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

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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.

**KEY WORDS:** MAGUK, Cell junction, Basolateral, Epithelia, Drosophila, Trachea

**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 the organization of the β1 integrin cytoskeleton is altered in chitin-synthetase mutants (Tonning et al., 2005).
Beginning at stage 15, organization of the luminal matrix requires the luminal 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, luminal 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 Neuronal (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)g1 – 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 luminal 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 luminal (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 to adhesion complexes.

Verm and Serp are known to associate with SJs as seen by partial localization in vivo (Luschnig et al., 2006; Wang et al., 2006); gp anti-Verm 1:1000 (Wang et al., 2006). Embryos were fixed in formaldehyde, staining was 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.

**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 dfl-YFP balancer (Le et al., 2006) was used instead of a CyO actin-GFP balancer.

**Immunohistochemistry**

The following antibodies were used: anti-tracheal luminal 2A12 1:5 and anti-Arm-N2A71 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 (Wood et al., 1997); r anti-Nrv2.1 1:500 (Paul et al., 2007); r anti-Nrx 1:200 (Bauamgarter et al., 1996); r anti-Veli 1:500 (Bachmann et al., 2004); r anti-DE-cadherin DECAD2 1:20 (Oda et al., 1994); r anti-Simu 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), 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’-GCACCCCTTTTCCATTAAGGATGTTCAAGCCCAACATCGAATCTATTGGACTCATACCACCCAGGATGACAAAGGCATCGATTCCCT, 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 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, except 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).
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). 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., 2001). 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, 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, 2002). Vari is encoded by 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.

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 luminal 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. 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 (Fig. 1C-D). 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 and Table 1) and that CG9326 cDNAs could rescue vari mutations (Fig. 1G; 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 vertebrateDlg, 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

### Table 1. Summary of varicose allele characterization

<table>
<thead>
<tr>
<th>Allele</th>
<th>Classa</th>
<th>Mutation1</th>
<th>Domain</th>
<th>Tracheal phenotype</th>
<th>Junctional protein2</th>
<th>SJ barrier3</th>
<th>Isolation reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3BEFa2</td>
<td>Weak</td>
<td>AG→AA</td>
<td>1st common intron splice accept.</td>
<td>Mild</td>
<td>+/-</td>
<td></td>
<td>(Butler et al., 2001)</td>
</tr>
<tr>
<td>3953b</td>
<td>Intermediate</td>
<td>17 bp Δ</td>
<td>3rd common intron</td>
<td>Mild/Strong</td>
<td>-</td>
<td>-/+</td>
<td>(Beitel and Krasnow, 2000)</td>
</tr>
<tr>
<td>R2376</td>
<td>Strong</td>
<td>S178F</td>
<td>HOOK</td>
<td>Strong</td>
<td>N.D.</td>
<td></td>
<td>This work</td>
</tr>
<tr>
<td>HM2043</td>
<td>Strong</td>
<td>E182K</td>
<td>HOOK</td>
<td>Strong</td>
<td>–</td>
<td>–</td>
<td>This work</td>
</tr>
<tr>
<td>R979</td>
<td>Strong</td>
<td>R185C</td>
<td>HOOK</td>
<td>Strong</td>
<td>–</td>
<td>–</td>
<td>This work</td>
</tr>
<tr>
<td>R3</td>
<td>Null</td>
<td>Q179Stop</td>
<td>HOOK</td>
<td>Strong</td>
<td>–</td>
<td>–</td>
<td>This work</td>
</tr>
<tr>
<td>327</td>
<td>Null</td>
<td>AG→AA</td>
<td>4th common intron splice accept.</td>
<td>Strong</td>
<td>–</td>
<td>–</td>
<td>This work</td>
</tr>
<tr>
<td>F033f</td>
<td>Null</td>
<td>Transposon</td>
<td>TATTAA_AATAAG</td>
<td>1st common intron</td>
<td>Strong</td>
<td>–</td>
<td>(Thibault et al., 2004)</td>
</tr>
</tbody>
</table>

1Classification based on genetic behavior in Table 2 and nature of the mutation.
2PDZ, SH3, HOOK and GUK domains, as well as a possible C-terminal PDZ-binding motif (Fig. 1K).
3The ‘−’ indicates that Vari staining at the SJ region was not detectable; N.D., not determined.
4§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.
5¶ Null Transposon 1st common intron Strong – – (Thibault et al., 2004)
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 Vari proteins in Drosophila, a model system for investigating the roles of MAGUKs in epithelial cell junctions. Vari is localized to septate junctions (SJs) in Drosophila and vertebrates and is likely to be more representative than C. elegans as a model system for investigating the roles of MAGUKs in epithelial cell junctions.

**Vari is required for septate junction formation**

To determine if Vari organizes SJs, we tested all five SJ mutants for SJ barrier function and examined the subcellular localization of five SJ proteins in three epithelial tissues of three different organisms: human, mouse, and Drosophila. Vari co-localized with the canonical SJ markers Cor and Nrx (Baumgartner et al., 1996; Fehon et al., 1994) in the trachea, hindgut, and 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 extends the similarity between invertebrates and vertebrates and vertebrate basolateral regions first evidenced by the similar localizations of Scrib, Dlg and Lgl in both vertebrates and Drosophila (Knust and Bossinger, 2002). 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 in septate junctions and morphogenesis**

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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^{SKEFH2} 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^{2953b}, Dlg and
the Na\textsuperscript{+}/K\textsuperscript{+}-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\textsuperscript{+}/K\textsuperscript{+}-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\textsuperscript{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\textsuperscript{F033} and data not shown). Thus, in 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\textsuperscript{4646}, sinu\textsuperscript{p907}, cor\textsuperscript{2} and nrv2\textsuperscript{22b} 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 Ncli-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...
DEVELOPMENT

Development 134 (5)

1006 RESEARCH ARTICLE

Fig. 5. vari is required for accumulation of Verm and Serp in the tracheal lumen. (A–E) Luminal 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\(^{953b}\) and Lac\(^{1102b}\), Verm accumulates in the cytoplasm (arrows in C and E). vari\(^{953b}\) 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) Luminal 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\(^{953b}\) allele. Scale bar: in H, 2.5 μm for A–H.

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\( \times 10^6\) 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\(^{953b}\) allele (Beitel and Krasnow, 2000) and the vari\(^{953Fa2}\) and vari\(^{953Fa2}\) 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\(^{953b}\) 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\(^{953b}\) 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; Table 1). vari\(^{953b}\) appears to be a null mutation that results from a transposable element insertion into the first common intrinsic region (Fig. 1K). vari\(^{953b}\) behaves as a deficiency in genetic crosses, and in vari\(^{953b}\) mutants Vari protein levels are not distinguishable from background (Fig. 1M; Fig. 3O; Table 1; Table 2; and data not shown).

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.
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\textsuperscript{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\textsuperscript{+} 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 Puls2/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\textsuperscript{R2376} 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\textsuperscript{null} null mutants (Fig. 1G,J) and restore normal SJ localization of both Cor and Veli (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\textsuperscript{null} 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 luminal matrix (Luschning et al., 2006; Wang et al., 2006). In vari and other SJ mutants, Verm is not secreted, the luminal 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

### Table 2. Genetic characterization of varicose alleles

<table>
<thead>
<tr>
<th>Allele</th>
<th>Allele type</th>
<th>% Viability (n)*</th>
<th>% Viability (n)*</th>
<th>% Viability (n)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>38Fa2</td>
<td>Weak</td>
<td>74% 636 w*</td>
<td>83% 763 w*</td>
<td>0% 680</td>
</tr>
<tr>
<td>395sB</td>
<td>Intermediate</td>
<td>73% 798 w*</td>
<td>0% 688 w*</td>
<td>0% 674</td>
</tr>
<tr>
<td>R979</td>
<td>Strong</td>
<td>6% 1256 w*</td>
<td>0% 672 w*</td>
<td>0% 677</td>
</tr>
<tr>
<td>HM2043</td>
<td>Strong</td>
<td>29% 700 w*</td>
<td>0% 678 w*</td>
<td>0% 633</td>
</tr>
<tr>
<td>R2376</td>
<td>Strong</td>
<td>18% 602 w*</td>
<td>0% 604 w*</td>
<td>0% 675</td>
</tr>
<tr>
<td>327</td>
<td>Null</td>
<td>0% 737 w*</td>
<td>0% 609 w*</td>
<td>0% &gt;999</td>
</tr>
<tr>
<td>R3</td>
<td>Null</td>
<td>0% 784 w*</td>
<td>0% 669 w*</td>
<td>0% 622</td>
</tr>
<tr>
<td>F033</td>
<td>Null</td>
<td>2% 721 w*</td>
<td>0% 697 w*</td>
<td>0% &gt;999</td>
</tr>
<tr>
<td>Df(2L)1T2</td>
<td>Deficiency</td>
<td>0% 824 w*</td>
<td>0% 708 w*</td>
<td>0% &gt;999</td>
</tr>
</tbody>
</table>

* % Viability (n)=[# straight wing adults/# curly wing adults+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%.

†∞ Crossovers 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\textsuperscript{F033} is considered a null allele despite having a very low percentage of viable progeny when crossed to vari\textsuperscript{R2376}. Vari\textsuperscript{null} phenotype.

§Vari\textsuperscript{R3} because of their complete lethality in trans to vari\textsuperscript{R2376} occasionally cause somewhat more severe phenotypes than Df(2L)1T2 and thus may be slightly antimorphic. In addition, Df(2L)1T2 is unhealthy as a heterozygote in trans to WT, which is likely to mask any minimal viability of the vari\textsuperscript{R2376}/null phenotype.
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 functional motifs, 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 Crb-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.

We are grateful to M. Ternet for initial mapping of vari mutations, C. Gottardi, I. T. Helenius and members of the Beitel and Bhat labs for comments on the manuscript, to the Bloomington Stock Center, the Developmental Studies Hybridoma Bank, the Drosophila Genome Resource Center and many members of the Drosophila community for fly stocks, antibodies and cDNAs. Additional thanks to R. Engen, A. Graff, M. Kelaita, E. Klostermann, R. Lefkowitz, H. Patel and M. VanGompel for isolating alleles, and W. Russin of the Northwestern Biological Imaging Facility for assistance with confocal imaging. S.M.P. was supported by NIH Lung Biology training grant S T22 HL706139-03. M.A.A. was supported by NIH GM62047 and N505356 and funds from the state of North Carolina. G.J.B. was a recipient of a Burroughs Wellcome Fund Career Award in the Biomedical Sciences, NSF Career Award IBN-0133411 and NIH R01 GM069540.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/5/999/DC1

**References**


