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Drosophila Varicose, a member of a new subgroup of basolateral MAGUKs, is required for septate junctions and tracheal morphogenesis

Victoria M. Wu[1,*], **Marcus H. Yu**[1,*], **Raehum Paik**[2], **Swati Banerjee**[2], **Zhiguo Liang**[3], **Sarah M. Paul**[1], **Manzoor A. Bhat**[2,4], and **Greg J. Beitel**[1,†]

1 *Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL 60208, USA*

2 *Department of Cell and Molecular Physiology, University of North Carolina School of Medicine, Chapel Hill, NC 27599, USA*

3 *Department of Microbiology and Immunology, University of Illinois-Chicago, Chicago, IL 60612, USA*

4 *UNC Neuroscience Center, Curriculum in Neurobiology, Neurodevelopmental Disorders Research Center, University of North Carolina School of Medicine, Chapel Hill, NC 27599, USA*

Abstract

Epithelial tubes are the functional units of many organs, but little is known about how tube sizes are established. Using the *Drosophila* tracheal system as a model, we previously showed that mutations in *varicose* (*vari*) cause tubes to become elongated without increasing cell number. Here we show *vari* is required for accumulation of the tracheal size-control proteins Vermiform and Serpentine in the tracheal lumen. We also show that *vari* is an essential septate junction (SJ) gene encoding a membrane associated guanylate kinase (MAGUK). In vivo analyses of domains important for MAGUK scaffolding functions demonstrate that while the Vari HOOK domain is essential, the L27 domain is dispensable. Phylogenetic analyses reveal that Vari helps define a new MAGUK subgroup that includes mammalian PALS2. Importantly, both Vari and PALS2 are basolateral, and the interaction of Vari with the cell-adhesion protein Neurexin IV parallels the interaction of PALS2 and another cell-adhesion protein, Necl-2. Vari therefore bolsters the similarity between *Drosophila* and vertebrate epithelial basolateral regions, which had previously been limited to the common basolateral localization of Scrib, Dlg and Lgl, proteins required for epithelial polarization at the beginning of embryogenesis. However, by contrast to Scrib, Dlg and Lgl, Vari is not required for cell polarity but rather is part of a cell-adhesion complex. Thus, Vari fundamentally extends the similarity of *Drosophila* and vertebrate basolateral regions from sharing only polarity complexes to sharing both polarity and cell-adhesion complexes.

Keywords

MAGUK; Cell junction; Basolateral; Epithelia; *Drosophila*; Trachea

† Author for correspondence (e-mail: beitel@northwestern.edu)
*These authors contributed equally to this work

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INTRODUCTION

The function of organs such as the lung, kidney and vascular system depends on epithelial and endothelial tubes of specific sizes. However, the cell biological and molecular processes that control tube sizes are largely unknown. The *Drosophila* tracheal system is a network of ramifying epithelial tubes that serves as a combined pulmonary-vascular system to directly deliver oxygen to tissues (reviewed by Uv et al., 2003). The comparative simplicity and genetic tractability of the tracheal system has made it one of the best models of tubular epithelial morphogenesis. The tracheal system develops from a series of sacs into a complex network of branches through a highly orchestrated series of cell migrations, cell shape changes and rearrangements of cell-cell junctions (Samakovlis et al., 1996). An important element of these morphogenetic events is that changes in tube size occur reproducibly during specific developmental periods (Beitel and Krasnow, 2000). Each tracheal branch has a specific size that results from the action of branch-specific signaling events that at least in some branches are known to act through transcription factors such as Spalt-Major (Spalt) (Chen et al., 1998;Chihara and Hayashi, 2000;Franch-Marro and Casanova, 2002;Myat et al., 2005;Ribeiro et al., 2004). At least one additional transcription factor, Grainyhead, is required to control tube length and apical cell surface in the major tracheal branches, but the transcriptional targets that more directly mediate these functions remain to be identified (Hemphala et al., 2003). Recent work by multiple groups has produced a basic molecular framework of the mechanisms that execute the size changes of 'tube expansion', a process that increases the diameter – but not the length – of the major tracheal tubes over a 2 hour period, and then gradually lengthens the tubes without changing their diameters (reviewed by Swanson and Beitel, 2006). These tube size changes result from changes in cell shape and possibly cell size, but do not involve changes in cell number (Beitel and Krasnow, 2000).

The tube expansion mechanism depends upon a fibrillar, chitin-based extracellular matrix that is assembled in the tracheal lumen at the beginning of the diameter dilation (Tonning et al., 2005). As development progresses, chitin at the apical cell surface is organized into a highly patterned, multilayered cuticle. Lumenal chitin is eliminated before hatching. Defects in chitin synthesis or organization cause tracheal tube diameters to become either too large or too small, and tube lengths to become over-elongated (Araujo et al., 2005;Devine et al., 2005;Moussian et al., 2006;Tonning et al., 2005). The exact role of the chitin-based matrix in controlling tracheal cell shape is unclear. Although the lumenal matrix and cuticle may serve as structural forms or 'mandrils' that mechanically shape the tracheal cells and tubes, an instructive or signaling role for the matrix is suggested by the observation that theorganization of the β_H-spectrin cytoskeleton is altered in chitin-synthetase mutants (Tonning et al., 2005).

Beginning at stage 15, organization of the lumenal matrix requires the lumenal secretion of the putative chitin deacetylases, Vermiform (Verm) and Serpentine (Serp). In *verm* and *serp* mutants, the chitin-based matrix becomes disorganized and tracheal tubes become too long (Luschnig et al., 2006;Wang et al., 2006). Surprisingly, lumenal secretion of Verm requires a cell-cell junction termed the septate junction (SJ) (Wang et al., 2006). Septate junctions are complex cell adhesion junctions that have at least 15 known components (reviewed by Knust and Bossinger, 2002;Margolis and Borg, 2005;Wu and Beitel, 2004). These include transmembrane cell-adhesion proteins such as Neurexin IV (Nrx-IV; herein referred to as Nrx) and Neuroglian (Nrg), cytoplasmic proteins such as the FERM-domain protein Coracle (Cor; Cora – Flybase), the basal polarity proteins Scribbled (Scrib), Discs large (Dlg; Dlg1 –Flybase), and Lethal giant larvae (Lgl; L(2)gl – Flybase), and proteins with roles that remain to be determined, such as the Na^+/K^+-ATPase (Genova and Fehon, 2003;Paul et al., 2007;Paul et al., 2003). Mutations in most known SJ components cause tracheal phenotypes indistinguishable from the *verm* mutant phenotype, consistent with the failure of Verm to be secreted into the tracheal lumen in the SJ mutants so far examined (Wang et al., 2006). Secretion

of other apical lumenal markers appears normal in SJ mutants, indicating that Verm is secreted by a specialized pathway, the mechanism of which remains to be determined.

Although the role of SJs in lumenal (apical) secretion is not understood, other SJ functions are well defined. SJs have functional and molecular similarity to vertebrate tight junctions (TJs), in that both junctions require members of the claudin protein family to create the paracellular diffusion barriers between epithelial cells that are essential to the survival of multicellular animals (Anderson et al., 2004;Behr et al., 2003;Wu and Beitel, 2004;Wu et al., 2004). However, SJs are not simply the homologs of TJs, because there are significant ultrastructural, molecular and functional differences between SJ and TJs (reviewed by Wu and Beitel, 2004). For example, TJs are apical of adherens junctions (AJs) and contain conserved apical polarity complexes, while SJs are basal of AJs and contain the polarity proteins Scrib, Dlg and Lgl, which have vertebrate homologs that also localize basolaterally (reviewed by Knust and Bossinger, 2002). Thus, in some respects SJs are more related to complexes found in the basolateral regions of vertebrate epithelial cells than to TJs.

Although Scrib, Dlg and Lgl establish and currently define the similarity between SJ and vertebrate basolateral regions, it is notable that these proteins are not representative of most SJ components. *Drosophila* Scrib, Dlg and Lgl are maternally contributed and constitute a distinct subgroup of proteins required for initial epithelial cell polarization during embryonic stages 5–8 (Bilder and Perrimon, 2000;Strand et al., 1994;Tanentzapf and Tepass, 2003;Woods et al., 1996). By contrast, most SJ components are not maternally expressed, are not required for cell polarity and only function relatively late in development when SJs begin forming during stage 13 (reviewed by Bilder, 2004;Knust and Bossinger, 2002;Tepass et al., 2001). Whether the Scrib, Dlg and Lgl proteins nucleate SJ assembly, or whether the nascent SJ recruits and incorporates Scrib, Dlg and Lgl has not been determined. It also has not yet been determined how Scrib, Dlg and Lgl are localized to the basolateral membrane in either *Drosophila* or vertebrate epithelia. Thus the similarity between *Drosophila* SJ and vertebrate basolateral regions has been limited to polarity complexes, and has not extended to cell adhesion complexes.

In this report we show that *vari* encodes a previously uncharacterized, membrane-associated, guanylate kinase (MAGUK) scaffolding protein that is required for SJ organization and that directly binds the cell adhesion protein Neurexin IV. Importantly, Vari helps define a new subgroup of MAGUKs that includes vertebrate PALS2. Both Vari and PALS2 localize basolaterally in epithelial cells and both interact through a PDZ domain with a basolateral adhesion protein. Thus, Vari is the first late-expressed SJ component to have a vertebrate homolog, and together Vari and PALS2 extend the similarity of *Drosophila* and vertebrate basolateral regions from polarity complexes to adhesion complexes.

MATERIALS AND METHODS

Fly stocks and phenotypic assays

Stocks were obtained from the Bloomington *Drosophila* Stock Center and published sources. SJ barrier function was determined as previously described (Lamb et al., 1998;Paul et al., 2003), with the exception that a CyO *dfd*-YFP balancer (Le et al., 2006) was used instead of a CyO actin-GFP balancer.

Immunohistochemistry

The following antibodies were used: anti-tracheal lumenal 2A12 1:5 and anti-Arm N27A1 1:100 (Developmental Studies Hybridoma Bank); mouse anti-Cor C566.9c and C615.16B 1:500; guinea pig (gp) anti-Cor 1:10000 (Fehon et al., 1994); rabbit (r) anti-Dlg 1:500 (Woods

et al., 1997); r anti-Nrv2.1 1:500 (Paul et al., 2007); r anti-Nrx 1:200 (Baumgartner et al., 1996); r anti-Veli 1:500 (Bachmann et al., 2004); rat anti-DE-cadherin DECAD2 1:20 (Oda et al., 1994); r anti-Sinu 1:500 (Wu et al., 2004); r anti-Verm 1:300 and Serp 1:300 (Luschnig et al., 2006); gp anti-Verm 1:1000 (Wang et al., 2006). Embryos were fixed in formaldehyde (Samakovlis et al., 1996), except for Sinu and Arm staining, which were heat fixed (Miller et al., 1989). Rat anti-Vari was used at 1:250 with formaldehyde fixation (although heat fixation can also be used) and was produced by cloning cDNA RE01836 into the pBad/His vector (Invitrogen) followed by expression in *Escherichia coli*, solubilization in 8 mol/l urea, purification with Ni-agarose beads, dialysis against 2 mol/l urea or PBS buffers and inoculation into rats. Guinea pig anti-Vari was produced by inoculating animals with purified 6XHis:Vari PDZ-SH3-GUK (see Protein interactions below, except pET28a, Novagen was used instead of pGEX-4T1). Secondary antibodies were used at 1:200 (Jackson ImmunoResearch and Molecular Probes). Confocal images were acquired on a Leica TCS SP2. To estimate relative levels of staining, heterozygous and homozygous embryos were imaged on the same slide in the same session and image adjustments were applied equally to matched images.

Molecular biology

RNAi was performed as previously described (Kennerdell and Carthew, 1998;Wu et al., 2004) using the *vari* common ORF primers 5′-GCACCCTTTCCATTAAGAGATG, TTCAAGCCAAACATCGAACTTA, ATTGGACTCATACCATCCCAAG and ATGACAAAAGGCATCAGTTCCT, each of which was preceded by a T7 promoter sequence.

The genomic sequence of each *vari* allele was determined from at least 35 bp 5′ of the first common exon and through the polyadenylation site, as well as 35 bp 5′ and 3′ of each spliceform-specific exon. UAS *vari* short and long transgenes were constructed by insertion of cDNAs RE01836 and RE31492, respectively, into pUAST followed by germline transformation. cDNAs were obtained from the *Drosophila* Genome Resource Center and sequenced using an ABI dye-terminator system. GenBank accession numbers: RE35569, DQ787101; RE47555, DQ787102; RE51859, DQ787103; RE54628, DQ787104; RE58272, DQ787105; RE60702, DQ787106; RE61615, DQ787107; RH14941, DQ787108.

Sequence comparisons and phylogenetic analyses

ClustalW and phylogenetic tree analyses were performed using the MacVector program (Accelrys) using representative full-length sequences downloaded from GenBank protein databases, expect for zebrafish ZO-1, for which a C-terminal sequence was predicted from genomic DNA sequences to produce a hypothetical protein that had more conservation with other vertebrate ZO-1 sequences. The ClustalW alignment of the sequences is presented in FASTA format (see Fig. S1 in the supplementary material). The phylogenetic tree in Fig. 2 was generated from 1000 bootstrap repetitions using the neighbor-joining method, gap site ignored, random tie breaking of branches with equal values and an uncorrected ‘p’. Human Carma3 was used as an outgroup to root the displayed tree.

Yeast two-hybrid screen

The two-hybrid interaction screen of a 0–20 hour *Drosophila* library was previously described in Bhat et al. (Bhat et al., 1999). Two of the 15 clones that interacted with the Nrx C-terminus encoded Vari fragments that completely contained the Vari PDZ domain and started with the 5′ sequences AATCCGACCGAGCCG and GGAGGCTACCTGTTC. Both clones ended in polyA sequences.

Precipitation assays

For protein interaction experiments, *vari* cDNA RH61449 (GenBank Accession number AY121709) was used as a template to amplify 1323 and 251 bp fragments (nt #805–1056 and nt #805–2127) that encode the PDZ, SH3 and GUK domains or only the PDZ domain of Vari, respectively. These fragments were cloned in frame into GST expression vector (pGEX4T1, Pharmacia) to generate fusion proteins of GST-Vari or GST-Vari.PDZ, which were used in the binding experiments. A DNA fragment that encodes the Nrx C-terminal 48 amino acids was cloned in a maltose binding protein (MBP) expression vector to generate MBP-Nrx-CT fusion protein. All proteins were expressed according to the vector manufacturer's instructions and the binding assays were carried out as previously described (Bhat et al., 1999). The Nrx-CT peptide was cleaved from the MBP fusion protein with thrombin and purified over spin column and the purified peptide of approximately 7 kDa used for binding with GST, GST-Vari or GST-Vari.PDZ. The peptide was identified by anti-NRX antibody in western blotting.

RESULTS

Varicose encodes multiple isoforms of a MAGUK

The *vari* gene was originally defined by the $vari^{3953b}$ mutation, which causes tracheal tubes to become too long and have some diameter abnormalities (Fig. 1B, F, I) (Beitel and Krasnow, 2000). To understand the molecular functions of *vari*, we used positional cloning to identify the *vari* transcription unit. Deficiency mapping narrowed the *vari* interval to an 80 kb region containing at least 36 genes. One of these, *CG9326*, was predicted to encode a MAGUK expressed at the time of SJ formation (Tomancak et al., 2002). MAGUKs are scaffolding proteins that contain SH3, HOOK, PDZ and catalytically inactive guanylate kinase (GUK) domains, and often contain other protein-protein interaction domains, such as the L27 domain (reviewed by Harris and Lim, 2001). As several known MAGUK proteins localize to, and have key roles at, cell junctions (reviewed by Harris and Lim, 2001;Knust and Bossinger, 2002;Margolis and Borg, 2005), *CG9326* was a strong candidate to be *vari*. Consistent with this prediction, RNAi knockdown of *CG9326* caused tracheal dorsal trunk length increases resembling those caused by $vari^{3953b}$ (Fig. 1D). We definitively demonstrated that *CG9326* was *vari* by showing that the sequence of *CG9326* was altered by all eight *vari* mutations (Fig. 1K, L, Table 1), that the *CG9326* protein was reduced or eliminated in *vari* mutants (Fig. 1M, N; Fig. 3P, S and Table 1) and that *CG9326* cDNAs could rescue *vari* mutations (Fig. 1G, J; Fig. 4G–L).

Sequencing of 11 cDNAs revealed that the *vari* locus generates two major transcript forms that share seven exons encoding the core PDZ, SH3, HOOK and GUK domains, as well as a possible C-terminal PDZ-binding motif (Fig. 1K). The longer splice forms also encode an N-terminal L27 protein-protein interaction domain absent from the shorter isoforms. Together, these results show that *vari* encodes multiple isoforms of a MAGUK required for tracheal morphogenesis.

Vari and PALS2/VAM-1 define a new MAGUK subgroup

To gain insight into possible cell biological roles of Varicose, we investigated the relationship between Vari and other MAGUKs. The MAGUK family can be divided into evolutionarily conserved subgroups, and in several cases it has been shown that subgroup members have similar functions (Fig. 2) (reviewed by Funke et al., 2005). For example, the Stardust (Sdt)/PALS1 and Dlg subgroups organize apical and basolateral cell polarity complexes in both flies and vertebrates (reviewed by Bilder, 2004;Margolis and Borg, 2005). We therefore aligned the Vari amino acid sequence with representative sequences from known MAGUK subgroups and MAGUKs that had not previously been assigned to specific subgroups. Phylogenetic trees were then generated using a 'bootstrap' algorithm that more robustly indicates relationships than do

'best tree' approaches (see Materials and methods). As shown in Fig. 2A, Vari does not belong to any of the previously characterized epithelial MAGUK subgroups such as the Dlg, ZO-1, Sdt/Par-3 or Lin-2/CASK subgroups, but instead belongs to a new subgroup of MAGUKs that includes PALS2 (Kamberov et al., 2000), VAM-1 (Tseng et al., 2001), MPP6 and MPP2. This subgroup has at least two members each in zebrafish, mice and humans, but to date the in vivo functions of the vertebrate members of this subgroup have not been determined. Importantly, although the domain structure of members of the Vari/PALS2 subgroup resembles that of P55 (MPP1) subgroup members, at the amino acid level the P55 subgroup is much more closely related to the LIN-2/CASK/Caki subgroup and is clearly distinct from the Vari/PALS2/VAM-1 subgroup (Fig. 2A; see Fig. S1 in the supplementary material). Thus, Vari is a founding member of a new subgroup of MAGUKs, the functions of which have not been previously determined.

Caenorhabditis elegans epithelial MAGUKs are significantly diverged from those of *Drosophila* and vertebrates

Several additional results from the phylogenetic analysis are also notable. First, while the P55 subgroup initially appears to be vertebrate-specific because there are no corresponding genes in *Drosophila* or *C. elegans* that have the PDZ-SH3-GUK structure of the P55 subgroup members, the invertebrate equivalents of P55 may be alternative splice products of the LIN-2/Caki subgroup that lack the CAM kinase and one or both L27 domains, and thus have close amino acid sequence and domain organization similarity to the P55 subgroup (Fig. 2A). Second, it is apparent that zebrafish Humpback (Konig et al., 1999) also defines a previously unrecognized subgroup of MAGUKs that is most similar to the Sdt subgroup (Fig. 2A). Third, some MAGUK subgroups, such as the Vari/PALS2 and Lin-2/Cask, have characteristic sequences at their C-termini that could be PDZ-binding motifs, while members of other subgroups, such as the Sdt/PALS1 and Humpback families, do not have conserved C-termini and lack potential PDZ-binding motifs (Fig. 2B). Fourth, although the Dlg, Sdt, ZO-1 and Lin-2 subgroups have clear representatives in vertebrates, *Drosophila* and *C. elegans*, the Vari and Humpback subgroups do not appear to have *C. elegans* members. Further, *C. elegans* DLG lacks the conserved C-terminal amino acids present in *Drosophila* and vertebrate Dlgs, and the *C. elegans* ZO-1 C-terminus is also considerably divergent from the *Drosophila* and vertebrate ZO-1 C-termini (Fig. 2B). Together, these observations show that while some MAGUK subgroups have been strongly conserved, other subgroups are diverging. A practical consequence of this divergence is that *Drosophila* is likely to be more representative than *C. elegans* as a model system for investigating the roles of MAGUKs in epithelial cell junctions.

Vari localizes to septate junctions

To begin investigating the functions of Vari, we determined the tissue and subcellular distribution of Vari using antibodies raised to domains common to all Vari isoforms. Vari protein was predominantly expressed in the hindgut and trachea starting at stage 14 (Fig. 4A), but was also clearly expressed in the dorsal epidermis by stage 12 (data not shown). This expression pattern corresponds to the RNA expression pattern of *CG9326* determined by the Berkeley *Drosophila* Genome Project (Tomancak et al., 2002). By stages 15 and 16, when SJ junction assembly has been completed, Vari co-localized with the canonical SJ markers Cor and Nrx (Baumgartner et al., 1996;Fehon et al., 1994) in the trachea, hindgut, salivary gland and epidermis (Fig. 1M,N;Fig. 3A–C;Fig. 4B; and data not shown). That Vari localizes to SJs is important, because the mouse protein most closely related to Vari is PALS2, and like Vari, PALS2 localizes to the basolateral region of epithelial cells (Kamberov et al., 2000). Thus, Vari and PALS2 extend the similarity between *Drosophila* SJs and vertebrate basolateral regions first evidenced by the similar localizations of Scrib, Dlg and Lgl in both vertebrates and *Drosophila* (Knust and Bossinger, 2002).

Vari is required for septate junction formation

To determine if Vari organizes SJs, we tested all *vari* mutants for SJ barrier function and examined the subcellular localization of five SJ components in three epithelial tissues of three different *vari* mutants: an intermediate allele $vari^{3953b}$, a strong allele $vari^{327}$ and a putative null allele $vari^{F033}$ (mutants described below). The dye exclusion assay of Lamb et al. (Lamb et al., 1998) showed that all *vari* mutations except the semi-viable $vari^{38EFa2}$ mutation caused SJ barrier defects (Table 1). As is typical of SJ mutants, in animals homozygous for the strong or null alleles of *vari* the SJ components Cor, Nrx, Sinuous (Sinu) and the Na^+/K^+-ATPase were all mislocalized basally in the trachea, hindgut and salivary glands (Fig. 1M, N;Fig. 3F, F′,I,I′; and data not shown). However, although Dlg levels were greatly reduced in strong and null *vari* mutants, Dlg was nonetheless localized correctly (Fig. 3K, K′). In $vari^{3953b}$, Dlg and the Na^+/K^+-ATPase β-subunit Nrv2 were correctly localized, but Cor and Nrx were mislocalized basally (Fig. 3G, G′,L,L′ and data not shown). By contrast to the tissue-specific effects of *sinu* mutations (Wu et al., 2004), but like mutations in the Na^+/K^+-ATPase (Paul et al., 2003), the SJs of all tissues examined were similarly affected by *vari* mutations.

We next investigated whether Vari was required for assembly or maintenance of septate junctions by following the subcellular localization of the canonical SJ marker Cor (Fig. 4A–F) during embryonic SJ assembly. In wild-type animals, Cor predominantly localizes to the SJ at stage 14. As SJs mature and develop barrier function during stages 15 and 16 (Paul et al., 2003), the amount of Cor localized to the SJs progressively increases (Fig. 4A–C). Some increase in the amount of Cor localized to the basolateral and basal membrane surfaces is also observed. By contrast, in $vari^{F033}$ null mutants, specific localization of Cor to the SJ region was not observed at any stage. At stage 14 Cor localized fairly uniformly to the basolateral and basal surfaces and a significant amount of cytoplasmic staining was also seen (Fig. 4D). At stages 15 and 16, little cytoplasmic staining was observed, but Cor still had a basolateral distribution, and abnormal basal accumulations of Cor became apparent (Fig. 4E, F). Thus, in the absence of Vari, SJs did not begin to assemble, indicating that Vari is required for SJ formation rather than maintenance.

To investigate if Vari had functions typical of other SJ components expressed after establishment of apical-basal polarity, we asked whether Vari was required for epithelial apical-basal polarity or for the recently identified apical secretory function of SJs (Wang et al., 2006). As is the case for late SJ components such as Sinu and Lachesin (Lac), Vari is not necessary for AJ formation or for establishing apical-basal polarity, because the AJ markers Armadillo/β-catenin (Arm) and DE-Cadherin (E-cad; Shotgun –Flybase), and the apical markers DPatJ (PatJ) and Veli were localized properly in all *vari* mutants (Fig. 3P, R and data not shown). Similarly, and as reported for other SJ mutants (Wang et al., 2006), the lumenal levels of the matrix-associated protein Verm were reduced and punctate cytoplasmic accumulations of Verm were frequently observed in *vari* mutants (Fig. 5B–D). Verm cytoplasmic staining was particularly strong and penetrant in $vari^{3953b}$ mutants compared with *vari* null and other SJ mutants, although the expressivity of the cytoplasmic accumulations varied considerably (Fig. 5C, D). Interestingly, the Verm-related protein Serp behaved differently from Verm in *vari*, *cor* or *Lac* mutants, because no lumenal or cytoplasmic staining of Serp was observed (Fig. 5G, H and data not shown). Together, these results show that Vari localizes to SJs and is required for their organization and function.

Localization of Vari to SJs depends on many other SJ components

Because Vari is a scaffolding protein involved in SJ assembly, we determined the extent to which Vari localization depends on other SJ components. In nrx^{4846}, $sinu^{nwu7}$, cor^{5} and $nrv2^{23b}$ null mutants, Vari levels were greatly reduced and the remaining Vari protein was mislocalized basally (Fig. 3M, M′,N,N′ and data not shown). Thus, as for all other SJ

components examined to date, there is an interdependence between Vari and other SJ components for subcellular localization and SJ assembly.

Vari binds to the cytoplasmic domain of Nrx

If Vari and PALS2 share functional as well as sequence similarity, one would expect them to interact with similar proteins. As the PDZ domain of PALS2 binds to the C-terminal PDZ-binding motif EYFI of the basolateral cell adhesion protein Necl-2 (Shingai et al., 2003), we investigated whether Vari's PDZ domain binds the SJ component Nrx, a basolateral cell-adhesion protein with a C-terminus that ends in the related sequence EIFI (Baumgartner et al., 1996). In a yeast two-hybrid screen using the cytoplasmic 48 amino acids of Nrx as bait, we recovered 15 positive clones from 2×10^6 colonies (Bhat et al., 1999). Two of these clones contained cDNAs encoding Vari, seven encoded the multi-PDZ-domain protein dPATJ, and the remaining six encoded proteins did not bear recognizable binding motifs (Materials and methods). The recovery of only Vari and dPATJ, but not others of the more than 125 PDZ-binding proteins in the *Drosophila* genome, suggests that the interaction between the Nrx C-terminus and Vari is quite specific.

To confirm the yeast two-hybrid results, we performed pull-down assays using either the short isoform of Vari or the Vari PDZ-domain fused to GST (see Materials and methods). Both Vari fusion constructs, but not GST alone, could precipitate purified Nrx C-terminus, demonstrating that the Vari PDZ-domain can directly bind Nrx (Fig. 6A). Consistent with Vari and Nrx also interacting directly in vivo, both Vari fusion constructs, but not GST or GST fused to the Vari SH3 domain, precipitated Nrx from whole embryo lysates (Fig. 6B). Significantly, the apical protein Crbs was not precipitated by Vari or the Vari-PDZ fusions, despite Crbs having the C-terminal PDZ-binding motif ERLI, which closely resembles that of Nrx EIFI. Similarly, neither Vari nor the Vari-PDZ domain precipitated the SJ component Nrg, which has isoforms that also end in potential PDZ-binding motifs (ATYV or RKGL). These results indicate that there is significant binding specificity between the Vari PDZ-domain and the Nrx C-terminus. However, we also found that a subset of SJ components, including Cor and Nrv2, co-precipitated with Vari and Nrx and remained associated even in high salt washes (Fig. 6B). While these results are consistent with previous work by Genova and Fehon (Genova and Fehon, 2003) showing that Nrx, Cor and Nrv2 co-immunoprecipitate from embryo lysates and support the hypothesis that Vari scaffolds SJ complexes, these results also leave open the possibility that the in vivo interaction between Nrx and Vari could be indirect. Attempts to use double-mutant analysis to investigate the role(s) of interactions between Vari and Nrx have been unsuccessful, because we have been unable to establish lines of the double balanced heterozygotes using a variety of balancer chromosome combinations. Despite these caveats, Vari binding to Nrx in vitro parallels PALS2 binding to Necl2 in vitro, thus extending the similarity between Vari and PALS2, and between SJs and vertebrate basolateral regions.

The SH3 HOOK but not L27 domain is required for Varicose function

To investigate the functions of the different domains of Vari, we characterized the original $vari^{3953b}$ allele (Beitel and Krasnow, 2000) and the $vari^{38EFa2}$ and $vari^{F033}$ alleles (Butler et al., 2001;Thibault et al., 2004), as well as five *vari* mutations that we generated in an ethylmethanesulfonate (EMS) non-complementation screen (Fig. 1; Table 1). $vari^{3953b}$ has a 17 bp deletion in the intron after the second common exon and is an intermediate allele that is viable in certain trans-heterozygous combinations (Table 2). In $vari^{3953b}$ homozygotes, Vari protein does not significantly accumulate at the SJ region and frequently has a tracheal phenotype similar to that caused by a *vari* null mutation (Fig. 3P,Q; Table 1). $vari^{F033}$ appears to be a null mutation that results from a transposable element insertion into the first common intronic region (Fig. 1K). $vari^{F033}$ behaves as a deficiency in genetic crosses, and in $vari^{F033}$

mutants Vari protein levels are not distinguishable from background (Fig. 1M; Fig. 3O; Table 1; Table 2; and data not shown).

Surprisingly, all four EMS mutations that cause amino acid changes affect the HOOK domain and are, to our knowledge, the first genetic mutations in a MAGUK HOOK domain (Table 1). The HOOK domain is a unique feature of MAGUK-type SH3 domains and is an unusually long loop interposed between the fourth and fifth beta strands of the standard SH3 domain (McGee et al., 2001;Tavares et al., 2001). This loop can interact with proteins such as the FERM-domain band 4.1 protein and may enable MAGUKs to homo- or hetero-multimerize via interfolded SH3 domains. Animals homozygous for the Vari HOOK domain mutations have severe tracheal defects resembling those of the $vari^{F033}$ null mutants and lack detectable accumulations of Vari at the SJs. Whether total Vari protein levels are reduced by these mutations is unclear, as immunohistochemical staining using the current anti-Vari antibodies produces a variable background. This variability makes it difficult to distinguish between the Vari protein levels appearing to be reduced because Vari is mislocalized to the cytoplasm and across the basolateral membrane, as is seen in SJ mutants such as *nrv2* (Fig. 3, compare N,N′ with S), or because Vari is destabilized and degraded in the HOOK-domain mutants. Attempts to assess Vari protein levels directly using western blotting have been unsuccessful with the current antibodies. Regardless, the strong tracheal phenotypes of the HOOK-domain mutants indicate that the HOOK domain has a crucial role for Vari function.

We also investigated the role of the L27 protein-protein interaction domain in Vari function. L27 domains are distinguishing features of several MAGUK subgroups that in vitro evidence suggests are important mediators of MAGUK scaffolding functions (e.g. Kamberov et al., 2000;Tseng et al., 2001) (reviewed by Funke et al., 2005). However, in only a few (e.g. Nakagawa et al., 2004) cases has the in vivo importance of L27-mediated interactions been confirmed. For Vari, a two-hybrid screen of the *Drosophila* proteome detected a significant interaction between Vari and Veli (Giot et al., 2003), which paralleled the L27-mediated in vitro interactions between Pals2/Vam-1 and vertebrate Veli (Kamberov et al., 2000;Tseng et al., 2001). Despite this corroborative evidence, the functional importance of the Vari-Veli interaction in epithelial cells was suspect, because Veli localization is unaffected by *vari* mutations and because the subcellular distributions of Vari and Veli do not overlap (Fig. 3O). Veli localizes apically, while Vari localizes to the basolateral SJs. We confirmed that the Vari L27 domain is nonessential by showing that the short isoform of Vari, which lacks the L27 domain, can completely rescue the tracheal and lethal phenotypes of *vari* null mutants (Fig. 1G, J) and restore normal SJ localization of both Cor and Vari (Fig. 4G, I). Further, despite yeast two-hybrid data indicating that the Vari L27 domain interacts with Veli, expression of the long isoform of Vari rescued *vari* null mutants to viability, and in rescued animals the L27-containing Vari-long isoform localized to SJs rather than the apical domain where Veli is localized (Fig. 4J–L). Together, these results show that although the HOOK region of Vari has an essential role, the L27 domain is dispensable.

DISCUSSION

Vari was originally identified as a gene required for regulating the size of epithelial tubes. In *vari* mutants, tracheal tubes become too long without changes in tracheal cell number (Beitel and Krasnow, 2000). Here we show that Vari encodes multiple isoforms of a MAGUK that helps define a new subgroup of MAGUKs. Vari functions in the assembly of the septate junctions and is required for the apical secretion of the protein Verm, which is thought to be responsible for modifying a chitin-based lumenal matrix (Luschnig et al., 2006;Wang et al., 2006). In *vari* and other SJ mutants, Verm is not secreted, the lumenal matrix becomes abnormal and tracheal tubes become elongated.

Vari organizes septate junctions

The protein-protein interaction domains present in Vari suggest it acts as a scaffolding protein that helps bring together different components of the SJ complex. This hypothesis is supported by our GST-pull down assay results showing Vari's PDZ domain can directly bind the intracellular domain of Nrx, a transmembrane SJ adhesion protein. Binding of the Vari PDZ domain to Nrx would leave Vari's SH3, GUK and predicted C-terminal PDZ-binding motif available to anchor other SJ components to the membrane, or to bring together different transmembrane SJ components. One model is that Vari may help bring the Dlg-Scib complex to the membrane through interfolding of the Vari and Dlg SH3 domains, which is made possible by the unique HOOK domain insert in the MAGUK SH3 domains (McGee et al., 2001;Tavares et al., 2001). Whether or not Vari anchors the Dlg complex to the rest of the SJ, genetic evidence indicates that Vari has functions beyond simply bridging between transmembrane Nrx and intracellular SJ complexes, because *vari* mutations can strongly enhance the phenotypes caused by mutations in the *Drosophila* claudin *sinuous*, whereas *nrx* mutations do not enhance *sinuous* mutations (Wu et al., 2004).

Vari extends the similarity between *Drosophila* SJ and vertebrate basolateral regions from polarity to adhesion complexes

By itself, the finding that Vari encodes a MAGUK was not unexpected, as many MAGUKs are associated with cell-cell junctions (reviewed by Harris and Lim, 2001). However, it is significant that Vari helps define a new subgroup of MAGUKs that includes mammalian PALS2, because Vari and PALS2 both localize basolaterally and bind the C-termini of basolateral cell adhesion proteins. Thus, Vari and PALS2 bolster the similarity between *Drosophila* and vertebrate epithelial basolateral regions that was first evidenced by the common basolateral localization of the Scrib, Dlg and Lgl early polarity proteins. However, by contrast to the polarity proteins, Vari is not required for cell polarity but rather is expressed late in embryonic development and is part of a cell-adhesion complex. Thus, Vari fundamentally extends the similarity of *Drosophila* and vertebrate basolateral regions from containing only conserved polarity complexes to containing both conserved polarity and cell-adhesion complexes.

Epithelial junctions as modular entities

The finding of more extensive similarity between SJ and vertebrate basolateral regions suggests that continued study of *Drosophila* SJs will provide insight into vertebrate epithelial basolateral regions. Further, these results support the idea that during evolution there has been conservation of different junctional functions, such as forming paracellular barriers and anchoring of polarity complexes. However, the comparison of TJs and SJs also makes it clear that there has been limited conservation of which particular functions have assorted to different junctions. An attractive explanation for these somewhat contradictory observations is that junctional functions are modular, and that the disparate junctions in different species represent alternative combinations of functional modules. For example, *Drosophila* SJs could be considered a combination of the claudin-based paracellular-barrier function and the basolateral polarity proteins Dlg, Scrib and Lgl. Alternatively, vertebrate TJs could be considered a combination of the claudin-based paracellular-barrier function and the apical polarity complexes of Crbs-Baz and Sdt-aPKC-Par-6. Thus, when comparing junctions between species, it is likely to be more useful to compare specific junctional functions, such as molecular details of polarity or barrier functions, than to attempt to directly compare junctions in their entirety.

If complex junctions such as TJs and SJs are comprised of functional modules, one would expect that these junctions should contain distinct molecular subcomplexes that mediate distinct functions. Consistent with this proposal, extensive work by many labs has shown that the polarity proteins of Crb-Sdt and Baz-cdc42-aPKC form specific complexes (reviewed by

Margolis and Borg, 2005). Claudin proteins appear to be part of a 'barrier complex' because claudins are required for and co-localize with the paracellular barrier in both *Drosophila* and vertebrates. Functional demonstration of the independence of the barrier and polarity complexes in both species is provided by the observations that cell polarity is not affected by selective disruption of the barrier complex in either mammals by knockdown of ZO-1 and ZO-2 (Umeda et al., 2006), or in *Drosophila* by mutations in claudin genes (Behr et al., 2003;Wu et al., 2004). The Vari/PALS2 proteins could play a pivotal role in allowing cytoplasmic subcomplexes to associate different adhesion-junctional complexes, either in different cell types or during evolution, because changing which adhesion complex Vari or PALS2 associate with could be as simple as changing the four amino acid PDZ-binding motifs of one or a few transmembrane proteins. It seems likely that evolving a few unstructured amino acids would be significantly easier than evolving three-dimensional binding surfaces. Thus, Vari and its homologs could provide crucial – but malleable – links between conserved intracellular complexes and the divergent transmembrane junctional complexes found across the animal kingdom.

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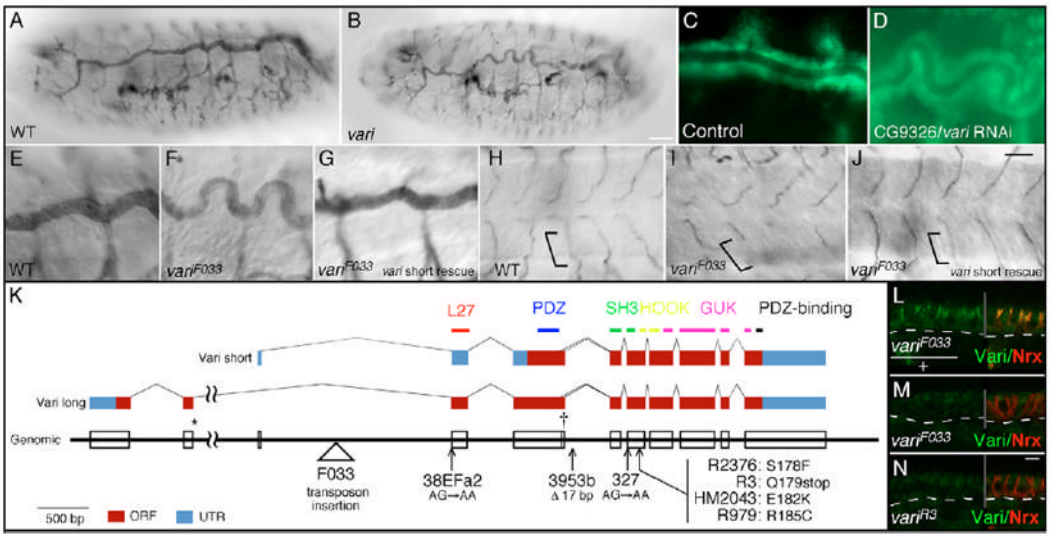

Fig 1. *vari*/*CG9326* encodes a MAGUK required for tracheal tube size control

(**A**,**B**,**E**,**F**,**H**,**I**) Compared to wild type, *vari* mutant trachea are long (B,F) and are missing lumenal segments of the ganglionic branches (bracket in I). (**C**,**D**) RNAi knockdown of *CG9326* phenocopies *vari* mutations. (**G**,**J**) Ubiquitous expression of the short *vari* isoform rescues *vari* null mutant phenotypes. (**K**) The *vari*/*CG9326* genomic locus, transcript organization, protein domains and mutations. †, alternative splicing generates either a 35 or 14 amino acid linker between the PDZ and SH3 domains. Note the PDZ-SH3 linker in PALS2, a mouse homolog of Vari, is also alternatively spliced to produce linkers of different length (Kamberov et al., 2000); *, splicing at a GC splice donor site was confirmed by sequencing seven independent cDNAs and the genomic DNA of multiple wild-type and lab stocks. (**L–N**) Varicose (green) co-localizes with the SJ marker Nrx (red) in wild-type ($vari^{F033}$/+) surface epithelia (L), but is absent in genetically null mutants $vari^{F033}$ (M) and $vari^{R3}$ (N). Left and right panels show the same image, but left panels show guinea pig anti-Vari (green channel), whereas right panels show Vari and Nrx (composite red/green). Trachea visualized in A,B,F–J with monoclonal 2A12, in C,D with *btl::gal4* UAS GFP. Genotype in G,J is $vari^{F033}$ *da::gal4*/ $vari^{F033}$ UAS *vari* short. (A–J,L–N) Stage 16 animals. Dashed lines indicate location of basal cell surfaces; L and M are cropped from the same image of adjacent heterozygous and homozygous animals. Scale bars: in B, 25 μm for A,B; in J, 50 μm for C–J; in N, 1.5 μm for L–N. ORF, open reading frame; UTR, untranslated region; WT, wild type.

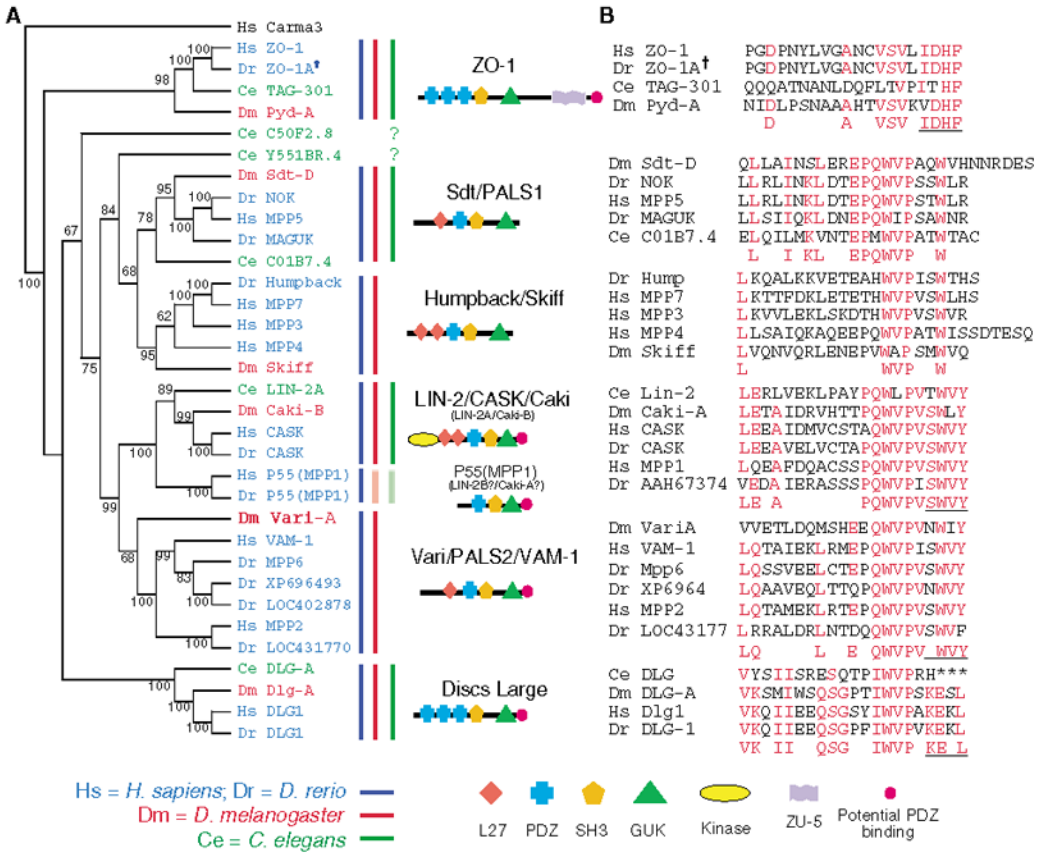

Fig 2. Vari and its homologs define a new subgroup of epithelial MAGUKs

(**A**) A dendrogram showing the sequence similarities between Vari and other MAGUKs. Conserved subgroups are delineated by colored lines with subgroup names or members and representative domain structures adjacent. Colored lines show species representation. PALS2 is the mouse homolog of VAM-1 (Kamberov et al., 2000). Semitransparent lines beside the P55 (MPP1) subgroup indicate that while invertebrates lack distinct P55 genes, alternative splice forms of the LIN-2/Caki subgroup may be the functional equivalent. ?, MAGUKs that cannot be assigned to conserved subgroups. Numbers at dendrogram node points show the percentage of dendrograms created in 1000 bootstrap iterations containing the displayed grouping. Human Carma3 was used as an outgroup to root the displayed dendrogram. (**B**) C-terminal alignments of the MAGUK subgroups. Residues conserved between more than 75% of shown subgroup members are marked in red. Potential PDZ-binding motifs for each subgroup are underlined in the consensus. †, Dr_ZO-1 C-terminal sequence predicted from genomic sequence and is absent in current gene annotations; ***, end of coding sequence.

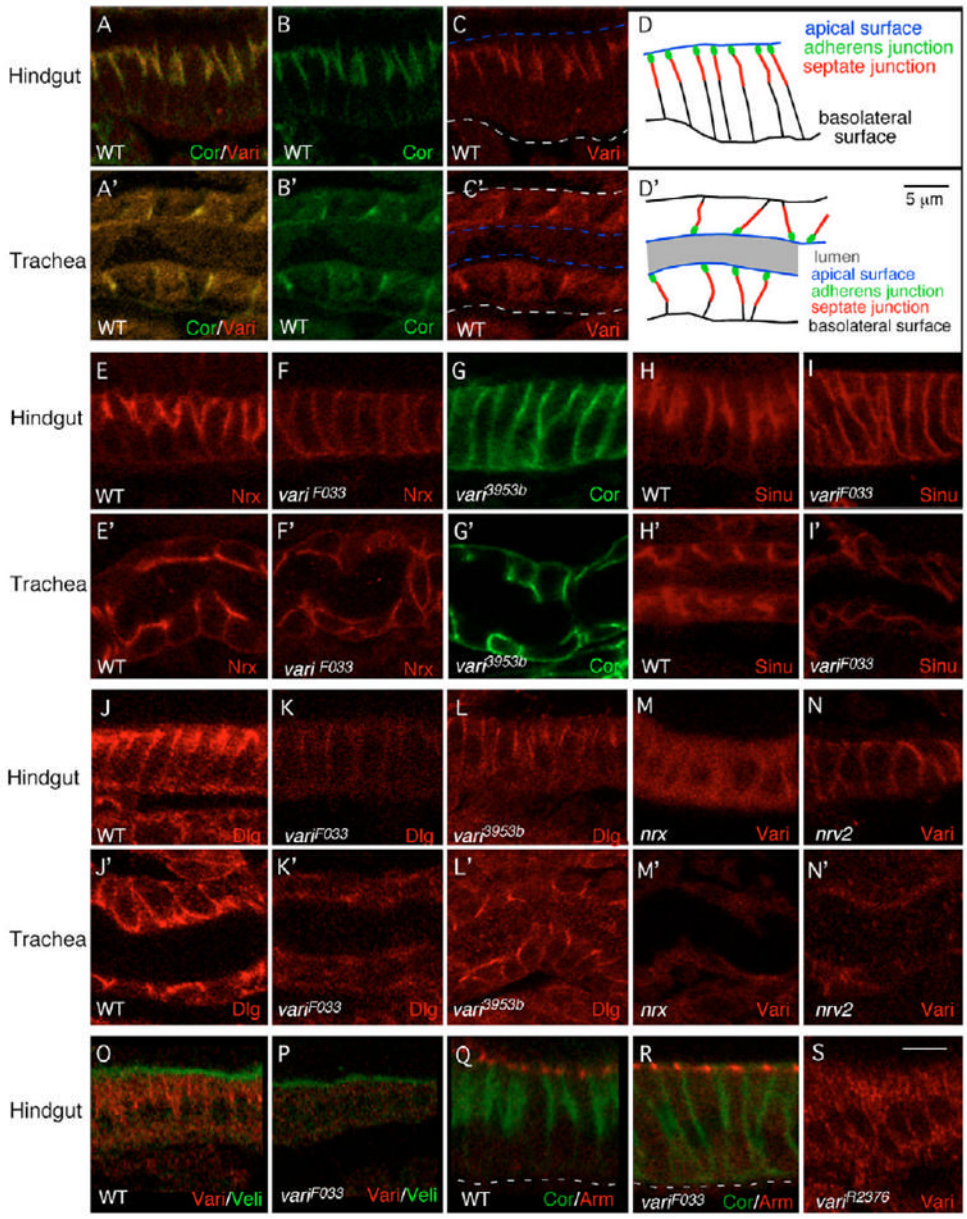

Fig 3. Vari is an SJ component

(**A–D′**) Vari localizes to epithelial SJs in the hindgut (A,C) and trachea (A′,C′) of wild-type animals where it co-localizes with the canonical SJ marker Cor (A,A′,B,B′). (D) Schematic diagrams showing the organization of membrane domains and cell-cell junctions in the hindgut (D) and trachea (D′). (**E–K**) Vari is required for stable formation of SJs, as in *vari*^{F033} null mutants the SJ components Nrx, Sinu and Dlg do not accumulate in a specific region, but instead are mislocalized along the basolateral membrane (F,F′,I,I′,K,K′). In *vari*^{3953b} partial loss-of-function mutants, some SJ components such as Cor (G,G′) are almost entirely mislocalized, whereas others such as Dlg only show reduced localization (**L**,**L′**). (**M–N′**) Localization of Vari to the SJ depends on other SJ components, as Vari does not specifically localize to the SJ region in *nrx* (M,M′) or nrv2 (N,N′) mutants. (**O–R**) Vari is not required for apical-basal polarity, as localization of the adherens junction and apical markers Arm and Veli is not disrupted in *vari*^{F033} null mutants. (**S**) Vari protein does not accumulate at SJ regions in

animals homozygous for *vari* alleles that affect the HOOK domain (Table 1). All animals stage 16. Scale bar: in S, 5 μm for all images. WT, wild type.

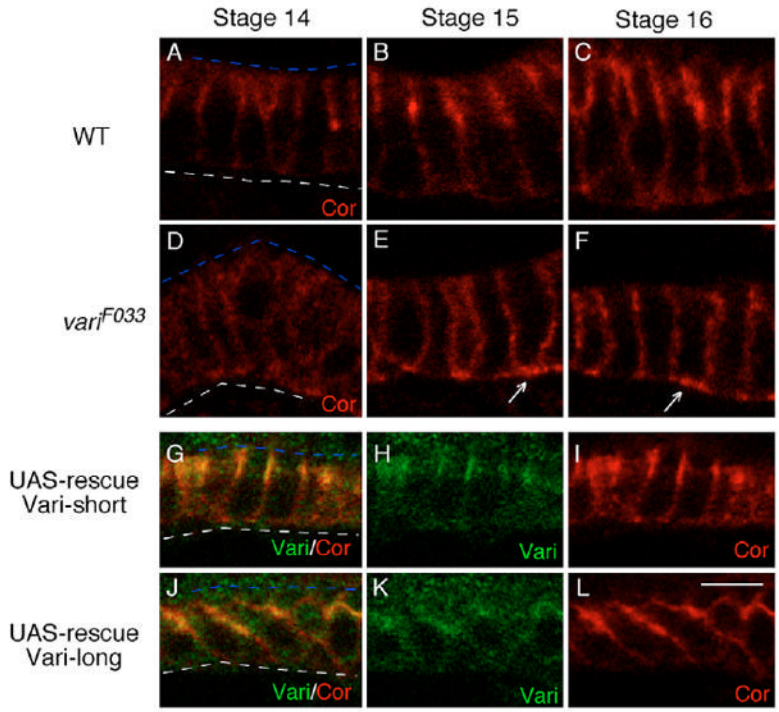

Fig 4. Vari is required for the assembly rather than stability of SJs

(**A–F**) In wild-type animals, Cor localizes predominantly to SJs during stage 14, and then continues to accumulate in SJs during stages 15 and 16 (A–C). In *vari*^{F033} null mutants SJs do not assemble, as indicated by the failure of Cor to localize specifically to the SJ region (D–F). Instead, Cor shows diffuse basolateral and basal localization at all stages. Abnormal accumulations of Cor at the basal surface are visible during stages 15 and 16 (arrows in E and F). (**G–L**) In *vari*^{F033} null homozygotes expressing either the short (G–I) or long (J–L) isoforms of Vari, SJ junction barrier function is restored (data not shown) and the Vari isoforms (green) and Cor (red) show their normal localization to SJs. G–L are stage 16 animals of genotype *vari*^{F033} UAS::Vari-isoform/*vari*^{F033} *da*-Gal4. Hindgut shown in all images. Blue lines, apical cell surface; white lines, basal cell surface. Scale bar: in L, 5 μm for A–L.

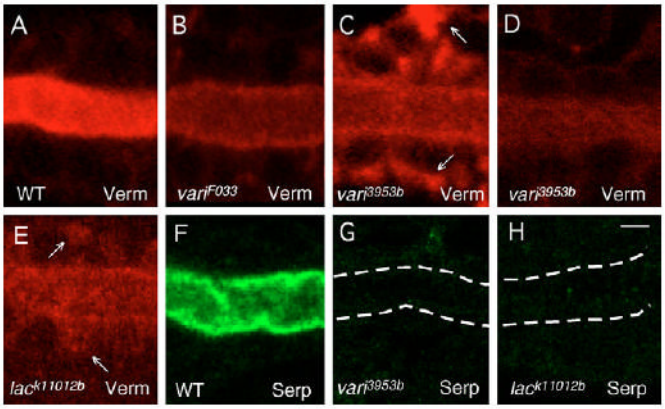

Fig 5. *vari* is required for accumulation of Verm and Serp in the tracheal lumen

(**A–E**) Lumenal secretion and accumulation of the putative matrix modifying protein Verm is disrupted in *vari* (B–D) and other SJ mutants such as *Lac* (E). Notably, in some mutants such as $vari^{3953b}$ and $Lac^{k11012b}$, Verm accumulates in the cytoplasm (arrows in C and E). $vari^{3953b}$ mutants show variable expressivity of the Verm secretion defect, with some animals showing extensive cytoplasmic Verm accumulation (C) and others showing little (D). (**F–H**) Lumenal accumulation of the Verm-related protein Serp is defective in *vari* and *Lac* mutants, but in contrast to Verm, Serp is completely absent in *vari* and *Lac* mutants, including in the hypomorphic $vari^{3953b}$ allele. Scale bar: in H, 2.5 μm for A–H.

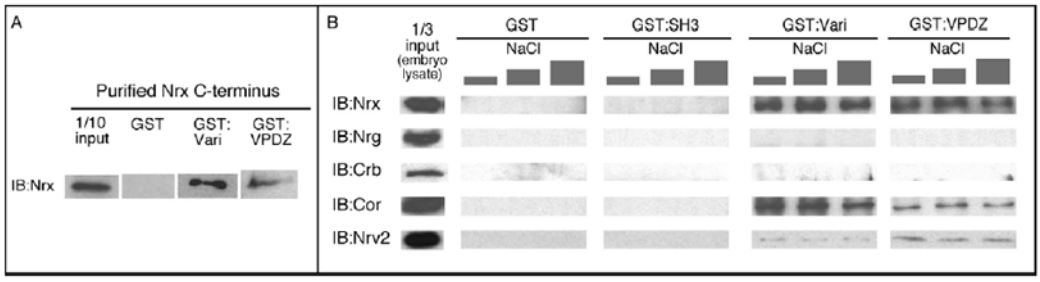

Fig 6. Vari interacts with Nrx through the PDZ domain

(**A**) A purified C-terminal fragment of Nrx is co-precipitated by purified Glutathione S-transferase fused to the short isoform of Vari and the Vari PDZ domain, but not GST or GST fused to the Vari-SH3 domain. (**B**) GST-Vari and GST-VPDZ co-precipitate Nrx but not Crb from 12–18-hour-old embryo lysates, despite the similarity of the Crb and Nrx C-terminal PDZ-binding motifs (ERLI vs EIFI). NaCl concentrations: 150, 350 and 550 mmol/l. GST, Glutathione S-transferase; IB, immunoblot antisera; VPDZ, Vari PDZ domain.

Table 1

Summary of *varicose* allele characterization

Allele	Class*	Mutation†	Domain	Tracheal phenotype	Junctional protein‡	SJ barrier§	Isolation reference
38EFa2	Weak	AG→AA TTCCAACTGAA	1st common intron	Mild	−/+	+	(Butler et al., 2001)
3953b	Intermediate	17 bp Δ CTTGC_AGTTG	3rd common intron	Mild/Strong	−	−/+	(Beitel and Krasnow, 2000)
R2376	Strong	S178F ACCATTCCAAG	HOOK	Strong	N.D.	−	This work
HM2043	Strong	E182K AGCTGAAGGAG	HOOK	Strong	−	−	This work
R979	Strong	R185C AGCGGTGCAAA	HOOK	Strong	−	−	This work
R3	Null	Q179Stop CATCCTAAGAG	HOOK	Strong	−	−	This work
327	Null	AG→AA TTGCAAATCAT	4th common intron splice accept.	Strong	−	−	This work
F033¶	Null	Transposon TATTAA_AAATAG	1st common intron	Strong	−	−	(Thibault et al., 2004)

*Classification based on genetic behavior in Table 2 and nature of the mutation.

†The genomic sequence of the allele is shown with differences from the WT sequence underlined (most alleles) or marked with a '−' to indicate a deletion in *vari*^{3953b} or the transposable element insertion in *vari*^{F033} (amino acid numbering is from ATG of short isoform).

‡The '−' indicates that Vari staining at the SJ region was not detectable; N.D., not determined.

§SJ paracellular barrier function was assayed using the 10 kDa dye exclusion assay of Lamb et al. (Lamb et al., 1998) with '+' indicating normal barrier function, '−/+' indicating variable penetrance and expressivity of the barrier defect depending on the genetic background, and '−' indicating defective barrier function.

¶Full designation: *PBac{WH}CG9326*^{f00033}.

Table 2

Genetic characterization of *varicose* alleles

Allele	Allele type	% Viability (*n*)*				
		38EFa2†	*3953b*†	*327*	*F033*	*Df(2L)TW2*
38EFa2	Weak	**74% 638 w^{-}**‡ **56% 658 w^{+}**‡	**83% 763 w^{-}** **98% 944 w^{+}**	0% 680	0% 860	0% 880
3953b	Intermediate	**73% 798 w^{+}**	0% 688 w^{-}	0% 674	0% 653	0% 661
R979	Strong	**6% 1256 w^{-}** **8% 912 w^{+}**	0% 672 w^{-}	0% 677 0% 683 w^{+}	0% 632	0% 623
HM2043	Strong	**29% 700 w^{-}** **16% 720 w^{+}**	0% 678 w^{-} 0% 670 w^{+}	0% 633	0% 776	0% 636
R2376	Strong	**18% 602 w^{-}** 0% 604 w^{+}	0% 604 w^{-} 0% 811 w^{+}	0% 675	0% 730	0% 904
327	Null§	0% 737 w^{+}	0% 609 w^{+}	0% >999	0% 626	0% 607
R3	Null§	0% 784 w^{-} 0% 617 w^{+}	0% 669 w^{-} 0% 796 w^{+}	0% 622	0% 643	0% 628
F033	Null§	2%§ 721 w^{+}	0% 697 w^{+}	0% 626	0% >999	0% 626
Df(2L)TW2	Deficiency§	0% 824 w^{+}	0% 708 w^{+}	0% 607	0% 626	0% >999

*% Viability=[# straight wing adults÷(# curly wing adults+# straight wing adults)÷0.33]×100; *n*, number of progeny counted. Unless otherwise specified, alleles were balanced with CyO dfd-YFP w^{-} (Le et al., 2006); bold text, viability >5%; remainder, viability <5%.

†Crosses were set up using females of the column genotype and males of the row genotype.

‡The genotype of the female balancer chromosome is indicated. w^{-}, CyO dfd-YFP w^{-}; w^{+}, CyO dfd-YFP w^{+}.

§$vari^{F033}$ is considered a null allele despite having a very low percentage of viable progeny when crossed to $vari^{38EFa2}$ because $vari^{327}$ and $vari^{R3}$, which might otherwise be considered null because of their complete lethality in *trans* to $vari^{38EFa2}$, occasionally cause somewhat more severe phenotypes than Df(2L)TW2 and thus may be slightly antimorphic. In addition, Df(2L)TW2 is unhealthy as a heterozygote in trans to WT, which is likely to mask any minimal viability of the $vari^{38EFa2}$/null phenotype.