to be necessarily bigger to generate an increase in tracheal branch length. A major contribution to the increase in organ size could lie in the fact that cells become more elongated. Indeed, *Lac* mutant tracheal cells appear more elongated, identifying the control of cell shape as one of the regulatory mechanisms of tracheal tube size. It remains an open question whether, in this case, the control of cell length is a direct consequence of the role in cell adhesion. Thus, for example, in a pure mechanical model, the lessening of the tight contact between cells could abrogate a constraint for their elongation. Alternative explanations are also possible; for instance, tight contact among the tracheal epithelial cells would be required for coordinated signalling, allowing polarized conduction of information. Moreover, some of the SJ components themselves could control cell and tissue size via intercellular and intracellular cell signalling, as has been hypothesized, for example, for Cora and Nrg (Genova and Fehon, 2003; Lamb et al., 1998). An unexpected nuclear function for the vertebrate tight junction component ZO2, structurally and functionally related to *Drosophila* Dlg, has been recently suggested (Islas et al., 2002), possibly linking the permeability barrier and signalling functions of these structures. In this respect, other features of the Lac protein not necessarily related to its adhesion properties could be relevant to the control of tube size. For instance, Lac could also have a role in signalling, as is known for other GPI-linked Ig proteins (Stefanova et al., 1991; Walsh and Doherty, 1997).

Although the available data indicate that the mechanisms of tubulogenesis share many basic strategies across diverse animal groups (Affolter et al., 2003; Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003), the generality of our results could be hampered by the fact that SJs have not been reported in vertebrates. However, the localization of the Lac protein at the SJs, and the function of SJs in morphogenesis, could be of functional significance to other systems for several reasons. First, some of the roles of SJs are assumed to be performed by tight junctions in vertebrates. Second, some tight junction components are structurally related to, and others are homologues of, SJs proteins. Third, many homologues of the *Drosophila* SJ proteins, as well as structures that are similar to the SJs, have been identified in mammals (Bellen et al., 1998; Girault and Peles, 2002; Tepass et al., 2001). In this context, SJs-like proteins could be similarly involved in defining proper organ size and morphogenesis in these other systems, by influencing cell adhesion properties and cell shape.

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