The Na^+/K^+ ATPase is required for septate junction function and epithelial tube-size control in the *Drosophila* tracheal system

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Summary

Although the correct architecture of epithelial tubes is crucial for the function of organs such as the lung, kidney and vascular system, little is known about the molecular mechanisms that control tube size. We show that mutations in the *ATPα* α and *nrv2* β subunits of the Na^+/K^+ ATPase cause *Drosophila* tracheal tubes to have increased lengths and expanded diameters. *ATPα* and *nrv2* mutations also disrupt stable formation of septate junctions, structures with some functional and molecular similarities to vertebrate tight junctions. The Nrv2 β subunit isoforms have unique tube size and junctional functions because Nrv2, but not other *Drosophila* Na^+/K^+ ATPase β subunits, can rescue *nrv2* mutant phenotypes. Mutations in known septate junctions genes cause the same tracheal tube-size defects as *ATPα* and *nrv2* mutations, indicating that septate junctions have a previously unidentified role in epithelial tube-size control. Double mutant analyses suggest that tube-size control by septate junctions is mediated by at least two discernable pathways, although the paracellular diffusion barrier function does not appear to involved because tube-size control and diffusion barrier function are genetically separable. Together, our results demonstrate that specific isoforms of the Na^+/K^+ ATPase play a crucial role in septate junction function and that septate junctions have multiple distinct functions that regulate paracellular transport and epithelial tube size.

Key words: Tracheal system, Na^+/K^+ ATPase, Na^+ pump, Septate junctions, Tight junctions, Epithelial tubes, Epithelial morphogenesis, Tube-size control, Tubulogenesis, *Drosophila*

Introduction

Epithelial and endothelial tubes are the essential functional units of many organs including the vascular system, kidney and lung. All of these organs contain tubes of characteristic but widely ranging sizes because efficient flow through any tubular network requires the size of a tube be matched to the flow it supports. A tube that is too small in diameter will have insufficient flow (e.g. obstructive heart diseases), whereas a tube that is too large in diameter can impinge on surrounding tissue (e.g. enlarged kidney tubules in polycystic kidney disease) or have an undesirably slow rate of flow (e.g. blood clotting in cavernous hemangiomas). Thus, control of tube architecture presents fundamental cell biological and developmental problems with medical implications. However, the molecular mechanisms by which epithelial cells measure, change, and then maintain tube diameter and length are not known.

To identify and study the functions of genes required for epithelial tube-size control we are using molecular genetic approaches with the *Drosophila* tracheal system. The tracheal system is the gas exchange organ of the fly, but also resembles and performs some of the same functions as the vertebrate vascular system by directly delivering oxygen through a ramifying network of tubes (Manning and Krasnow, 1993). The tracheal system is created from clusters of invaginated epithelial cells that organize into branches which extend and interconnect to create a complex tubular network (Affolter and Shilo, 2000; Samakovlis et al., 1996). The genetic tools available in *Drosophila*, coupled with the reproducible and rapid development of the tracheal system, make it a powerful system for studying the genetic and molecular basis of tube-size control (Beitel and Krasnow, 2000; Lubarsky and Krasnow, 2003).

Epithelial tube morphogenesis has been shown to require coordinated cell shape changes and dynamic adjustments of cell junctions (Lubarsky and Krasnow, 2003; O'Brien et al., 2002; Hogan and Kolodziej, 2002). Adherens junctions have been shown to play an important role in tracheal morphogenesis (Tanaka-Matakatsu et al., 1996; Uemura et al., 1996; Oda and Tsukita, 1999; Chihara et al., 2003; Lee and Kolodziej, 2002) and in vertebrate epithelial tube formation (Pollack et al., 1997; Pollack et al., 1998). However, to date, the involvement of *Drosophila* septate or vertebrate tight junctions in epithelial tube morphogenesis has not been examined.

Insect septate junctions form the trans-epithelial diffusion barrier that regulates passage of solutes through the spaces between adjacent cells in an epithelium (Tepass et al., 2001). In vertebrates, the paracellular diffusion barrier in epithelial cells is provided by tight junctions (Tsukita et al., 2001; Anderson, 2001). Despite their common barrier function, septate and tight junctions are generally referred to as

analogous structures because they have very different morphologies when examined using electron microscopy and because septate junctions are located basolateral to the adherens junction while tight junctions lie apically. Nonetheless, *Drosophila* septate and vertebrate tight junctions contain some similar proteins that are crucial for their function (Tepass et al., 2001). For example, *Drosophila discs large* encodes a PDZ domain-containing MAGUK scaffold protein similar to vertebrate ZO-1; *coracle* is similar to band 4.1; and *scribble* to human scribble (Willott et al., 1993; Takahisa et al., 1996; Fehon et al., 1994; Bilder and Perrimon, 2000; Nakagawa and Huibregtse, 2000). These observations raise the possibility that the functional similarities of septate and tight junctions may reflect similarities in molecular architecture.

In previous work, we showed that the tracheal system undergoes highly regulated tube-size increases during development and identified eight 'tube expansion' genes that are specifically required for remodeling the size of the tracheal tubes once the initial network has formed (Beitel and Krasnow, 2000). We show that one of those genes is the *nrv2* locus, which encodes two isoforms of a β subunit of the Na^+/K^+ ATPase, and that mutations in the *ATPα* α subunit (*Atpalpha* – FlyBase) locus also cause similar tracheal tube-size defects. Further, we show *nrv2* and *ATPα* mutations disrupt septate junction function.

The Na^+/K^+ ATPase is an α/β heteromultimer that creates the essential electrochemical gradient across the plasma membrane by transporting two K^+ into and three Na^+ out of the cell for each ATP hydrolyzed (Blanco and Mercer, 1998; Chow and Forte, 1995). The α subunit is a ten transmembrane-domain protein containing the Na^+ and K^+ channels and the phosphorylation and nucleotide binding sites (Chow and Forte, 1995). The single transmembrane domain β subunit is required for transport of the α subunit out of the ER (Geering et al., 1996; Hasler et al., 1998) and is thought to modulate the affinity of the α subunit for Na^+ and K^+ (Blanco and Mercer, 1998; Chow and Forte, 1995; Hasler et al., 1998). In flies there are three β subunit loci, *nrv1* and *nrv3* (CG8663), each of which produce one isoform, and *nrv2* which encodes two isoforms, Nrv2.1 and Nrv2.2. There are two α subunit loci, *ATPα* that produces at least 12 α subunit isoforms (Palladino et al., 2003), and CG17923, which appears to be minimally expressed because its transcripts are poorly represented in existing cDNA libraries. Of these loci, only *nrv1*, *nrv2* and *ATPα* have been characterized in detail, primarily for their roles in the nervous system (Lebovitz et al., 1989; Palladino et al., 2003; Sun and Salvaterra, 1995b; Sun et al., 1998). A tracheal phenotype was recently described for *ATPα* mutants, although β-subunit mutants were not analyzed (Hemphala et al., 2003).

In vertebrates, different Na^+/K^+ ATPase isoforms have been proposed to have unique functions because they are expressed with tissue and temporal specificity, and because different isoforms have distinct biochemical and pharmacological properties in vitro (Blanco and Mercer, 1998). However, in vivo experiments have so far failed to provide support for this hypothesis (Weber et al., 1998). Our results show that septate junctions and specific Na^+/K^+ ATPase isoforms have previously unidentified roles in tracheal tube-size control and that the tube-size control and trans-epithelial barrier functions of septate junctions are distinct.

Materials and methods

Fly strains, genetic manipulations and EMS mutagenesis

Flies were obtained from the Bloomington Stock Center or published sources, except for the following: *nrv2*^{23B} and *nrv2*$^{l(2)k13315}$e22c-GAL4 from J. Genova and R. Fehon (personal communication) and *btl*-GAL4 UAS-GFP from M. Metzstein and M. Krasnow (unpublished). *nrv2*^{nwu3} was generated by imprecise excision of the l(2)k04223 P-element insertion and deletes the first two and most of the third common exons. The genomic sequence across the deletion breakpoints is AAGGCCCTCGCTATAACCAAGGAGAATAAC. This allele is likely to be a molecular null because the deleted region encodes transmembrane domain and extracellular regions, which are required for assembly with the α subunit and for export of the Na^+/K^+ ATPase from the endoplasmic reticulum (Geering et al., 1996; Hasler et al., 1998). Germline clones were produced with the *nrv2*^{nwu3}, FRT 40A chromosome using the FLP-ovoD method (Chou and Perrimon, 1996). EMS mutagenesis generated two *nrv2* nonsense mutations, one in the first common exon (*nwu5*: CTGCATGTGG→CTGCATGTGA) and one in the second common exon (*nwu6*: CAAGCACTGG→CAAGCACTAG) that were identified by non-complementation with *nrv2* $^{l(2)k4223}$. Third chromosome lines were balanced by either currently available balancers or the TM6B *Hu Sb*^{1} *e Tb*^{1} *ca*^{1} chromosome onto which we mobilized the *dfd* Hz2.7lacZ element (McGinnis et al., 1990).

Immunohistochemistry, microscopy and morphometric analysis

Antibodies 2A12 and TL1, embryo fixation, staining and staging procedures are described elsewhere (Samakovlis et al., 1996). Other antibodies used were AS55 (Reichman-Fried et al., 1994), H5F7 (Sun and Salvaterra, 1995a), anti-α5 (Lebovitz et al., 1989), anti-Armadillo (Riggleman et al., 1990), anti-Coracle (Fehon et al., 1994), anti-DLG (Woods et al., 1996), anti-DLT (Bhat et al., 1999), anti-Neurexin (Baumgartner et al., 1996) and anti-β-Galactosidase (Capel). Embryos stained with α5 anti-α were fixed in 4% paraformaldehyde and hand devitellinized. For H5F7 anti-β, standard formaldehyde/heptane fixation and methanol devitellinization produced basolateral staining that overlapped with Coracle and Neurexin, while paraformaldehyde fixation and hand devitellinization yielded cytoplasmic/perinuclear staining. Using either method, ectodermal staining was reduced to background levels in *nrv2* null mutants. H5F7 staining of embryos expressing UAS- *nrv*X transgenes revealed that for the *btl*-Gal4 and e22c-Gal4 drivers, *nrv2.1*, *nrv2.2* and *nrv3* had equivalent staining levels and subcellular localizations, but that although detectable, *nrv1* staining was significantly weaker.

Dorsal trunk and transverse connective lengths were measured as described by Beitel and Krasnow (Beitel and Krasnow, 2000), except Metamorph software (Universal Imaging) was used for the morphometric analysis. Confocal images were captured using a Leica TCS SP2 maintained and supported by the Northwestern Biological Imaging Facility. To assess protein levels, heterozygotes and homozygous mutants were imaged at the same settings on the same slide in the same session. Adjustments performed in Photoshop were applied equally to all images. All embryos were stage 16 unless otherwise noted.

Dye exclusion and RNAi

Texas Red-conjugated 10 kDa dextran was injected into embryos as described by Lamb et al. (Lamb et al., 1998). We believe that abnormally rapid leakage across tracheal epithelia indicates septate junction defects and not other tracheal defects as the disconnected tracheal segments of *hnt* mutant or homozygous balancers embryos do not leak dye (data not shown).

Double-stranded RNAs were generated by PCR amplification of templates with primers containing the T7 promoter sequence, in vitro transcription, and were injected into early embryos following standard

protocols (Kennerdell and Carthew, 1998). *btl*-Gal4 UAS-GFP homozygote embryos were used to visualize the tracheal system. RNAi-injected embryos were allowed to develop until stage 16-17, injected with 10 kDa dye and viewed with a Zeiss Axioplan2 microscope. Common exon regions were chosen for dsRNAs templates for all genes tested, except for nrv2.1 and nrv2.2 where unique 5′ exons were targeted. Specificity of isoform-targeted dsRNAs was demonstrated by injecting nrv2.1 or nrv2.2 dsRNA into *btl*-GAL4 UAS-*nrv2.2* embryos. nrv2.2 dsRNA injections caused tracheal phenotypes and reduced levels of the overexpressed Nrv2.2 (5/5 embryos), while nrv2.1 dsRNA injections caused tracheal morphology defects but did not affect Nrv2.2 levels (9/9 embryos).

Molecular biology

The sequence of *nrv3*, the predicted Na^+/K^+ ATPase β subunit CG8633, was determined from cDNA GH12088 (GenBank Accession Number AY314744). UAS-*nrv1*, UAS-*nrv2.1*, UAS-*nrv2.2* and UAS-*nrv3* were constructed by inserting *nrv1*, *nrv2.1*, *nrv2.2* (Sun and Salvaterra, 1995b) and *nrv3* cDNAs into the pUAST vector (Brand and Perrimon, 1993). *nrv2* alleles were sequenced by PCR amplification of genomic DNA from heterozygous flies, followed by cycle dye termination sequencing.

Results

nrv2, a Na^+/K^+ ATPase β subunit locus, is required for tracheal tube size control

In previous work, we showed that embryos homozygous for the l(2)k04223 strain had tracheal tube-size regulation defects (Beitel and Krasnow, 2000). Using inverse PCR, we determined that the transposable element in this strain was inserted in an intron in the *nervana2* (*nrv2*) locus, which encodes two alternatively spliced isoforms of a Na^+/K^+ ATPase β subunit (Sun and Salvaterra, 1995a; Sun and Salvaterra, 1995b). Two lines of evidence demonstrate that the tracheal phenotype of l(2)k04223 homozygotes results from disruption of the *nrv2* locus. First, an independent transposable element insertion in the *nrv2* locus, l(2)k13315, causes the same tracheal phenotypes as l(2)k04223 and fails to complement l(2)k04223 for tracheal phenotypes and viability (data not shown). Second, in an EMS non-complementation screen we isolated two additional mutations that fail to complement l(2)k04223 (see Materials and methods). Both of these mutants contain a single nonsense base change in the exons common to both *nrv2* isoforms, and both have the same tracheal phenotypes as l(2)k04223. Together, these results show that a Na^+/K^+ ATPase β subunit is required for tracheal tube-size control.

To better define the role of *nrv2* in epithelial morphogenesis, we generated a putative *nrv2* null allele, $nrv2^{nwu3}$, that deletes the first three common *nrv2* exons which encode transmembrane and extracellular domains (see Materials and methods). In addition, Genova and Fehon (Genova and Fehon, 2003) provided a second putative null allele, $nrv2^{23B}$, that removes all *nrv2* common exons. The phenotypes caused by $nrv2^{nwu3}$ or $nrv2^{23B}$ do not become more severe in trans to a chromosomal deficiency known to delete *nrv2*, providing genetic evidence that these are null alleles.

In *nrv2*-null embryos, beginning at the time of tracheal tube expansion, multicellular tracheal tubes become increasingly abnormal so that most tube lengths are significantly increased and all tube diameters are irregular with expansions and constrictions along their lengths (Fig. 1B,F-I; Table 1). Defects are also present in regions of single cell tubes formed by autocellular junctions, particularly near the ends of the ganglionic branches where there are lumenal staining discontinuities (Fig. 1G).

Although the process of tracheal tube expansion is drastically disrupted in *nrv2*-null mutants, the earlier of phases of tracheal tube morphogenesis, including early tube-size regulation, are normal (Table 1). Furthermore, overall embryonic morphogenesis of *nrv2*-null mutant embryos also appears to be grossly normal as evidenced by the correct outgrowth of all tracheal branches to their target tissues and the absence of major patterning or morphogeneic defects as assessed using DIC optics (Fig. 1B,G). One possible explanation for the apparent specific morphogenic requirement of *nrv2* in tracheal tube expansion is that there is a maternal contribution of *nrv2* that provides enough activity to support early morphogenic processes, but not enough to support tracheal tube expansion, which occurs late in embryonic development. However, in situ hybridization and microarray analysis did not reveal any significant maternal *nrv2* transcript (data not shown and Berkeley Drosophila Genome Project) and embryos lacking both maternal and zygotic *nrv2* are indistinguishable from *nrv2* zygotic null embryos (Fig. 1H). Thus, *nrv2* does not play a role in early epithelial formation or general embryonic morphogenesis, but instead appears to be specifically required for remodeling the length and diameter of tracheal tubes.

ATPα, a Na^+/K^+ ATPase α subunit locus, is required for tracheal tube size control

To test whether *nrv2* functions as part of the Na^+/K^+ ATPase to control tracheal tube size, we examined the embryonic phenotypes of mutations in the major Na^+/K^+ ATPase α subunit locus, *ATPα* (Lebovitz et al., 1989; Sun et al., 1998; Palladino et al., 2003). The transposable element insertions $ATP\alpha^{l(3)1278}$, $ATP\alpha^{l(3)04694}$ and $ATP\alpha^{l(3)07008}$ caused tracheal defects similar or identical to *nrv2*-null mutations, including tube length increases, diameter expansions and ganglionic branch discontinuities (Fig. 1M,N). The *ATPα*–null mutations $ATP\alpha^{DTS1R1}$ and $ATP\alpha^{DTS1R2}$ (Palladino et al., 2003) also caused *nrv2*-like length and ganglionic branch defects, but caused only mild diameter defects (Fig. 1O). Although one would normally expect the *ATPα*-null mutations to cause more severe phenotypes than partial loss-of-function mutations, the hypomorphic *ATPα* mutations might cause strong *nrv2*-like phenotypes by producing inactive α subunits that could unproductively interact with Nrv2 and deplete the pool of Nrv2 available for productive interactions with other binding partners, such as α subunits expressed from the secondary Na^+/K^+ ATPase α subunit locus CG17923. However, despite some differences between the phenotypic effects of different *ATPα* mutations, the observation that both null and partial loss-of-function mutations cause *nrv2*-like tracheal tube-size defects demonstrates that the *ATPα* locus is required for tracheal tube-size control and suggests that the *nrv2* β and *ATPα* α subunits function together in this process.

The Na^+/K^+ ATPase is required for septate junction function

During our investigations of the role of the Na^+/K^+ ATPase in tube-size control, J. Genova and R. Fehon reported that Na^+/K^+ ATPase mutants had salivary gland septate junction defects

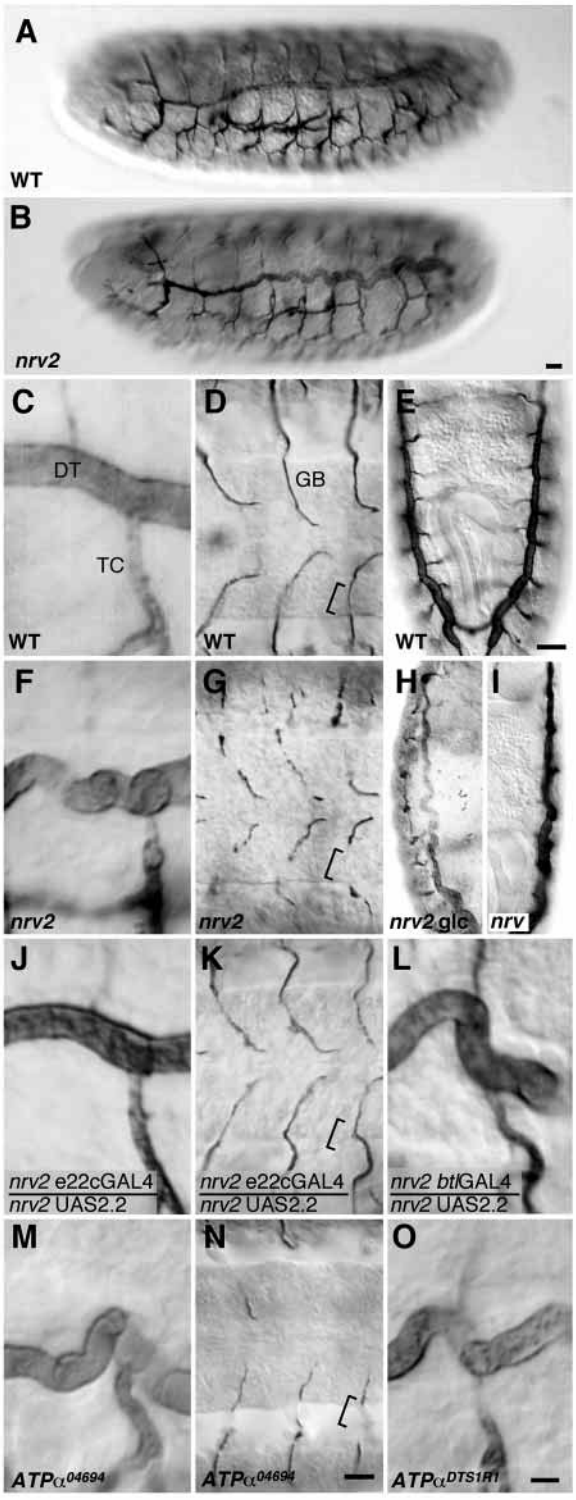

Fig. 1. *nrv2* and *ATPα* mutations cause tracheal length and diameter defects. When compared with wild-type (WT) animals (A,C-E), *nrv2* (B,F-I) and *ATPα* (M-O) mutant trachea have increased length, diameter expansions and missing lumen segments. The tracheal defects in *nrv2* homozygotes can be completely rescued by expressing the *nrv2.2* isoform using the e22c-Gal4 driver (J,K) or partially rescued using the *btl*-Gal4 driver (L). Similarly, expression of the *nrv2.1* isoform by the e22C-Gal4 driver could also completely rescue all tracheal defects in *nrv2* mutants (Fig. 6H), whereas the *btl*-Gal4 driver produced only the same partial rescue seen with *nrv2.2* (data not shown). (A-C,F,J,L,M,O) Lateral views of the dorsal trunk (DT) and transverse connective (TC). (D,G,K,N) Ventral views of the ganglionic branches (GB). (E,H,I) Dorsal views of the dorsal trunk. The animal in H lacks both zygotic and maternal *nrv2*. All images are of stage 16 embryos, except E,H,I, which show stage 15 embryos. Examples of lumenal gap regions are indicated with brackets. UAS-*nrv2.1* or UAS-*nrv2.2* expressed under the control of the e22c driver rescued the *nrv2* tracheal phenotype to wild type in 8/9 and 19/21 animals respectively. Genotypes: (A,C-E) Oregon R; (B,F,G,I) $nrv2^{nwu3}$; (H) $nrv2^{nwu3}/nrv2^{-23B}$ from $nrv2^{nwu3}$ germline clone (glc); (J,K) $nrv2^{k13315}$e22C-Gal4/$nrv2^{l(2)k04223}$ UAS nrv2.2; (L) $nrv2^{l(2)k04223}$ *btl*-Gal4/$nrv2^{l(2)k04223}$ UAS nrv2.2; (M,N) $ATP\alpha^{04694}$; (O) $ATP\alpha^{DTS1R1}$. Scale bar: in B, 10 μm for A,B; in O, 5 μm for C,F,J,L,M,O; in N, 5 μm for D,G,K,N; in E, 10 μm for E,H,I.

(personal communication). We therefore tested whether tracheal septate junction barrier function was defective in Na^+/K^+ ATPase mutants using the dye exclusion assay of Lamb et al. (Lamb et al., 1998), which tests the ability of an epithelium to exclude a 10 kDa dye. In wild-type embryos, tracheal septate junction barriers become functional and excluded dye starting at late stage 15 (e.g. Fig. 2a). However, the tracheal and salivary gland epithelia in *nrv2* and *ATPα* mutants do not acquire the ability to regulate paracellular transport and cannot prevent the dye from inappropriately diffusing into the tracheal and salivary gland lumens (Fig. 2b,c).

To understand the nature of the septate junction defects in Na^+/K^+ ATPase mutants, we determined the subcellular distribution of septate junction and cell polarity components in stage 16 *nrv2* and *ATPα* null mutants. We examined three ectodermally derived epithelia – trachea, epidermis and salivary glands – and found similar defects in all three tissues. The effects were most clearly seen in the large columnar cells of the salivary gland in which the septate junction occupies only a small region of the lateral surface of the cell (Fig. 3) (Tepass and Hartenstein, 1994). By contrast, septate junctions occupy most of the lateral surface of tracheal cells, making visualization of mislocalized septate junction components more difficult. In both *nrv2* and *ATPα* mutants, the septate junction components Coracle, Neurexin and Discs Large mislocalize along the lateral and sometimes the basal cell surfaces, rather than being tightly localized to the apicolateral septate junction (Fig. 3A-D and data not shown). In some cases, the levels of these proteins appeared to also be reduced (e.g. Fig. 3D). The septate junction component Fasciclin III was undetectable in *nrv2* and *ATPα* mutant salivary glands and strongly reduced in trachea (Fig. 3F and data not shown).

Although every septate junction marker tested is severely affected by the Na^+/K^+ ATPase mutations, the effects appear to be specific for septate junctions since the localizations and levels of the adherens junction components E-cadherin (Shotgun) and β-catenin (Armadillo), and the apical determinant Discs Lost were unaffected in *nrv2* and *ATPα* null mutants (Fig. 3F,H). Together, these data demonstrate that Na^+/K^+ ATPase mutations specifically disrupt septate junctions.

The Na^+/K^+ ATPase localizes to septate junctions independent of Coracle

The function of the Na^+/K^+ ATPase in the septate junction

Table 1. Summary of tracheal phenotypes of septate junction mutants*

Gene (alleles)†	DT length‡	TC length‡	DT diameter defects	Diameter defects in other primary branches	2A12 staining defects§	AS55 staining defects§	Missing lumen in ganglionic branches§	Onset of phenotypes
nrv2 (*nwu3*)	**113±2%**	99±3%	++	++	+	+	+	Stage 15
(*23B*)	**126±2%**	109±6%	++	++	+	+	+	Stage 15
coracle (*4, 5, 4/5*)	**122±2%**	104±3%	++	++	+	+	+	Stage 15
neurexin IV (*4865, 4025*)	**116±4%**	N/D	++	++	++	++	+	Stage 15
neuroglian (*14, 17*)	**121±2%**	94±3%	++	++	+	+	+	Stage 15
gliotactin (*J29-41bl, AE2-4*)	**146±6%**	N/D	+	+	+/–	N/D	–	Stage 15

*Phenotypes of stage 16 embryos. For all mutants, early tracheal development, including primary branch budding and outgrowth, lumen formation and morphology, and TL1 antigen expression occurred normally during stages 11-14. In all cases, tracheal branches fused at the correct stages to form the contiguous tubular networks. –, normal; +, mild; ++, moderate; +++, severe, N/D, not determined.

†Where multiple alleles are listed, all alleles of each gene had comparable tracheal phenotypes.

‡Values shown (mean±s.e.m., n>5) have been normalized to stage 16 wild-type values (embryo length, 361±4 μm; DT length, 194±1 μm; TC length, 47±1 μm) as described by Beitel and Krasnow (Beitel and Krasnow, 2000). Values different from wild type (P<0.005) are in bold. Measurements were made on *cor[4]/cor[5]*, *nrx[4865]/nrx[4025]*, *nrg[17]*, and *gli[J29-41bl]* embryos. Average lengths of embryos were *nrv2*[*nwu3*], 349±6 μm; *nrv2*[*23B*], 365±10 μm; *cor*, 372±4 μm; *nrx*, 375±6 μm; *nrg*, 330±3 μm; *gli*, 377±19 μm.

§Defects included non-staining regions of ganglionic branches and/or delayed or reduced staining in the dorsal or other branches.

could be direct as structural component, indirect through its generation of the electrochemical gradient, or both direct and indirect. To assess a possible direct role, we investigated the subcellular localization of the ATPα and Nrv2 proteins. In salivary glands, epidermis and trachea, ATPα staining predominantly colocalizes with Coracle and Neurexin at the apicolateral septate junction region, although some variable, low intensity ATPα staining of the basolateral cell surfaces that did not correlate with genetic background was also observed (Fig. 4A-C and data not shown). In *nrv2* null mutants, ATPα levels are significantly reduced in the trachea and essentially absent in salivary glands (Fig. 4D). Where ATPα staining is

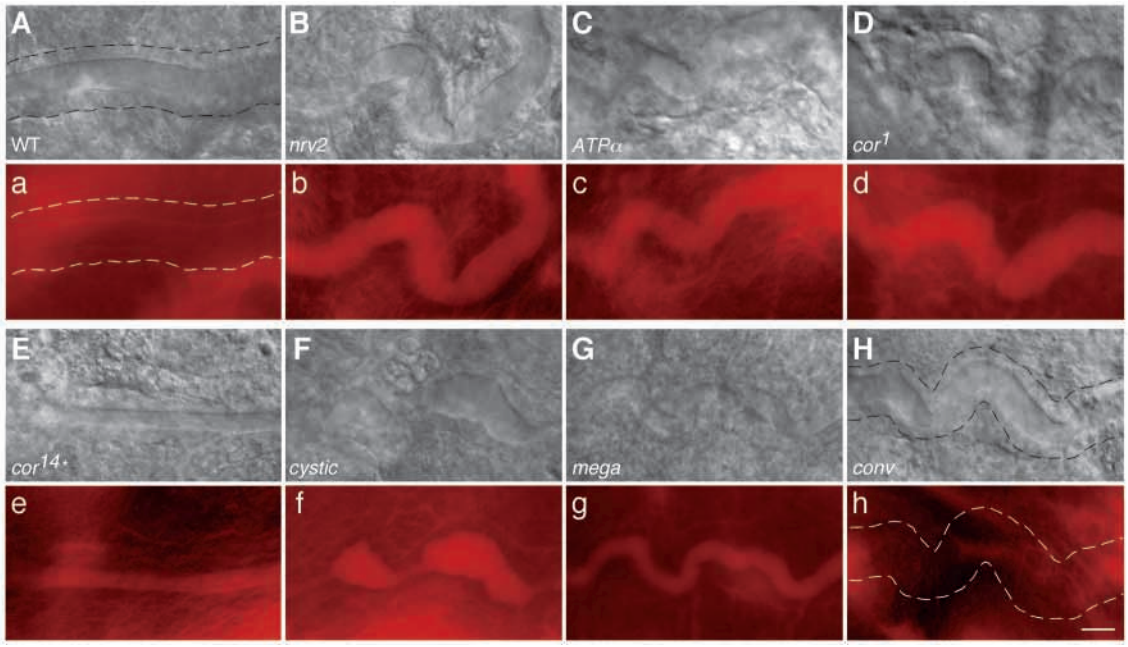

Fig. 2. Tracheal tube size defects do not result from trans-epithelial diffusion barrier defects. The integrity of the septate junction diffusion barrier in tracheal and septate junction mutants was assessed by a dye permeability assay. For each mutant, DIC images of the trachea are shown in black and white (A-H) and the matched fluorescence image taken in the same focal plane is shown in color (a-h). Wild-type trachea exclude fluorescently labeled 10 kDa dextran dye injected into the body cavity (a; dotted lines outline tracheal tubes), while the trachea of Na^+/K^+ ATPase and other mutants are permeable to the dye which enters and fills their lumens (b-g). Permeability defects do not cause the observed tube-size defects as cor^{14}* mutants have defective diffusion barriers (e) but have normal tracheal morphology (E). Furthermore, *convoluted* (*conv*) mutants have the identical tube-size defects as *nrv2* (compare H with B) but do not have permeability defects (h). All mutants that failed to exclude dye from the trachea also failed to exclude it from the salivary gland. Genotypes: $nrv2^{nwu3}$, $ATP\alpha^{DTS1R2}$, cor^{14}* is $coracle^{14}$ plus additional unidentified genetic background, $cystic^{k13717b}$, $megatrachea^{EA97}$, $convoluted^{k6507b}$. In addition, $coracle^{5}$, $varicose^{3953b}$, $neurexin\ IV^{14}$, $neuroglian^{17}$, $gliotactin^{J29\text{-}41bl}$, $nrv2^{l(2)k04223}$ *btl*-Gal4/$nrv2^{l(2)k04223}$ UAS-nrv2.1 and $nrv2^{l(2)k04223}$ btlGal4/$nrv2^{l(2)k04223}$ UAS nrv2.2 animals have tracheal diffusion barrier defects, while $coracle^{15}$, $hindsight^{1142}$, $nrv2^{k13315}$ e22C-Gal4/$nrv2^{l(2)k04223}$ UAS-nrv2.2, $nrv2^{k13315}$ e22C-Gal4/$nrv2^{l(2)k04223}$ UAS nrv2.1 and $coracle^{14}$(backcrossed) do not (data not shown). Scale bar: 10 μm.

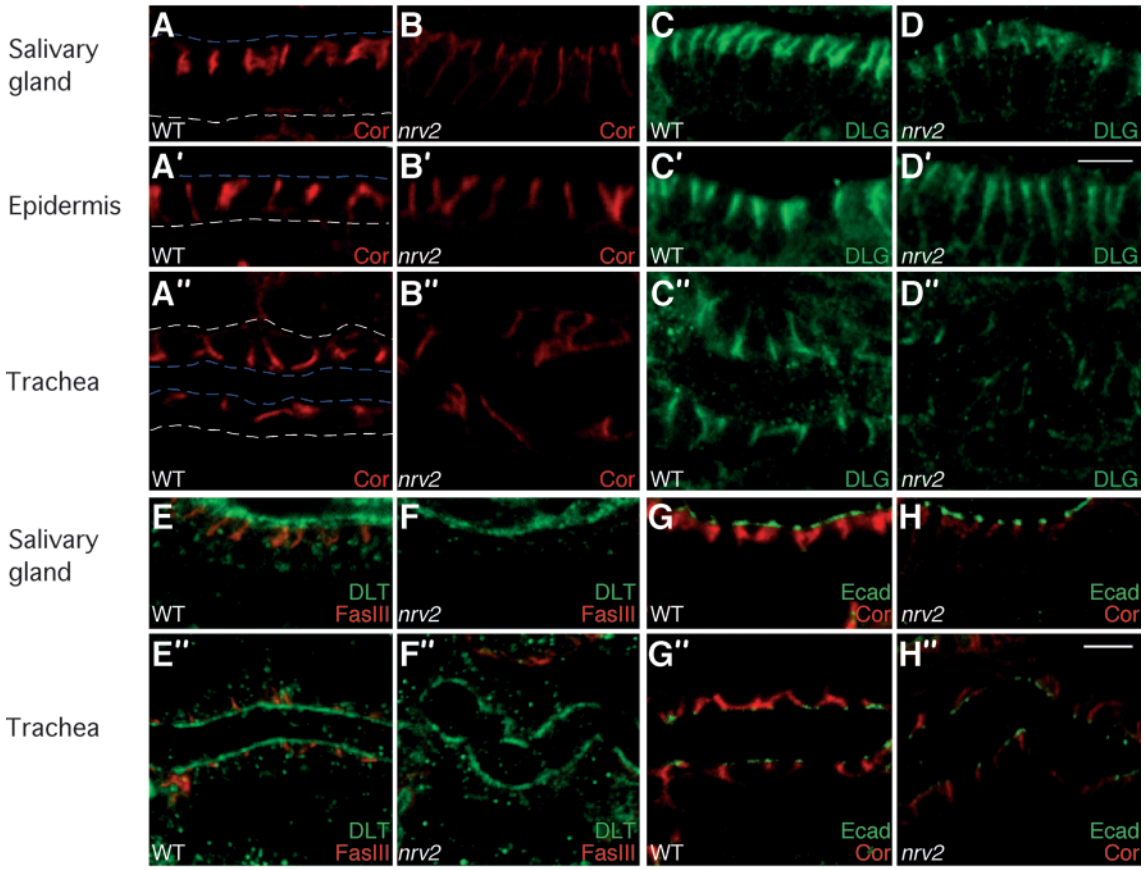

Fig. 3. Septate junction components are mislocalized or dramatically reduced in Na^+/K^+ ATPase mutants. In *nrv2* mutants (B,D,F,H), Coracle (red, B,H), Discs Large (green, D) and FasIII (red, F) appear to be reduced and/or no longer have the septate junction localization seen in wild-type animals (A,C,E,G). Neurexin staining closely resembled Coracle staining and was mislocalized in *nrv2* mutants (data not shown). Localization of Discs Lost (green, E,F), E-cadherin (green, G,H) and Armadillo (data not shown) was unaffected by *nrv2* mutations (F,H). *ATPα* null mutations caused the same mislocalization and defects in protein levels as do *nrv*2 mutations (data not shown). Basal surfaces are outlined in white, apical/lumenal surfaces in blue. For each marker examined, the wild-type and mutant images are matched pairs (e.g. A and B, and C and D), where the wild type is a heterozygous embryo imaged with the same settings, on the same slide, and in the same session as the mutant embryo to provide an internal reference for protein levels. Scale bar: in H″, 5 μm for A-D,A″-D″,E-H″; in D′, 5 μm for A′-D′.

visible, it is no longer localized to the apicolateral septate junction region, but instead along the entire lateral surface. Thus, the ATPα localizes to the septate junction, and this localization is Nrv2 dependent.

Staining *nrv2*-null heterozygotes and homozygotes with an anti-β subunit antibody (Sun and Salvaterra, 1995a) revealed that Nrv2 accounts for the essentially all of anti-β subunit staining in ectodermally derived tissues, but the subcellular localization of Nrv2 could not effectively be assessed because β subunit localization depended on fixation conditions and ranged from basolateral to perinuclear (see Materials and methods). As localization of the ATPα subunit is dependent on Nrv2 (Fig. 4D-D″), and the *ATPα* and *nrv2* mutants have the same phenotype, it seems likely that the functional Nrv2 protein will be localized to the septate junction.

Significantly, although Coracle and Neurexin are mislocalized and their levels may be reduced in *ATPα* and *nrv2* mutants (Fig. 3B and data not shown), the localizations and levels of ATPα are unaltered in *coracle* mutants (Fig. 4E). Together, the above results suggest that the Na^+/K^+ ATPase is a structural component of the septate junction, and that it is required for stable formation of a complex containing Coracle and Neurexin.

Tracheal tube size control requires septate junctions but not paracellular barrier function

To investigate the relationship between septate junctions and tube-size control, we determined the effects of mutations in other septate junction components on tracheal morphology. Null mutations in *coracle* and *neurexin* cause tracheal morphology defects that are essentially identical to those of *nrv2* and the tube expansion mutants *varicose*, *sinuous* and *convoluted* (Fig. 5C-E,I,K; Table 1) (Beitel and Krasnow, 2000). Furthermore, mutations in *gliotactin* and *neuroglian*, two genes required for the blood-brain barrier formed by the septate junction (Auld et al., 1995; Dubreuil et al., 1996; Genova and Fehon, 2003; Schulte et al., 2003), also cause tracheal morphology defects similar to those caused by mutations in the tube-expansion genes (Fig. 5B,G; Table 1). Thus, the septate junction complex appears to be required for tracheal tube-size control.

To determine whether the paracellular barrier function of the

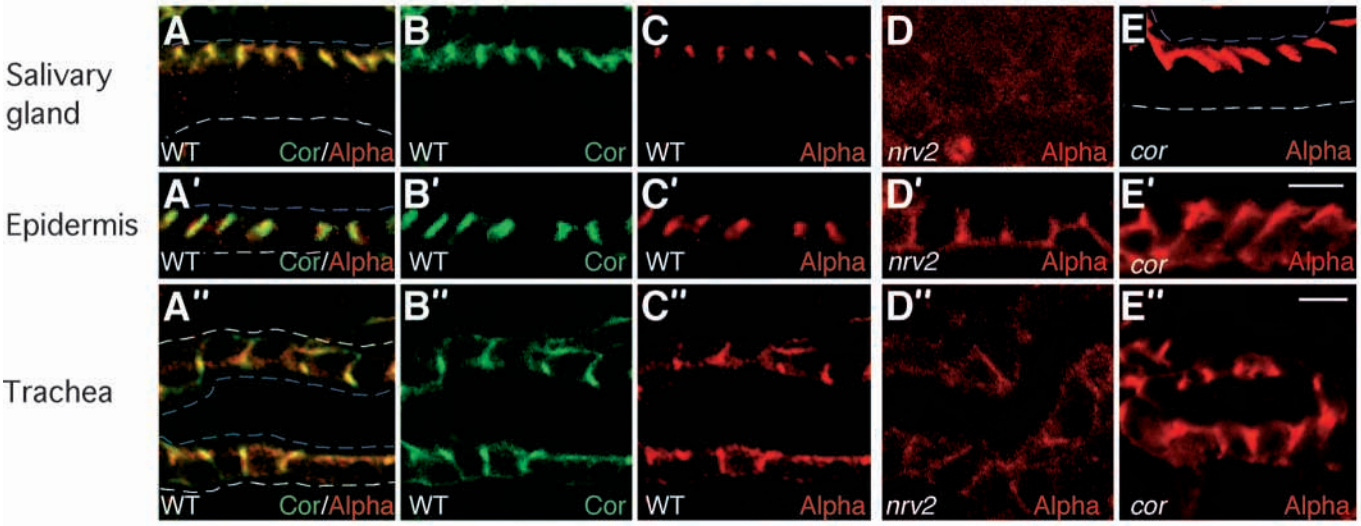

Fig. 4. Na^+/K^+ ATPase localization to the septate junction depends on Nrv2 but not Coracle. In wild-type (A,B,C) and *coracle* mutant animals (E), the Na^+/K^+ ATPase (red A,C,E) localizes to the septate junction marked by Coracle in wild-type animals (green A,B). However, in *nrv2* mutants (D) Na^+/K^+ ATPase staining is reduced and/or not localized to the septate junction. Na^+/K^+ ATPase staining (α5 anti-α subunit monoclonal) is undetectable in ATPα mutants (data not shown). Basal surfaces are outlined in white, apical/lumenal surfaces in blue. Scale bar: in E″, 5 μm for A-E,A″-E″; in E′, 5 μm for A′-E′.

septate junction is important for tracheal tube-size control, we examined the correlation between the tracheal and paracellular barrier phenotypes caused by tube expansion and septate junction mutants. In mutants with paracellular barrier defects, there is no relationship between paracellular defect and the type or severity of tracheal morphology defect. For example, although *megatrachea* and *cystic* mutant epithelia are both permeable to a 10 kDa dye (Fig. 2f,g), they have strikingly different types of tracheal phenotypes – *megatrachea* mutants have extremely long tubes with almost no diameter defects, whereas *cystic* mutants have dramatic diameter expansions and constrictions without any increase in length (Fig. 2F,G) (Beitel and Krasnow, 2000). Furthermore, a strain containing a weak *coracle* allele (Ward et al., 2001) has minimal tracheal morphological defects despite having epithelia permeable to a 10 kDa dye (Fig. 2E). If septate junction permeability defects caused the tracheal defects, then mutants with similar paracellular barrier defects should cause similar tracheal phenotypes. Because we find no correlation between the type or severity of tube-size and barrier defects, it appears that tracheal tube-size control by the septate junction does not depend on paracellular diffusion barriers.

Nrv2.1 and Nrv2.2 both have tracheal tube-size control and junctional barrier activity

Although the above results demonstrate that the *nrv2* locus plays a crucial role in tracheal tube-size control and septate junction function, they do not address possible functional differences between the two Nrv2 protein isoforms, Nrv2.1 and Nrv2.2, because the available *nrv2* mutations affect exons common to both (Fig. 6A; see Materials and methods). Nrv2.1 and Nrv2.2 share the same predicted transmembrane and extracellular domains, but differ dramatically in their ~49 amino acid intracellular domains in which they are only 29% identical and 48% similar. To investigate possible functional differences between the isoforms, we used dsRNAs corresponding to either the *nrv2* common exons or to *nrv2.1*- or *nrv2.2*-specific exons in RNAi experiments to ‘knockdown’ either both nrv2 transcripts or the nrv2.1 or nrv2.2 transcripts specifically (see Material and methods). We found that injection of any of nrv2 common, nrv2.1 or nrv2.2 dsRNAs caused the same tracheal tube length and diameter defects as *nrv2* null mutations, and all three dsRNAs caused defects in septate junction barrier function (Fig. 6D-F). These results suggest that in normal development, both Nrv2 isoforms are required for tracheal tube-size control and septate junction function.

To determine whether the apparent requirement for both Nrv2.1 and Nrv2.2 resulted from inherent functional differences between the two isoforms, we used the two component expression system of Brand and Perrimon (Brand and Perrimon, 1993) to express *nrv2.1* and *nrv2.2* transgenes using a variety of drivers. Driving one or two copies of either UAS-*nrv2.1* or UAS-*nrv2.2* or both together specifically in the tracheal system of *nrv2* homozygotes using one or two copies of the *btl*-Gal4 driver (Shiga et al., 1996) resulted in the same partial rescue of tracheal defects (Fig. 1J; Fig. 2, legend). Specifically, the diameter defects and ganglionic branch lumenal gaps were almost completely rescued, but the length and paracellular transport defects were not significantly rescued. Driving UAS-*nrv2.1* or UAS-*nrv2.2* with the e22c-Gal4 driver, which is expressed throughout the ectoderm and ectodermally derived tissue and begins expressing much earlier in development than *btl*-Gal4 (stage 5 versus stage 10), completely rescued all tracheal morphological and junctional barrier defects in the large majority of *nrv2* homozygotes (Fig. 1J,K; Fig. 2, legend; Fig. 6H). These results suggest that for full rescue, *nrv2* is required at a window of time extremely early in tracheal development, possibly before ectodermal cells are specified to be tracheal cells.

The above results also demonstrate that both *nrv2.1* and *nrv2.2* have tube-size control and paracellular transport

activities. However, given that all combinations of Na^+/K^+ ATPase α and β subunits form functional ion pumps (Lemas et al., 1994; Schmalzing et al., 1991; Schmalzing et al., 1997), these results also raise the possibility that neither *nrv2* isoform has specific functions and that any β subunit could substitute for Nrv2. We therefore tested whether either of the two other *Drosophila* Na^+/K^+ ATPase β subunits, *nrv1* (Sun and Salvaterra, 1995b) or *nrv3* (CG8663), had tube-size or barrier activities. Driving UAS-*nrv1* or UAS-*nrv3* constructs with either *btl*-Gal4 or the e22c-Gal4 driver did not rescue any of the tracheal tube-size or septate junction barrier defects of *nrv2* mutants (e.g. Fig. 6I,J; Fig. 2, legend), despite the fact that expression from the UAS-*nrv2.1*, UAS-*nrv2.2* and UAS-*nrv3* transgenes appeared equivalent as assessed by immunohistochemical staining (see Materials and methods). Consistent with these results, injection of dsRNA corresponding to *nrv1* or *nrv3* into wild-type embryos did not cause either the characteristic tube-expansion defects or septate junction barrier defects (Fig. 6G and data not shown).

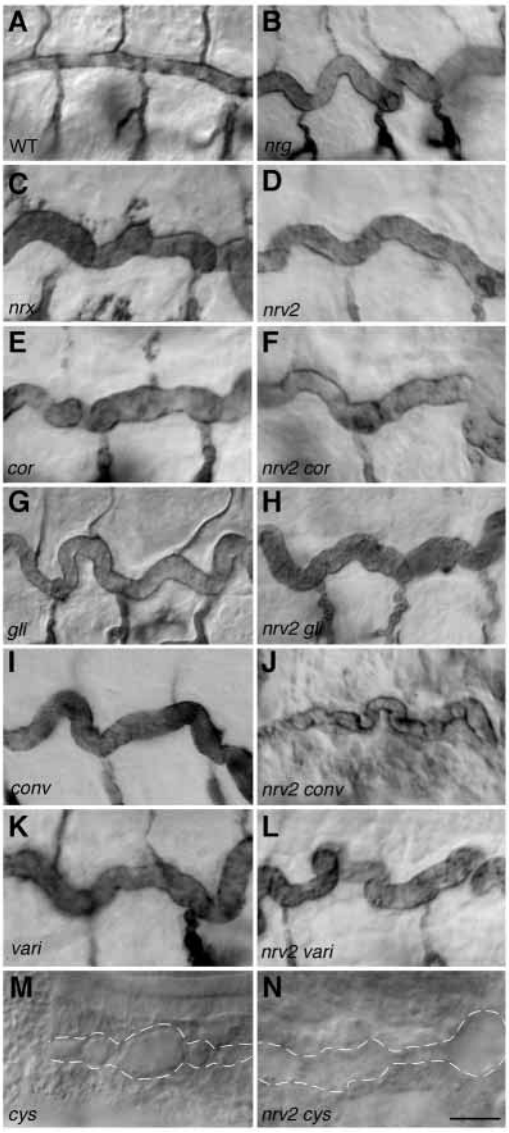

Fig. 5. Septate junctions control tracheal tube size through multiple pathways. Null mutations in the known septate junction genes *coracle* and *neurexin* cause the identical tracheal tube size phenotypes as null *nrv2* mutations (C-E). Double null mutant combinations of *nrv2* and *coracle* have the same phenotype as either single mutant (compare F with D and E), suggesting *nrv2* and *coracle* act in the same pathway. Similarly, double null mutant combinations of *nrv2* and *gli* are not more severe than the *nrv2*-null mutants (compare H with D). By contrast, *varicose*, *convoluted* and *cystic* mutations exacerbate the tracheal phenotypes of null *nrv2* mutations (compare J with D,F,H,I; L with D,F,H,K; N with D,F,H,M), suggesting that *varicose*, *convoluted* and *cystic* do not function only in a single linear pathway with *nrv2*. Dotted lines indicate tracheal lumens. Genotypes: (A) Oregon R; (B) nrg^{17}; (C) nrx^{4865}; (D) $nrv2^{23B}$; (E) cor^{5}; (F) $nrv2^{nwu3}cor^{5}$; (G) $gli^{AE2\text{-}45}$; (H) $nrv2^{nwu3}gli^{J29\text{-}7b}$; (I) $conv^{k6507b}$; (J) $nrv2^{nwu3}conv^{k6507b}$; (K) $vari^{3953b}$; (L) $nrv2^{k04223}vari^{3953b}$; (M) $cys^{k13717b}$; (N) $nrv2^{nwu3}cys^{k13717b}$. Scale bar: 5 μm.

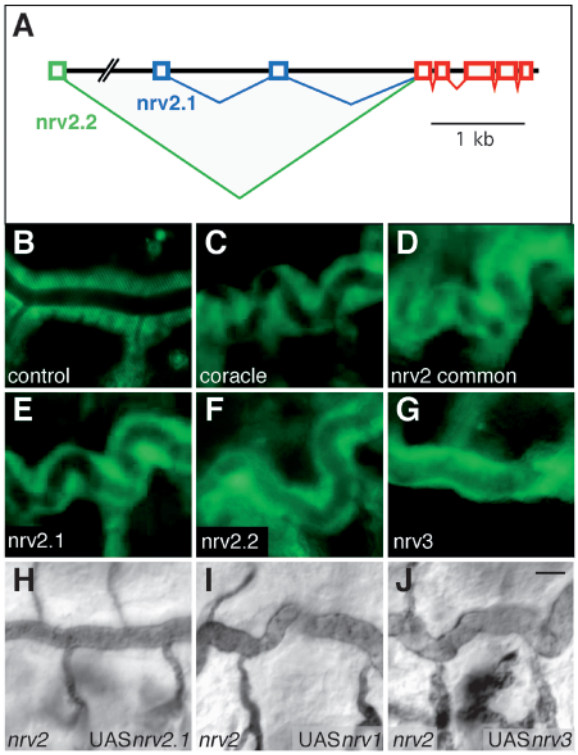

Fig. 6. Both *nrv2.1* and *nrv2.2*, but not *nrv1* and *nrv3*, are required for and possess tube-size control activity. (A) Organization of the *nrv2* locus. Exons common to both *nrv2* isoforms are shown as red boxes, while exons specific for the *nrv2.1* and *nrv2.2* isoforms are shown in blue and green, respectively. (B-G) Tracheal tube diameter and length defects are caused by the injection of dsRNA corresponding to *coracle* (C), *nrv2* common exons (D), *nrv2.1* and *nrv2.2* specific exons (E,F) into otherwise wild-type animals with tracheal GFP expression. Injection of these dsRNAs also causes septate junction barrier defects (data not shown). Injection of buffer (B) or *nrv3* dsRNA (G) did not cause tracheal tube-size defects. (H,I) Expression of *nrv2.1* in *nrv2* homozygotes using the e22C-Gal4 driver rescued all tracheal morphology defects (H), but expression of *nrv1* (I) or *nrv3* (J) did not detectably rescue the *nrv2* defects. Rescue by *nrv2.2* is shown in Fig. 1H. Genotypes: (B-G) *btl*-Gal4 UAS-GFP; (H) $nrv2^{k13315}$e22C-Gal4/$nrv2^{k04223}$ UAS-*nrv2.1*; (I) $nrv2^{k13315}$e22C-Gal4/$nrv2^{k04223}$UAS-*nrv1*; (J) $nrv2^{k13315}$e22C-Gal4/$nrv2^{23B}$UAS-*nrv3*. Scale bar: 5 μm.

We therefore conclude that Nrv2.1 and Nrv2.2 both have specific tube-size and septate junction barrier activities not present in other Na^+/K^+ ATPase β subunits.

Genetic interactions define multiple pathways for tracheal tube size control

The nearly identical tracheal morphological defects in *nrv2*, *coracle*, *varicose* and *convoluted* (Fig. 5D,E,I,K; Table 1) suggested that these genes may act in a single linear genetic pathway. If so, then double mutant combinations of *nrv2*-null alleles and mutations in other tube-expansion genes should have the same tracheal phenotypes as a *nrv2*-null single mutant. This prediction is true for *coracle* and *gliotactin* (Fig. 5F,H), and is consistent with Coracle mislocalization in *nrv2* mutants (Fig. 3B). However, the double mutant combination of a *nrv2*-null mutation and a *convoluted* mutation that does not cause septate junction barrier defects (Fig. 2h) causes more severe phenotypes than *nrv2*-null mutations (Fig. 5J). Similarly, the double mutant combination of a *nrv2*-null mutation and a *varicose* mutation that causes septate junction barrier defects (Fig. 2, legend) causes more severe phenotypes than *nrv2*-null mutants (Fig. 5L), indicating that these genes are unlikely to act in a simple linear pathway. The *nrv2 cystic* double mutant phenotype was indistinguishable from *cystic* (Fig. 5M,N), suggesting that *cystic* acts in parallel or downstream of *nrv2*.

Discussion

Despite the importance of epithelial tubes to the function of many of organs, little is known about the pathways and proteins responsible for controlling multicellular tube size. Our results show that the Na^+/K^+ ATPase and septate junctions have previously unidentified roles in *Drosophila* tracheal tube-size control. They further provide the first in vivo evidence for functional differences between Na^+/K^+ ATPase isoforms as Nrv2 but not other *Drosophila* β Na^+/K^+ ATPase subunits have tube-size and septate junction activity. Below, we discuss models for how tracheal tube size is controlled by septate junctions and implications of our results for vertebrate tube-size control.

Septate junction control of tube size is not mediated by paracellular barrier function

A simple explanation for the abnormal sizes of tracheal tubes in mutants having septate junction defects would be that ionic or hydrostatic disequilibria across the tracheal epithelium disrupts tracheal cell morphogenesis. If so, then all mutants with similar paracellular barrier defects should have equivalent tracheal morphologies. However, we found that barrier mutants had tracheal morphologies ranging from near wild type in the case of cor^{14*} to diameter- or length-specific defects in *cystic* and *megatrachea*. These results support the conclusion that septate junction control of tube size is not dependent on regulation of paracellular diffusion.

Multiple pathways of septate junction tube size control and paracellular transport

The mutant phenotypes and genetic interactions among tracheal tube-expansion and septate junction mutants suggest there are at least two genetic pathways by which septate

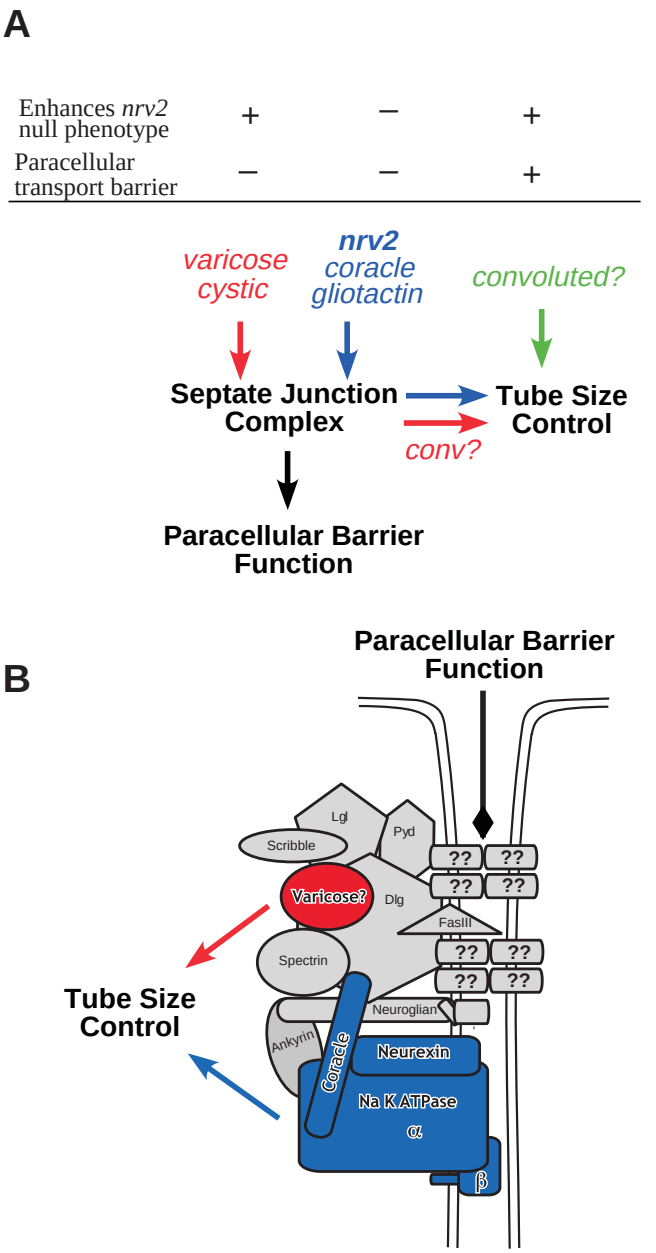

Fig. 7. Models for the role of the Na^+/K^+ ATPase in tube-size control via the septate junction. See text for detailed discussions. (A) One possible formulation of the genetic pathways controlling tracheal tube size and septate junction barrier function. This model is based on the barrier phenotypes of the shown mutants and on genetic interactions between these mutants and *nrv2*-null mutants. Comparison of these phenotypes and interactions divides the mutants into three classes. *nrv2* appears to act in a linear genetic pathway with *gliotactin* and *coracle* (shown in blue) but in a parallel, partially redundant, or branching pathway to *varicose* and *cystic* (shown in red). *convoluted* may act either downstream of a *varicose*/*cystic* pathway (indicated in red) and/or in parallel pathways (indicated in green). (B) A molecular representation of the pathways shown in A to illustrate how *nrv2*/*coracle* (blue) and *varicose* (red) could both be required for septate junction barrier function, but act in genetically distinguishable pathways for tube-size control. Question marks indicate uncertainty in identity or subcellular localization. Only a subset of known septate junction proteins are shown.

junctions regulate tracheal tube size (Fig. 7A). For example, *nrv2* and *coracle* appear to act in the same genetic pathway as *nrv2* and *coracle* null mutants have the same tracheal phenotypes as each other and as *nrv2 coracle* double null mutants (Fig. 7A, middle column). This genetic evidence is supported by our observations that the localization of Coracle to septate junctions is disrupted in *nrv2* and *ATPα* mutants. By contrast, although *nrv2*-null and *varicose* mutants have the same tracheal phenotypes, *nrv2* and *varicose* are unlikely to act in the same linear genetic pathway because *nrv2 varicose* double mutants have more severe tracheal phenotypes than *nrv2*-null mutants (Fig. 7A, left column). This result suggests that either *varicose* and *nrv2* function in separate pathways to control tube size, or there is redundancy between the functions of these genes. We present the separate pathway model as a formal logic diagram in Fig. 7A and as a molecular model in Fig. 7B.

Additional genetic pathways may also be revealed by the differential effects of tube-expansion mutations on septate junction barrier integrity. For example, in contrast to *nrv2* and other mutations, the existing *convoluted* mutations do not affect septate junction barrier integrity and therefore may define a size-control pathway that acts in parallel to septate junction pathways (Fig. 7A, right column, green 'convoluted?'). Consistent with this proposal, the double mutant combination of a *nrv2* null mutation and a *convoluted* mutation result in more severe tracheal morphology defects than either *nrv2*-null or *convoluted* mutations alone. Alternatively, genes such as *convoluted* may function in a branch of a septate junction pathway to link septate junctions to tube size control as shown in Fig. 7A (right column, red '*conv?*'). Although these models are necessarily incomplete, they offer testable predictions about gene interactions and subcellular localizations of uncharacterized gene products that should help define tube-size control and paracellular barrier pathways at the molecular level.

Possible mechanisms for Na^+/K^+ ATPase and septate junction regulation of tube size

A central issue raised by our findings is the nature of the molecular functions(s) of the Na^+/K^+ ATPase and septate junctions in tube-size control. Although the Na^+/K^+ ATPase has been studied intensively for more than 40 years for its function as an ion pump (Chow and Forte, 1995; Blanco and Mercer, 1998), our data indicate that the tracheal tube-size function of the Na^+/K^+ ATPase is intimately associated with its role in septate junction function. Furthermore, as described above, the paracellular barrier and tube-size control functions of the septate junction are separable.

In one class of model that accounts for these observations, the role of the Na^+/K^+ ATPase is to organize septate junctions, which control tube size by an undetermined mechanism. The many functions of vertebrate tight junctions provide possible examples of non-barrier mechanisms by which septate junctions could control tube size. In particular, tight junctions organize polarized apical secretion mediated by the exocyst (O'Brien et al., 2002), bind cytoskeletal components such as ankyrin and fodrin (Fanning et al., 1999), contain potential signaling molecules such as the tyrosine kinases Src, Yes and protein kinase C (Fanning et al., 1999), and have recently been shown to regulate the activity of a Y-box transcription factor (Balda et al., 2003). In addition, both septate and tight junctions complexes contain proteins that organize epithelial cell apical/basal domains (Tepass et al., 2001).

Of the tube-size control models that do not invoke ion-transport functions of the Na^+/K^+ ATPase, models involving apicobasal domain organization are particularly attractive. Apical surface regulation is a common theme in tubulogenesis (Lubarsky and Krasnow, 2003; Buechner et al., 1999; O'Brien et al., 2002), and has been shown to play an important role in tube-size control in the *Drosophila* salivary gland (Myat and Andrew, 2002). Several observations support the possibility that septate junctions control tracheal tube size through the apical cell surface. First, the differential regulation of tracheal apical and basal cell surfaces suggests that tracheal tube size control is mediated at the apical cell surface (Beitel and Krasnow, 2000). Second, the increased tracheal tube lengths and diameters present in tube-expansion mutants necessitate an increased apical cell surface area. Given that the Dlg/Scrib/Lgl complex normally present in septate junctions has an early embryonic function to negatively regulate the extent of the apical membrane domain (Bilder et al., 2003; Tanentzapf and Tepass, 2003), this complex could also act later to negatively regulate tracheal apical surface area.

In an alternative class of models that are not exclusive of the above possibilities, the ion-pump activity of the Na^+/K^+ ATPase may directly or indirectly mediate tube-size control. For example, pharmacologically blocking Na^+/K^+ ATPase ion-transport activity leads to increased intracellular Ca^{2+} levels in some cell types (Ravens and Himmel, 1999), and Ca^{2+} signaling abnormalities may be the molecular defect that causes the enlarged tubules of polycystic kidney disease (PKD) (Calvet, 2002; Hou et al., 2002; Yoder et al., 2002a; Yoder et al., 2002b). Another example is that the low intracellular Na^+ level maintained by the Na^+/K^+ ATPase is required for formation of tight junctions and stress fibers in Madin-Darby canine kidney (MDCK) cells, an epithelial cell line that can form tubules in response to hepatocyte growth factor (Rajasekaran et al., 2001). Septate junction formation might also require low intracellular Na^+ levels. Finally, disruption of the cellular Na^+/K^+ electrochemical gradient could impact secondary active transport of other solutes that may be important for proper tube-size regulation.

Although the exact biochemical roles of the Na^+/K^+ ATPase and septate junctions in tube-size control are unclear, identification of these complexes as parts of a tube-size control mechanism is an important step towards further understanding these mechanisms at the molecular level.

Na^+/K^+ ATPase in vertebrate epithelial tube-size disorders

The Na^+/K^+ ATPase has been implicated in vertebrate tube-size control by the abnormal subcellular localization of the Na^+/K^+ ATPase in the inappropriately expanded tubules in individuals with PKD and in several animal models of cystic kidney diseases (Avner et al., 1992; Carone et al., 1995; Ogborn et al., 1995; Wilson et al., 2000; Wilson et al., 1991). However, it has not yet been determined whether this mislocalization contributes to the progression of cystic diseases or whether it is merely a secondary effect of other cellular defects. Our finding that the Na^+/K^+ ATPase is required for normal tube-size control in the *Drosophila* tracheal system

suggests that the vertebrate Na^+/K^+ ATPase may play an important role in maintaining the normal size of kidney and other epithelial and endothelial tubes. Ultimately, a molecular understanding of the tube-size control mechanisms should allow development of new strategies for preventing and treating PKD and other diseases resulting from epithelial and endothelial tube defects.

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