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Neuroglian, Gliotactin, and the Na^+/K^+ ATPase are essential for septate junction function in *Drosophila*

Jennifer L. Genova and Richard G. Fehon

Department of Biology, Developmental, Cell, and Molecular Biology Group, Duke University, Durham, NC 27708

One essential function of epithelia is to form a barrier between the apical and basolateral surfaces of the epithelium. In vertebrate epithelia, the tight junction is the primary barrier to paracellular flow across epithelia, whereas in invertebrate epithelia, the septate junction (SJ) provides this function. In this study, we identify new proteins that are required for a functional paracellular barrier in *Drosophila*. In addition to the previously known components Coracle (COR) and Neurexin (NRX), we show that four other proteins, Gliotactin, Neuroglian (NRG), and both the α and β subunits of the Na^+/K^+ ATPase, are required for formation of the paracellular barrier. In contrast to previous reports, we demonstrate that the Na pump is not localized basolaterally in epithelial cells, but instead is concentrated at the SJ. Data from immunoprecipitation and somatic mosaic studies suggest that COR, NRX, NRG, and the Na^+/K^+ ATPase form an interdependent complex. Furthermore, the observation that NRG, a *Drosophila* homologue of vertebrate neurofascin, is an SJ component is consistent with the notion that the invertebrate SJ is homologous to the vertebrate paranodal SJ. These findings have implications not only for invertebrate epithelia and barrier functions, but also for understanding of neuron–glial interactions in the mammalian nervous system.

Introduction

Epithelial cells control and maintain distinct extracellular environments via a combination of selective membrane permeability and the formation of a paracellular diffusion barrier, a tight seal between epithelial cells that regulates solute flow between adjacent cells. Extensive studies of vertebrate epithelial cells have demonstrated that one function of the tight junction is the formation and regulation of the paracellular diffusion barrier (Tsukita et al., 2001). Paracellular barriers are found throughout an organism in tissues such as the intestines, the kidney, and the bladder, where they are required for proper nutrient absorption or secretion (Fasano, 2000; Firth, 2002). The importance of a paracellular barrier is demonstrated in diseases involving the endothelial blood–brain barrier where disruption of the barrier is associated with many central nervous system diseases, including Alzheimer's, multiple sclerosis, and bacterial meningitis (Huber et al., 2001).

In *Drosophila* epithelial cells, the paracellular barrier is formed by the pleated septate junction (SJ),* which first appears midway through embryogenesis (Tepass and Hartenstein, 1994; Tepass et al., 2001). Ultrastructurally, SJs are characterized by regions in the apical-lateral membranes of epithelial cells that maintain a constant distance between adjacent cells. These regions have electron-dense material, or septae, that are present in large numbers and spaced uniformly between the cell membranes. This regular alignment pattern of both the membranes and the septae gives the junction a ladder-like appearance (Tepass and Hartenstein, 1994).

Genetic analysis in *Drosophila* has identified several components of the SJ. In embryos mutant for either *coracle* (*cor*) or *Neurexin* (*Nrx*), the membranes of adjacent cells maintain their uniform spacing but the septae are lost (Lamb et al., 1998), giving the junction the appearance of a ladder that has no rungs. The structural loss of these septae corresponds to a functional loss of the paracellular barrier, which can be visualized by diffusion of a fluorescently labeled molecule across epithelial sheets (Lamb et al., 1998). *cor* encodes a *Drosophila* homologue of mammalian protein 4.1, a cytoplasmic protein that associates with the erythrocyte plasma membrane via interactions with the cytoplasmic tail of glycophorin C, a transmembrane protein (Marfatia et al., 1994, 1995). COR binds to NRX, a transmembrane protein with a cytoplasmic tail similar to that of glycophorin C (Baumgartner et al., 1996; Ward et al., 1998). Both NRX and COR localize to

Address correspondence to Richard G. Fehon, Box 91000, Duke University, Durham, NC 27708. Tel.: (919) 613-8192. Fax: (919) 613-8177. E-mail: rfehon@duke.edu

*Abbreviations used in this paper: ATPα, Na pump α subunit; COR, Coracle; DCAD2, *Drosophila* Cadherin 2; DLG, Discs large; DLT, Discs lost; EGFR, EGF receptor; FASIII, Fasciclin III; FERM, protein 4.1, ezrin, radixin, moesin; GLI, Gliotactin; IP, immunoprecipitation; NRG, Neuroglian; NRV, Nervana; NRX, Neurexin; PSJ, paranodal SJ; SJ, septate junction.

Key words: paracellular barrier; paranodal septate junction; glia; Na pump; epithelia

the SJ of ectodermally derived epithelial cells (Fehon et al., 1994; Baumgartner et al., 1996). In addition to disrupting the epithelial paracellular barrier, *cor* and *Nrx* mutations disrupt the blood–brain barrier, which is created by SJs located between individual glial cells that insulate neurons. Another gene, *Gliotactin (Gli),* has been characterized as being important for a proper blood–brain barrier (Auld et al., 1995).

The SJ has historically been thought of as an invertebrate-specific junction; however, recent studies of the vertebrate nervous system have identified a junction that is both molecularly and structurally homologous, the paranodal SJ (PSJ) (Einheber et al., 1997; Tepass et al., 2001). The PSJ occurs between neurons and the glial cells that myelinate them, the oligodendrocytes and Schwann cells. Each glial cell wraps around and contacts the neuron multiple times in a spiral pattern to form the paranodal loops. The PSJ forms between the paranodal loops and the neuron and keeps the node of Ranvier distinct from the internodal region by providing a seal between the neuron and glial cell. This seal provides a barrier within the neuronal membrane that separates Na^+ channels at the node of Ranvier from K^+ channels under the glial cells, and a paracellular diffusion barrier between the neuron and the ensheathing glial cell (Salzer, 1997; Arroyo and Scherer, 2000). Consistent with these structural and functional similarities, the invertebrate epithelial SJ and the vertebrate PSJ also display similarities at the molecular level. Caspr (contactin-associated protein; also known as paranodin), a mammalian homologue of NRX, is located on the neuronal face of the PSJ (Einheber et al., 1997), where it interacts with protein 4.1 (Menegoz et al., 1997), which is homologous to *Drosophila* COR.

To identify additional components of the *Drosophila* SJ, we screened a collection of *P* element insertion mutations for a previously identified phenotype attributable to a loss of the paracellular barrier (Lamb et al., 1998). Two genes, *Na pump α subunit* (*Atpα*) and *Nervana 2* (*Nrv2*) (which encodes the β subunit of the Na^+/K^+ ATPase) were identified as essential for the barrier function of the SJ. In addition, Neuroglian (NRG), which is homologous to known components of the PSJ, and GLI, which is necessary for the blood–brain barrier, were tested and found to be necessary for the paracellular barrier. Direct immunostaining, epitope-tagged expression constructs, and GFP-tagged proteins indicate that NRV2, ATPα, and NRG localize to the SJ, and that they are interdependent for this localization. In keeping with this finding, we demonstrate the existence of a protein complex containing COR, NRX, NRG, and NRV. Taken together, these results suggest a novel complex involving the Na^+/K^+ ATPase that is necessary for establishing and maintaining the primary paracellular barrier in invertebrate epithelia, the SJs. Thus our studies provide new insights into the structure and function of SJs in both invertebrate epithelial cells and in the homologous PSJ of the vertebrate nervous system.

Results

Identification of genes required for the paracellular barrier function of the *Drosophila* SJ

In polarized epithelial cells, the SJ forms a paracellular barrier that blocks diffusion between the apical and basolateral epithelial surfaces (Tepass et al., 2001). This barrier function is disrupted by mutations in *cor* and *Nrx*, two known binding partners that localize to the SJ (Baumgartner et al., 1996; Lamb et al., 1998). The paracellular barrier function can be assayed by injecting a fluorescently labeled dextran into the body cavity of developing embryos and then scoring for diffusion across the epithelium into the lumen of the salivary gland (Lamb et al., 1998). We used this simple assay to identify additional genes required for a functional SJ barrier.

To identify novel SJ components, 1,274 lethal *P* element insertion lines from the Bloomington Stock Center were screened for mutations that affect dye diffusion across the salivary gland epithelium. From this screen, we identified *P* element insertions in two genes that were previously unknown to have roles in SJ function but displayed dye diffusion phenotypes (Fig. 1, D and E). The first, *l(3)01453a* (Fig. 1 D), is reported to disrupt the *Atpα* gene (Spradling et al., 1999). The identity of *l(3)01453a* as an allele of *Atpα* was confirmed using previously identified alleles of this gene (Feng et al., 1997). As described below, we determined that the second, *l(2)k13315*, is inserted within *Nrv2*, which encodes a β subunit of the Na^+/K^+ ATPase (Fig. 1 E; Sun and Salvaterra, 1995b; Xu et al., 1999). Together, these two proteins form the functional Na^+/K^+ ATPase.

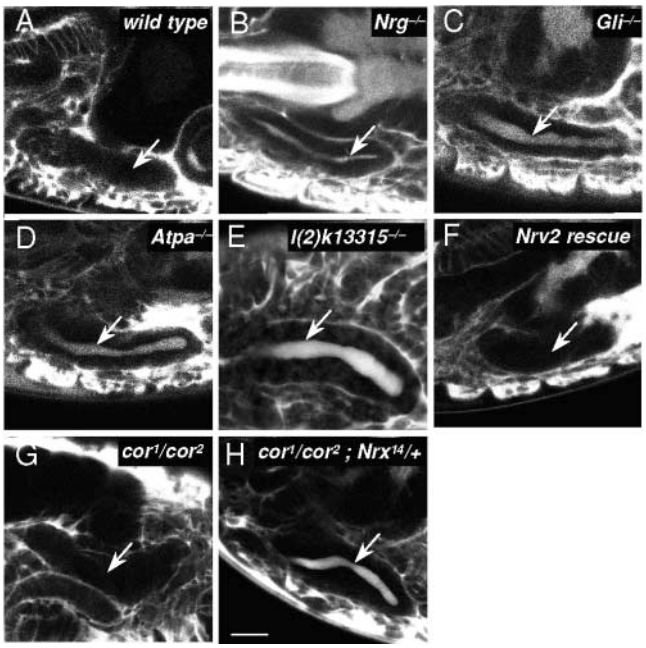

Figure 1. **Identification of genes essential for formation of the paracellular barrier.** Confocal sections of the salivary glands of live, late stage, embryos. Arrows indicate the lumen of the salivary glands in all panels. Embryos were injected posteriorly with a fluorescently labeled dextran that is restricted from entering the lumen of the salivary gland by the paracellular diffusion barrier in wild-type embryos (A). However, dye enters into the lumen of the salivary gland, indicating that this barrier is lost in embryos homozygous mutant for Nrg^{14} (B), Gli^1 (C), $Atp\alpha^{01453a}$ (D), and *l(2)k13315* (E). The dye diffusion phenotype of *P* element mutation *l(2)k13315* is rescued when UAS–Flag Nrv2.2 is expressed under the e22C-GAL4 driver in embryos (F). cor^1/cor^2 mutant embryos have an effective diffusion barrier (G) that is lost when one copy of *Nrx* is removed (H). Bar, 25 μm.

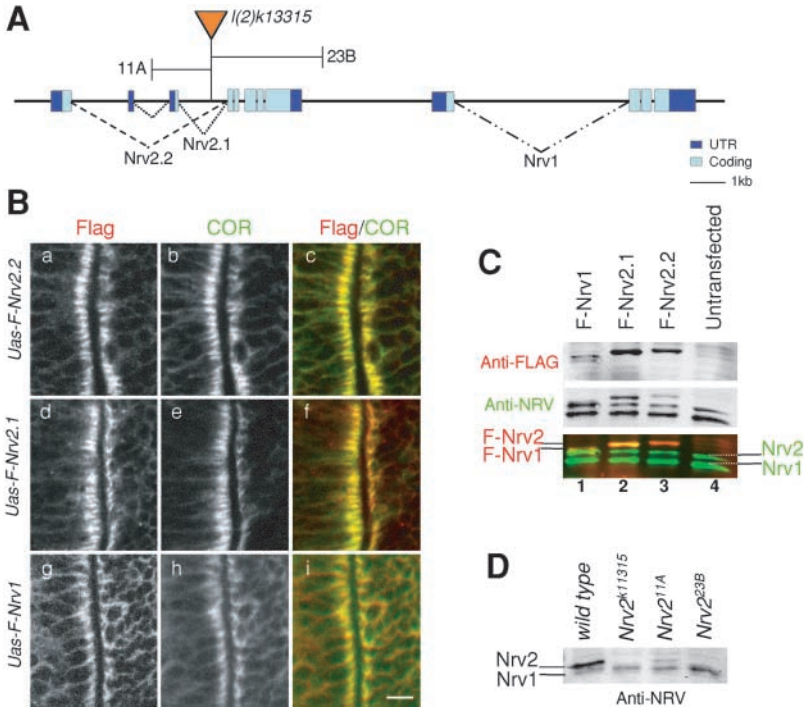

Figure 2. **The *Nrv2* locus encodes two proteins that localize to the SJ and are disrupted in the *l(2)k13315* mutant.** (A) The *Nrv2* locus encodes two transcripts, *Nrv2.1* and *Nrv2.2*. The proteins encoded by these transcripts are type 2 transmembrane proteins, which differ only in their cytoplasmic domains. Also indicated is *Nrv1,* a gene located ~3 kb downstream that also encodes an Na^+/K^+ ATPase β subunit. The schematic of the *Nrv2* locus includes the *P* element insertion *l(2)k13315* as well as two deletions created by imprecise excision of the *P* element. $Nrv2^{11A}$ is a deletion of 1,589 bp, which removes the *Nrv2.1* start site, and $Nrv2^{23B}$ is a deletion of 2,176 bp, which removes all of the *Nrv2* common exons. (B) The proteins encoded by the *Nrv2* and *Nrv1* loci were 5′ tagged with Flag and then expressed in the wing imaginal disc using the *Apterous*-GAL4 driver and UAS promoter. F-NRV2.2 (a, red in c), F-NRV2.1 (d, red in f), and F-NRV1 (g, red in i) all localize to the SJ where they colocalize with COR (b, e, and h; green in c, f, and i). Bar, 10 μm. (C) The anti-NRV antibody, mAb 5F7, which recognizes NRV2.1, NRV2.2, and NRV1, stains two protein bands from untransfected S2 cells (lane 4). The Flag-tagged *Nrv* transgenes were expressed in S2 cells using the *Ubiquitin*-GAL4 driver, and an immunoblot of these cells was probed using anti-Flag (top panel, red in bottom panel) and mAb 5F7 (middle panel, green in bottom panel). The banding pattern observed using the Flag-tagged NRV proteins indicates that NRV2.1 and NRV2.2 (lanes 3 and 4) run slower than NRV1 (lane 1). Overlaying these two antibodies shows which NRV protein corresponds to each band (bottom). (D) Protein from late stage embryos was examined for NRV protein by probing with mAb 5F7. Wild-type embryos have both NRV2 and NRV1 proteins; however the NRV2 protein band is completely absent in $Nrv2^{k13315}$ and $Nrv2^{23B}$ homozygous embryos. The NRV2 band is still present, although greatly reduced, in embryos homozygous for $Nrv2^{11A}$.

Inverse PCR and sequencing from the ends of the *l(2)k13315 P* element showed that it is inserted into the second intron of the *Nrv2* locus (unpublished data; Fig. 2 A). *Nrv2* encodes two transcripts that differ in their 5′ start sites but share common 3′ exons (Fig. 2 A). This results in isoforms of this type 2 transmembrane protein with different cytoplasmic domains. A similar gene, *Nervana 1* (*Nrv1*), also encodes an Na^+/K^+ ATPase β subunit and is located ~3 kb downstream of *Nrv2* (Fig. 2 A). NRV2.1, NRV2.2, and NRV1 are recognized by a monoclonal antibody, 5F7 (Sun and Salvaterra, 1995a). To determine which protein corresponds to the bands recognized by 5F7 on immunoblots, we expressed these proteins in *Drosophila* S2 cultured cells using Flag-tagged constructs coding for *Nrv2.1, Nrv2.2*, and *Nrv1*. Untransfected cells, which express both genes, were compared with cells expressing Flag-tagged NRV proteins to determine the relative migration patterns of the different proteins. All three proteins are shifted upward on the gel by the addition of the Flag tag (Fig. 2 C). We determined that the slower migrating band recognized by 5F7 corresponds to both NRV2.1 and NRV2.2, which differ in length by only one amino acid (Fig. 2 C; Sun et al., 1998). As expected, the faster-moving band corresponds to NRV1, which is 13 or 12 amino acids shorter than NRV2.1 and NRV2.2, respectively.

To confirm that the *l(2)k13315 P* element insertion disrupts only *Nrv2*, we showed first that precise excision of this *P* element reverted its lethality (unpublished data), indicating that the lethal phenotype of this stock is due to the *P* element insertion itself rather than to a different lesion elsewhere on the chromosome. Second, to determine if NRV expression is affected, NRV proteins from embryos homozygous for the *l(2)k13315* mutation, as well as two partial deletions of the *Nrv2* locus generated by imprecise excision of the *l(2)k13315* insertion (Fig. 2 A; unpublished data), were examined on immunoblots. NRV2 proteins are absent in *l(2)k13315* embryos as well as in embryos carrying a deletion of all the common exons of *Nrv2,* but NRV1 protein is unaffected (Fig. 2 D). Interestingly, a small amount of NRV2 protein is still present in $Nrv2^{11a}$, a deletion that removes the *Nrv2.1* start site but leaves *Nrv2.2* intact. To confirm that this insertion affects only the *Nrv* gene, genetic rescue experiments were performed using UAS-driven *Nrv* cDNA transgenes. When expressed under the control of the embryonically expressed e22c-GAL4 driver (Lawrence et al., 1995), *Nrv2.1* and *Nrv2.2* rescued the dye diffusion phenotype indicative of a barrier defect (Fig. 1 F; unpublished data). However, *Nrv1* did not rescue this phenotype, suggesting that although structurally quite similar, *Nrv1* and *Nrv2* have distinct functions. Together these results demonstrate that only *Nrv2* is affected by *l(2)k13315* and that *Nrv2* is required for a functional paracellular diffusion barrier.

In addition to screening *P* element mutations, mutations in two candidate genes, *Nrg* and *Gli*, were tested for functional SJs. *Drosophila Nrg* encodes a protein with similarity to vertebrate neurofascin-155, a protein found in paranodal loops (Bieber et al., 1989; Charles et al., 2002), and NRG has been previously reported to localize to the lateral membrane of epithelial cells (Dubreuil et al., 1997). Mutations in *Gli*, were of interest because they have been reported to disrupt the blood–brain barrier in the *Drosophila* nervous sys-

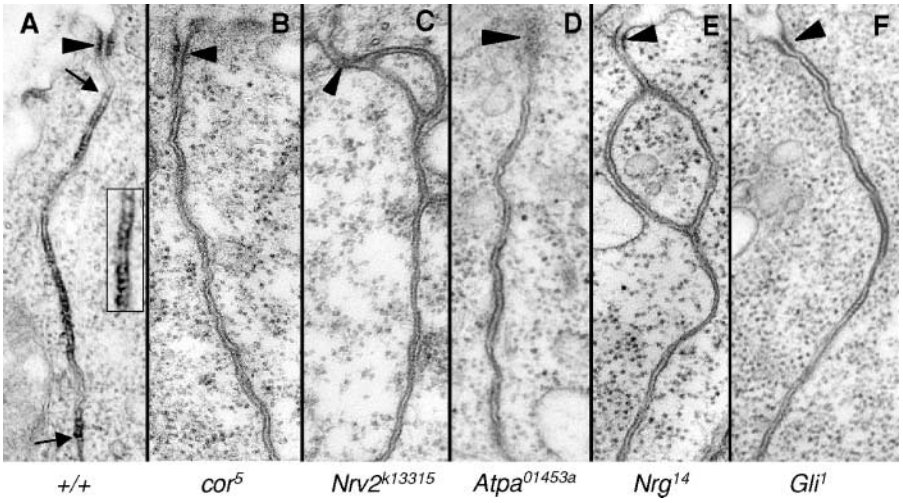

Figure 3. **The transverse septae are reduced or absent from the epidermal SJ of *cor*, *Nrv2*, *Atpα*, *Nrg*, and *Gli* mutant embryos.** Wild-type embryos have distinct junctional structures in the apical-lateral domain of polarized epithelial cells, including the adherens junction (A, arrowhead) and the SJ (A, between arrows). The inset in A demonstrates the alignment of septae between uniformly spaced membranes of adjacent cells (inset corresponds to region basal to the upper arrow). In cor^5, $Nrv2^{k13315}$, $Atp\alpha^{01453a}$, Nrg^{14}, and Gli^1 embryos, the adherens junction remains intact (B–F, arrowheads); however, the septae are disrupted.

tem (Auld et al., 1995), which is dependent on SJ function (Carlson et al., 2000). *Gli* encodes a transmembrane protein of the serine esterase family that is homologous to the neuroligin 3 protein expressed in vertebrate glial cells (Gilbert et al., 2001). The *Drosophila* blood–brain barrier is believed to consist of SJs that form in the perineural sheath that surrounds the central nervous system. As shown in Fig. 1, we found that previously identified mutations in both *Nrg* and *Gli* disrupt the paracellular barrier in the embryonic salivary gland (Fig. 1, B and C). In addition, *Nrv2*, *Atpα*, *Nrg*, and *Gli* mutant embryos all display decreased muscle contractions, a failure to properly inflate their trachea, and abnormal salivary gland morphology, as was described previously for *cor* and *Nrx* (Lamb et al., 1998; unpublished data).

Barrier-defective mutations display ultrastructural defects in the SJ

In cor^5 mutant embryos, the dye diffusion defect correlates with a structural loss of the septae characteristic of pleated SJs (Lamb et al., 1998). This phenotype suggested that the same structural loss might be found in the pleated SJs of embryos mutant for the newly identified SJ components. Wild-type SJs are found just basal to the adherens junctions and are characterized by a uniform spacing between the plasma membranes of adjacent cells and an extensive span of regularly aligned septae within this region (Fig. 3 A). In embryos homozygous for mutations in *Nrv2*, *Atpα*, *Nrg*, and *Gli*, this regular spacing of plasma membranes is maintained. However, the septae normally located between these membranes are reduced in number or absent (Fig. 3, B–F). Occasionally, some septae formed in these mutants, but we did not observe the extensive array found in wild-type embryos. This structural alteration of the SJ correlates with loss of the diffusion barrier and provides further confirmation that septae are required for this paracellular barrier.

NRV, ATPα, and NRG localize to SJs in epithelial cells

To further investigate possible functional interactions between *cor*, *Nrx*, and these new candidate SJ components, we asked if the proteins encoded by these genes localize to the SJ (we were unable to examine GLI localization because antibodies that recognize this protein were not available). Although NRV was reported previously as having only nervous system expression (Sun et al., 1998), we found that embryonic and wing imaginal epithelial cells abundantly express NRV, and that in these cells, NRV is largely localized to the SJ (Fig. 4, A–C; unpublished data). In addition, Flag-tagged versions of NRV2.1, NRV2.2, and NRV1 all localize to the SJ when expressed in imaginal epithelia (Fig. 2 B).

ATPα localization in embryonic epithelial cells had been described previously as basolateral (Lebovitz et al., 1989; Dubreuil et al., 1997, 2000). Surprisingly, instead we found it highly localized to the SJ in imaginal epithelia (Fig. 4, E–G). To confirm this subcellular localization, we performed two further tests. First, we found that whereas embryos devitellinized with methanol after fixation displayed diffuse basolateral staining in epithelial cells, embryos hand devitellinized without methanol showed striking SJ staining using monoclonal antibody α5 (unpublished data). Second, we made use of an existing GFP-tagged exon trap for *Atpα* (Morin et al., 2001). In living cells, GFP-tagged ATPα expressed under its endogenous promoter was localized to the SJ in embryonic (Fig. 4, M and N) and imaginal epithelia (Fig. 4 H). From these results, we conclude that ATPα is localized to the SJ in all epithelial cells, and that this localization is disrupted by the methanol devitellinization treatment commonly used for immunofluorescence in *Drosophila* embryos.

Immunolocalization of NRG in imaginal epithelial cells showed that this protein is also associated with the SJ (Fig. 4, I–K). Furthermore, GFP-tagged gene traps in both NRV2 and NRG (Morin et al., 2001) confirmed this observation in living epithelial cells (Fig. 4, D and L). Taken together, these results indicate that NRV2, ATPα, and NRG are all associated with the SJ, consistent with the genetic results that suggest that they are all functional SJ components.

Genetic interactions between SJ components

To determine whether the newly identified SJ components cooperate functionally in the formation of the SJ barrier, we looked for genetic interactions between *Nrv2*, *Atpα*, *Nrg*, *Gli*, and *cor*. This experiment made use of hypomorphic *cor* alleles, cor^1 and cor^2, to look for enhancement of an embryonic *cor* phenotype (Fehon et al., 1994). Both of these alleles

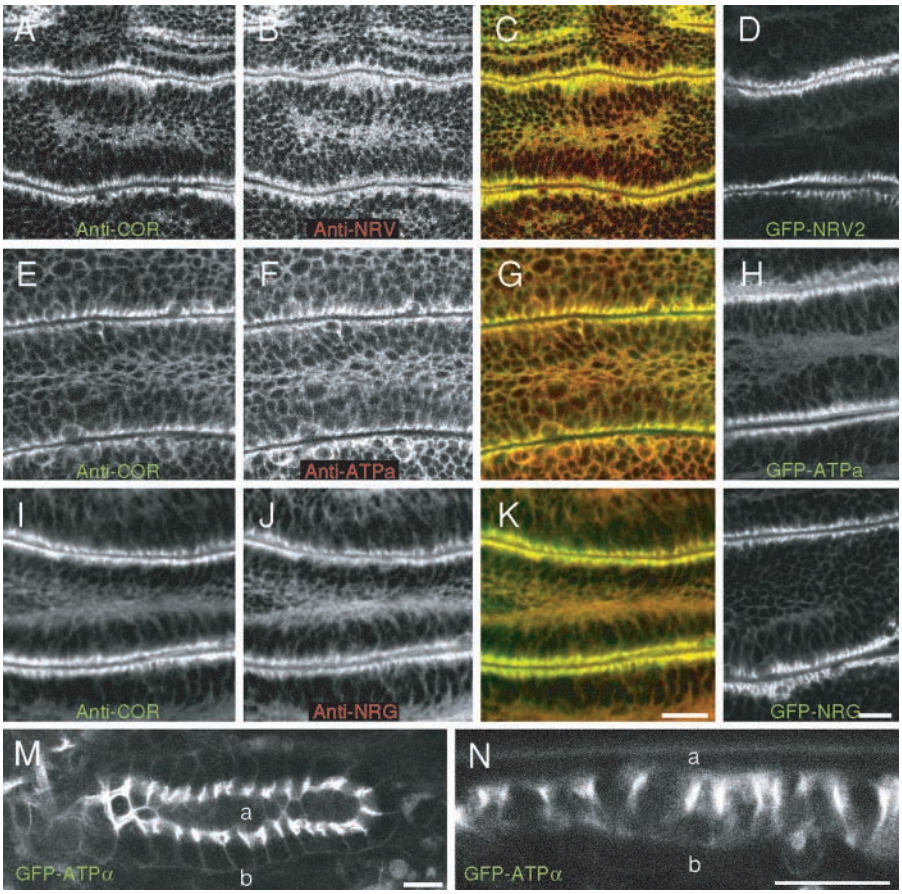

Figure 4. **NRV, ATPα, and NRG co-localize with COR at the SJ.** Anti-COR staining indicates the region of the SJ (A, E, and I). Antibodies recognizing NRV (B), ATPα (F), and NRG (J) localize to the region of the SJ and colocalize with COR (yellow indicates colocalization in C, G, and K). Gene trap lines (Morin et al., 2001) express GFP fused in frame with NRV2 (line G74) (D), ATPα (line G109) (H, M, and N), and NRG (line G305) (L). The GFP gene traps demonstrate SJ localization of these proteins in living wing imaginal disc (D,H, and L), embryonic salivary gland (M), and embryonic epidermal (N) cells. The apical (a) and basal (b) surfaces of the embryonic epithelia are indicated. Bars, 10 μm.

are associated with nonsense mutations just carboxy terminal to the protein 4.1, ezrin, radixin, moesin (FERM) domain. A previous study has shown that the FERM domain is sufficient to rescue the dye diffusion phenotype (Ward et al., 2001), and consistent with this observation, cor^1 and cor^2 homozygous mutant embryos have an intact diffusion barrier (Fig. 1 G). We attempted to enhance this embryonic phenotype to that of cor^5, a null mutant that lacks an effective barrier, by reducing the genetic dose of another SJ component. Removing one copy of *Nrx*, *Nrv2*, or *Atpα* in cor^2/cor^2 or cor^1/cor^2 mutant embryos disrupts the diffusion barrier (Fig. 1 H; unpublished data). The observed enhancement of this cor^- phenotype indicates that these SJ components interact functionally in vivo to establish the paracellular diffusion barrier.

Somatic mosaic analysis reveals interdependent relationships between some, but not all, SJ components

The newly identified SJ components *Nrv2*, *Atpα*, and *Nrg* are required functionally for the paracellular barrier and interact genetically with *cor*. These findings raise the possibility that these SJ proteins require one another for proper localization to the junction, as has been previously described for COR and NRX (Ward et al., 1998). To test this notion, we used somatic mosaic analysis to generate clones of mutant cells in wing imaginal discs and examined the localization of the SJ components in these mutant epithelial cells. We generated mutant clones for three of the SJ components, *cor*, *Nrg*, and *Nrv2*. No clones were observed in adult tissues for any of these mutations, as we have previously reported for *cor* mutants (Lamb et al., 1998).

In cor^5 clones, NRX staining is reduced and does not localize to the SJ (Ward et al., 1998). Similarly, we found that NRG (Fig. 5, I–L), ATPα (Fig. 5, E–H), and NRV (Fig. 5, M–P) staining was reduced and improperly localized in cor^5 cells. Likewise, in $Nrv2^-$ cells, COR, NRX, NRG, and ATPα staining was significantly reduced and mislocalized (Fig. 5, A–D; unpublished data), and in *Nrg*-null cells, COR, NRX, NRV, and ATPα were reduced and mislocalized (unpublished data). Together, analysis of these mutant clones demonstrates an interdependence of these SJ components for localization to the junction. Furthermore, this complex seems to be necessary in both cells that share a junction, because wild-type cells fail to properly localize NRX at points of contact with cor^5 mutant cells (Fig. 5, Y and Z).

To determine whether the absence of COR, NRG, or NRV2 resulted in a complete loss of the SJ, we examined the localization of SJ proteins not known to affect the diffusion barrier. Previous results have indicated that the SJ protein Discs large (DLG) is properly localized in cor^5 cells (Ward et al., 1998). DLG was also properly localized in *Nrg* and *Nrv2* mutant cells (Fig. 5, Q–T; unpublished data). In addition, the SJ protein Fasciclin III (FASIII; Patel et al., 1987) was properly localized to the SJ in all three mutant cell types (Fig. 5, U–X; unpublished data). The proper localization of both DLG and FASIII to the SJ indicates that at least part of the junction is intact in these mutant backgrounds and that

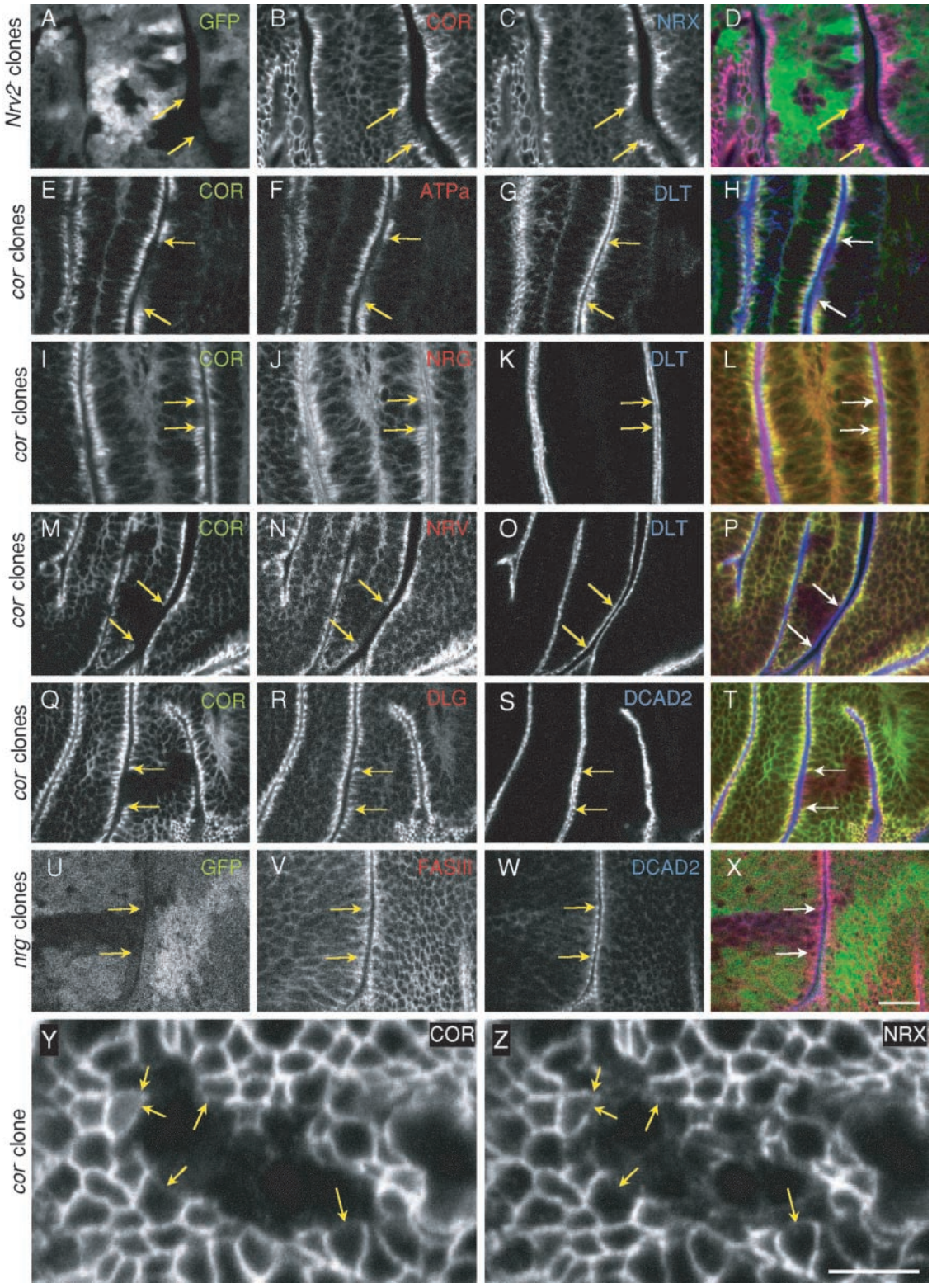

Figure 5. **Somatic mosaic analysis reveals interdependent relationships between some, but not all, SJ components.** (A–X) Cross sections of somatic mosaic clones in wing imaginal epithelia. Arrows indicate the clone boundary, with homozygous mutant cells found between the arrows. (A–D) *Nrv2*^{k11315} mutant cells were marked by an absence of GFP (A, green in D). *Nrv* mutant cells stained for COR (B, red in D) and NRX (C, blue in D) show these proteins reduced or absent. (E–P) *cor*^{5} mutant cells are identified by lack of staining for COR between the arrows (E, I, and M; green in H, L, and P). In *cor*$^{-}$ cells, ATPα (F, red in H), NRG (J, red in L), and NRV (N, red in P) protein levels are reduced or absent compared with expression in the surrounding wild-type cells. However, the apical marker DLT is unaffected (G, K, and O; blue in H, L, and P). Additional SJ markers, DLG (R, red in T) and FASIII (V, red in X), are not disrupted in *cor*^{5} or *Nrg*^{14} mutant cells, respectively, nor is the adherens junction marker DCAD2 (S and W; blue in T and X). (Y and Z) Tangential section of the apical region of the imaginal epithelium containing a *cor*^{5} clone. Both COR (Y) and NRX (Z) are reduced in wild-type cells where they contact mutant cells (indicated by arrows). Bars: (A–X) 20 μm; (Y and Z) 5 μm.

the mislocalization of individual SJ components is not due to disruption of the junction but rather to an interdependence for proper localization to the junction.

In addition to SJ components, apical markers were used to determine if the general apical–basal polarity was affected in mutant cells. In *cor*, *Nrv2*, and *Nrg* mutant clones, the apical marginal zone marker Discs lost (DLT) (Bhat et al., 1999) and the adherens junction marker *Drosophila* Cadherin 2 (DCAD2) (Oda et al., 1994) are still properly localized, suggesting that overall epithelial polarity is not affected in these mutant cells (Fig. 5, G, K, O, S, and W).

COR, NRX, NRV2, and NRG form a protein complex

Our findings indicate that COR, NRX, NRV2, ATPα, and NRG function in an interdependent manner at the SJ to form a diffusion barrier. These results suggest that the proteins encoded by these six genes may function as a complex at the SJ. To test this hypothesis, we immunoprecipitated embryonic lysates with anti-COR, anti-NRG, and anti-NRV antibodies and asked if the other proteins are coimmunoprecipitated. We were unable to immunoprecipitate NRX, ATPα, or GLI due to the lack of antibodies suitable for this experiment. In addition, ATPα and NRV could not be detected on immunoprecipitation (IP) immunoblots because they comigrated with antibodies used in the IP experiments.

Co-IP experiments demonstrate that these SJ components form a complex. When anti-COR was used as the precipitating antibody, we found that NRX and NRG also coimmunoprecipitate (Fig. 6 A). The reciprocal experiment using anti-NRG as the precipitating antibody also finds NRX and COR precipitating in a complex with NRG (Fig. 6 B). As we were unable to detect NRV or ATPα on these immunoblots, we used anti-NRV to precipitate the complex. Using this method, we found that both COR and NRX coimmunoprecipitate with NRV (Fig. 6 C); however, NRG was not detected (unpublished data). This result suggests that COR and NRX interact with the ATPase independent of an interaction with NRG. Together these IP results suggests that the Na^+/K^+ ATPase (both α and β subunits) is found in a complex with COR, NRX, and NRG.

We next asked whether the decreased staining for all components of the SJ-associated complex that we observed in somatic mosaic clones resulted from destabilization and degradation or merely mislocalization of the other components. To examine protein levels, we performed immunoblot analysis on mutant embryos of each genotype and probed for each protein of interest (Fig. 6 D). As expected, each of the mutations severely reduced levels of the proteins they encode. However, even though immunofluorescence staining suggests that all SJ components are destabilized, we found, generally, that levels of the other gene products were not significantly affected in each mutant genotype (NRV levels were partially reduced in *Atpα* mutants, NRX was reduced in *cor* mutants, and ATPα was reduced in Nrg^-). We conclude that although mislocalized in the different mutant backgrounds, the stability of most SJ proteins we examined was not affected.

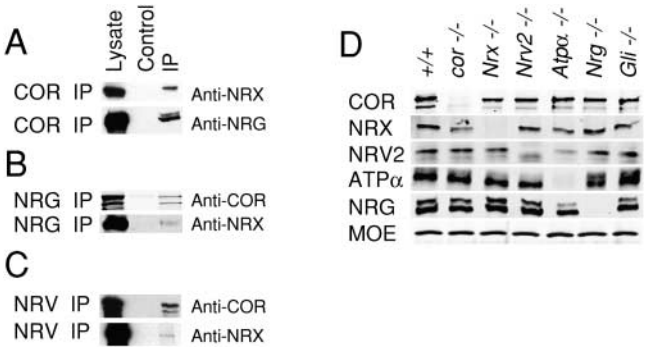

Figure 6. **COR, NRX, NRV, ATPα, and NRG interact in vivo to form a protein complex.** (A) NRX and NRG coimmunoprecipitate with COR in lysates from wild-type embryos, suggesting that these proteins are present in a complex. (B) COR and NRX precipitate with NRG, confirming this result. (C) NRV is a part of this complex because it precipitates both COR and NRX. (D) Embryo lysates from cor^5, Nrx^{14}, $Nrv2^{k13315}$, $Atpα^{01453a}$, Nrg^{14}, and Gli^1 mutant embryos were probed with antibodies that detect COR, NRX, NRV, ATPα, and NRG. In most cases, depletion of one component of the complex did not affect stability of other components. Anti-Moesin was used as a loading control.

Discussion

We have used a novel approach to identify components of the pleated SJ, which provides the barrier to paracellular diffusion in *Drosophila* epithelial cells. Three independent lines of evidence indicate that the proteins encoded by these genes are essential to the structure and function of epithelial SJs. First, mutations in all four identified loci, *Nrg*, *Gli*, *Nrv2*, and *Atpα*, disrupt the paracellular barrier of the salivary gland epithelium and alter the ultrastructure of epithelial SJs. Second, the proteins encoded by three of these genes localize to the region of the SJ as judged by antibody staining of fixed tissues and observation of GFP-tagged proteins expressed in living epithelial cells (reagents were unavailable for observations of the fourth protein, GLI). Third, somatic mosaic studies and IP experiments indicate that these proteins form an interdependent complex at the SJ. This complex also includes two previously identified SJ components, NRX, a transmembrane protein, and COR, a membrane-associated cytoplasmic protein with a FERM domain.

The Na^+/K^+ ATPase localizes to the SJ and is required for the paracellular diffusion barrier

One of the most intriguing results of this study is the identification of the Na^+/K^+ ATPase as a functional member of the SJ. We have shown that mutations in either the α subunit (ATPα; Fig. 1 H) or β subunit (NRV2; Fig. 1 D) disrupt the paracellular barrier of the embryonic salivary gland and that this functional loss corresponds to the structural loss of septae in the junction (Fig. 3, D and E). Although the SJ is localized just basal to the adherens junction near the apical end of the cell, previous characterizations of the Na^+ pump have described it as having a basolateral localization in *Drosophila* epithelial cells (Lebovitz et al., 1989; Dubreuil et al., 1997, 2000; Baumann, 2001). We examined the localization of the Na^+/K^+ ATPase using immunofluorescence and found that both subunits are highly concentrated at the SJ in imaginal epithelia. In embryonic epithelia, the results

differed depending upon the fixation and staining method; methanol treatment resulted in staining that appeared basolateral whereas staining of embryos fixed without methanol was localized to the SJ. Observations of GFP–exon trap lines (Morin et al., 2001) enabled us to confirm that both ATPase subunits localize to the SJ in live embryos and imaginal epithelia. Our results are limited to the examination of ectodermally derived epithelia such as the embryonic epidermis, foregut, hindgut, and salivary glands; however, previous examination of the ATPase was performed in the larval (Dubreuil et al., 2000) and adult (Baumann, 2001) midgut. Interestingly, the midgut does not contain pleated SJs but rather smooth SJs, and so subcellular localization of the ATPase may be cell type dependent.

The *Nrv2* and *Nrv1* genes encode β subunits of the Na^+/K^+ ATPase that differ in their cytoplasmic tails. The *P* element insertion (*l(2)k13315*) disrupts the *Nrv2* gene product but appears to have no affect on the *Nrv1* protein (Fig. 2 C). In addition, we find that both NRV2.1 and NRV2.2 are able to rescue the dye diffusion phenotype of *l(2)k13315* whereas NRV1 is not (Fig. 1). Together these results indicate that *l(2)k13315* is a mutation in the *Nrv2* locus, and that NRV2 normally functions in the SJ. Although both NRV2 and NRV1 were previously described as being nervous system specific (Sun et al., 1998), our evidence from immunostaining and from a GFP gene trap inserted within the *Nrv2* locus indicates that *Nrv2* is highly expressed in epithelial cells. Because NRV1 expression is not affected by the *l(2)k13315* mutation (Fig. 2 C) and *l(2)k13315* homozygous mutant cells in the wing imaginal disc lack NRV staining (unpublished data), we propose that *Nrv1* is nervous system specific and epidermal cells express only NRV2.

The observation that an *Nrv1* transgene cannot rescue the *Nrv2* dye diffusion phenotype, even though it localizes to the SJ when ectopically expressed in epithelial cells, suggests that the proteins encoded by these genes, although quite similar in structure, are functionally distinct. Given the sequence diversity within the cytoplasmic tail, the observation that when expressed ectopically (Fig. 2 B) all three proteins localize to the SJ strongly suggests that this localization is mediated by the extracellular or transmembrane domain, rather than by the intracellular domain. This complex pattern of β subunit expression and functional interactions suggests a surprising degree of functional regulation of the Na^+/K^+ ATPase in epithelial and neuronal cells.

The question still remains, What is the function of localizing the Na pump to such a specialized membrane domain, one of whose functions is to create a paracellular diffusion barrier? Several characteristics of the Na pump might be important in SJ function. Previous studies suggest that the Na^+/K^+ ATPase functions in cell adhesion (Pagliusi et al., 1990), though whether its role is structural or regulatory is unclear. Other studies suggest that the Na pump could function as a scaffold on which proteins essential for the paracellular barrier are organized. For example, both subunits bind to a variety of proteins, from those involved in signal transduction to cytoskeletal elements (Lopina, 2001). In addition, it is possible that the ion pumping activity of the Na pump actively participates in the formation or maintenance of the diffusion barrier. Studies in mammalian cells have demonstrated a requirement for the ATPase, and specifically the Na^+ gradient it produces, in cell polarity, adhesion, and the formation of tight junctions (Rajasekaran et al., 2001a,b). Because the tight junction is responsible for creating the paracellular barrier in vertebrate epithelial cells, the ATPase might perform a similar function in the paracellular barrier of the *Drosophila* SJ. Further experiments, using point mutations that specifically affect the pump function of the ATPase, could address these questions.

Proteins involved in the SJ's paracellular barrier form a complex

We have shown previously that COR binds to the cytoplasmic tail of NRX (Ward et al., 1998) in the SJ. Studies of the PSJ have shown that the mammalian homologues of NRX and NRG interact via their extracellular domains (Charles et al., 2002). Together, these observations suggest the existence of a multiprotein complex at the SJ in which COR binds to NRX, which in turn binds to NRG. Our finding that NRX and NRG coimmunoprecipitate when either anti-COR or anti-NRG antibodies are used to immunoprecipitate (Fig. 6, A and B) is consistent with this model. Because *Drosophila* epithelial cells express all three proteins, we cannot rigorously distinguish whether this interaction occurs within the same cell or between adjacent cells. However, our observation that wild-type cells are unable to efficiently assemble COR and NRX at the boundary with cor^- cells suggests that intercellular interaction with the same complex on adjacent cells is required for SJ assembly (Fig. 5, Y and Z). In addition, we find that NRV coimmunoprecipitates with both COR and NRX (Fig. 6 C). We were unable to detect NRG in this complex (unpublished data), suggesting that the interaction between NRV2 and the COR–NRX complex occurs independently of NRG, perhaps on the cytoplasmic side of the membrane. Although these results imply the possibility of an interaction between COR and the cytoplasmic tail of NRV2, this seems unlikely in light of our observations that NRV1, 2.1, and 2.2 all localize to the SJ, despite having different cytoplasmic tails. Thus, it is more likely that the interaction between COR and the ATPase occurs either through NRX or the α subunit.

Somatic mosaic analysis demonstrated that this complex of COR, NRX, NRV, ATPα, and NRG can be disrupted without affecting overall polarity, or other components of the SJ. We have not identified a component essential for the paracellular barrier that is unaffected in mutant cells, suggesting that we have yet to find the substrate upon which this complex assembles. Previous studies have demonstrated that Ankyrin binds both the cytoplasmic domain of NRG (Davis and Bennett, 1994; Dubreuil et al., 1996) and, as has been described in mammalian cells, the α subunit of NA^+/K^+ ATPase (Nelson and Veshnock, 1987). In addition, Ankyrin colocalizes with NRG at points of NRG-induced S2 cell adhesion complexes (Dubreuil et al., 1996). Thus, one candidate for a substrate upon which this complex assembles is Ankyrin, a well-known member of the membrane skeleton.

Other candidate proteins for this scaffold are Scribble and DLG. Both of these proteins are required early in *Drosophila* development for the establishment of epithelial cell polarity

and growth control (Woods et al., 1996; Bilder et al., 2000). If either is absent from epithelial cells, then the apical junctional complexes do not properly form and epithelial integrity is lost. Thus, Scribble and DLG may be among the first constituents of the SJ upon which the subsequently expressed SJ proteins assemble.

Is there a role for the SJ in signal transduction?

Previous studies have suggested that the SJ may function in intercellular signaling, particularly in the regulation of cell proliferation. For example, *dlg*, which encodes a PDZ repeat–containing, membrane-associated guanylate kinase protein, has tumor suppressor functions (Woods et al., 1996). Loss of function *dlg* mutations are characterized by disruption of apical–basal polarity and an overproliferation of the larval imaginal discs. However, it is not known whether this overproliferation is due to a direct involvement of DLG in a signal transduction cascade or to the disruption of apical–basal polarity within epithelial cells that could result in a disruption of apical signaling complexes. In addition to *dlg*, *cor* mutations were first isolated as dominant suppressors of a gain of function allele of the EGF receptor, $Egfr^{Elp}$ (also known as $Egfr^{E3}$), suggesting that COR may function to positively regulate EGFR pathway function. Interestingly, a recent study of NRG function in the developing *Drosophila* nervous system has proposed that it positively regulates EGF receptor function during axon guidance (Garcia-Alonso et al., 2000). The role of NRG in regulating EGFR function in epithelial cells has not been investigated, but our preliminary results indicate that *Nrg* mutations also dominantly suppress the rough eye caused by $Egfr^{Elp}$ (unpublished data). This result may suggest that NRG (or the entire complex) must be localized to the SJ in epithelial cells to regulate EGFR function. Alternatively, it is possible that the SJ complex is necessary to maintain polarized localization of the EGFR to the apical membrane (Fehon et al., 1994), though we have not observed an effect of *cor* mutations on EGFR localization (unpublished data).

The relationship between epithelial and paranodal SJs

The recent discovery of molecular, structural, and functional similarities between the invertebrate epithelial SJ and the vertebrate PSJ in the nervous system (Einheber et al., 1997; Tepass et al., 2001) gives added significance to the identification of new SJ components in *Drosophila*. In addition to COR/protein 4.1 and NRX/paranodin, we have shown that the SJ and PSJ share neurofascin-155 and a *Drosophila* homologue, NRG. This level of molecular homology strongly suggests that these two SJs are structurally and functionally homologous as well. It is therefore somewhat surprising that published reports (Ariyasu and Ellisman, 1987; Gerbi et al., 1999) indicate that the Na pump is uniformly distributed along the axonal membrane rather than being restricted to the PSJ. One possible explanation is that only a subset of the several Na^+/K^+ ATPase isoforms found in the mammalian genome is localized to the PSJ, and that these isoforms have not yet been studied. Similarly, it is not known if the mammalian homologues of *Drosophila* GLI, the neuroligins, might localize to the PSJ, or if the *Drosophila* homologue of contactin (FlyBase, 1996), a protein that interacts with NRX/paranodin (Einheber et al., 1997), localizes to the SJ. Although it is possible that the invertebrate epithelial SJ and vertebrate PSJ are fundamentally different in some respects, we feel that this is unlikely given the remarkable degree of similarity between these two junctions. In any case, it is clear that the genetic and genomic tools available in *Drosophila* can provide important insights into both the SJ and its vertebrate counterpart, the PSJ.

Materials and methods

Diffusion barrier permeability assay

Fluorescently labeled dextran was injected into stage 14 and older embryos as described previously (Lamb et al., 1998).

Immunofluorescence

Wing discs were dissected, fixed, and stained as described previously (Fehon et al., 1991). Primary antibodies were used at the following concentrations: anti-COR, 1:10,000; anti-DLG, 1:500; anti-NRX (provided by M. Bhat, Mount Sinai School of Medicine, New York, NY), 1:20,000; anti-NRV (mAb 5F7; provided by P. Salvaterra, City of Hope, Duarte, CA), 1:10,000; anti–ATPα (α5; provided by D. Fambrough, Developmental Studies Hybridoma Bank, Iowa City, IA), 1:200; anti-NRG (mAb 1B7; provided by M. Hortsch, University of Michigan, Ann Arbor, MI), 1:200; anti-Flag (mAb M2; Sigma-Aldrich), 1:20,000; anti-DLT (provided by M. Bhat), 1:20,000; anti-FASIII (provided by C. Goodman, University of California, Berkeley, CA), 1:1,000; anti-DCAD2 (provided by T. Uemura, Kyoto University, Kyoto, Japan), 1:500. Secondary antibodies (Jackson ImmunoResearch Laboratories) were used at 1:1,000. Confocal microscopy was performed either on an LSM 410 or LSM 510 system (Carl Zeiss MicroImaging, Inc.).

Transmission electron microscopy

Mutant embryos were genotyped using a GFP-labeled balancer chromosome. Embryos aged 20–22 h at 25°C were fixed using a previously described protocol (Prokop et al., 1996) with the exception that the primary fixative was injected into the body cavity of the embryos. Fixed embryos were embedded in eponate–araldite resin (Ted Pella, Inc.), sectioned on a Reichter-Jung Ultracut microtome, and analyzed on a Carl Zeiss MicroImaging, Inc. EM 10A electron microscope. At least five embryos of each genotype and at least 10 epidermal intercellular junctions per embryo were examined in detail. Negatives were scanned using a Sharp JX-320 scanner and Adobe Photoshop®.

Clonal analysis

To generate clones in the wing imaginal disc, the following three crosses were made: (1) *nrg^{14} P{ry$^{+t7.2}$=neoFRT}18A/FM6, w* virgin females mated to *y^1 w^{1118}, P{w^{+mC}=Ubi-GFP.nls}X P{ry$^{+t7.2}$=neoFRT}18A; P{ry$^{+t7.2}$=hsFLP}, Sb*/TM6, Tb* males, (2) *w^{1118}; $Nrv2^{k13315}$ P{ry$^{+t7.2}$=neoFRT}40A/CyO, P{w^{+mC}=ActGFP}JMR1* virgin females mated to *y^1 w*; P{w^{+mC}=Ubi-GFP}33 P{w^{+mC}=Ubi-GFP}38 P{ry$^{+t7.2}$=neoFRT}40A; P{ry$^{+t7.2}$=hsFLP}, Sb*/TM6, Tb* males, and (3) *y w; P{ry+7.2=neoFRT}43D cor^5/CyO, P{w^{+mC}=ActGFP}JMR1* virgin females mated to *w^{1118}; P{ry$^{+t7.2}$= neoFRT}43D P{w^{+mC}=piM}46F P{w^{+mC}=piM}47F; P{ry$^{+t7.2}$=hsFLP}, Sb*/TM6, Tb* males. The larvae resulting from these crosses were heat shocked at 72 ± 2 h after egg laying (at 25°C) using two 45-min heat shocks at 37°C, separated by 45 min at 25°C. Late third instar larvae were then dissected, fixed, and stained as described above.

Immunoprecipitation and immunoblot analysis

Embryos were aged to be 18–22 h old at 25°C, and from this point in the procedure, all steps were performed at 4°C. Embryos were washed three times in homogenization buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 0.01% BSA, protease inhibitor cocktail, Complete Mini [Roche], final solution pH is 7.6) and homogenized in 10× volume lysis buffer. The lysate was pulled through a 27-gauge needle five times and then centrifuged at 16,000 *g* at 4°C for 15 min. After removing the upper lipid layer, the supernatant was incubated for 3 h with protein A– or protein G–conjugated sepharose beads (Zymed Laboratories) that had been preabsorbed overnight with the precipitating antibody. The preabsorption solution was 1 ml 1× PBS, 30 μl sepharose beads (protein A conjugated for guinea pig anti-COR; protein G conjugated for mouse anti-NRG and mouse anti-NRV), and immunoprecipitat-

ing antibodies were added at the following concentrations: guinea pig anti-COR (1:250), mouse anti-NRG (mAb 1B7; 1:5), and mouse anti-NRV (mAb 5F7; 1:100). After incubation, the protein complex was precipitated at 6,000 *g* for 30 s, washed four times in lysis buffer, and washed for the last time in 1× PBS. The complex was then resuspended in 1× SDS sample buffer/1× PBS, boiled, and separated on a 3–15% SDS-PAGE gel followed by transfer to nitrocellulose.

Immunoblotting was performed using the Odyssey Infrared Imaging System and protocols (LI-COR). Primary antibodies were used at the following concentrations: guinea pig anti-COR, 1:20,000; rabbit anti-NRX (provided by M. Bhat), 1:20,000; mouse anti-NRG (mAb 3C10; provided by N. Patel, University of Chicago, Chicago, IL), 1:100. Secondary antibodies were used at the following concentrations: goat anti–mouse Alexa®680 (LI-COR), 1:5,000; goat anti–rabbit Alexa®680 (LI-COR), 1:5,000; and donkey anti–guinea pig CY5 (Jackson ImmunoResearch Laboratories), 1:500. Image files were exported from the Odyssey software in TIFF format.

Image preparation

Images acquired using Odyssey or Carl Zeiss MicroImaging, Inc. software were imported into Adobe Photoshop® or Improvision Volocity LE for cropping and contrast adjustment before being assembled into figures using Microsoft Powerpoint.

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References

Ariyasu, R.G., and M.H. Ellisman. 1987. The distribution of (Na+/K+)ATPase is continuous along the axolemma of unensheathed axons from spinal roots of 'dystrophic' mice. *J. Neurocytol.* 16:239–248.

Arroyo, E.J., and S.S. Scherer. 2000. On the molecular architecture of myelinated fibers. *Histochem. Cell Biol.* 113:1–18.

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

Baumann, O. 2001. Posterior midgut epithelial cells differ in their organization of the membrane skeleton from other *Drosophila* epithelia. *Exp. Cell Res.* 270: 176–187.

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

Bhat, M.A., S. Izaddoost, Y. Lu, K.O. Cho, K.W. Choi, and H.J. Bellen. 1999. Discs Lost, a novel multi-PDZ domain protein, establishes and maintains epithelial polarity. *Cell.* 96:833–845.

Bieber, A.J., P.M. Snow, M. Hortsch, N.H. Patel, J.R. Jacobs, Z.R. Traquina, J. Schilling, and C.S. Goodman. 1989. *Drosophila* neuroglian: a member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1. *Cell.* 59:447–460.

Bilder, D., M. Li, and N. Perrimon. 2000. Cooperative regulation of cell polarity and growth by *Drosophila* tumor suppressors. *Science.* 289:113–116.

Carlson, S.D., J.L. Juang, S.L. Hilgers, and M.B. Garment. 2000. Blood barriers of the insect. *Annu. Rev. Entomol.* 45:151–174.

Charles, P., S. Tait, C. Faivre-Sarrailh, G. Barbin, F. Gunn-Moore, N. Denisenko-Nehrbass, A.M. Guennoc, J.A. Girault, P.J. Brophy, and C. Lubetzki. 2002. Neurofascin is a glial receptor for the paranodin/Caspr-contactin axonal complex at the axoglial junction. *Curr. Biol.* 12:217–220.

Davis, J.Q., and V. Bennett. 1994. Ankyrin binding activity shared by the neurofascin/L1/NrCAM family of nervous system cell adhesion molecules. *J. Biol. Chem.* 269:27163–27166.

Dubreuil, R.R., G. MacVicar, S. Dissanayake, C. Liu, D. Homer, and M. Hortsch. 1996. Neuroglian-mediated cell adhesion induces assembly of the membrane skeleton at cell contact sites. *J. Cell Biol.* 133:647–655.

Dubreuil, R.R., P.B. Maddux, T.A. Grushko, and G.R. MacVicar. 1997. Segregation of two spectrin isoforms: polarized membrane-binding sites direct polarized membrane skeleton assembly. *Mol. Biol. Cell.* 8:1933–1942.

Dubreuil, R.R., P. Wang, S. Dahl, J. Lee, and L.S. Goldstein. 2000. *Drosophila* β-spectrin functions independently of α-spectrin to polarize the Na,K ATPase in epithelial cells. *J. Cell Biol.* 149:647–656.

Einheber, S., G. Zanazzi, W. Ching, S. Scherer, T.A. Milner, E. Peles, and J.L. Salzer. 1997. The axonal membrane protein Caspr, a homologue of Neurexin IV, is a component of the septate-like paranodal junctions that assemble during myelination. *J. Cell Biol.* 139:1495–1506.

Fasano, A. 2000. Regulation of intercellular tight junctions by zonula occludens toxin and its eukaryotic analogue zonulin. *Ann. NY Acad. Sci.* 915:214–222.

Fehon, R.G., K. Johansen, I. Rebay, and S. Artavanis-Tsakonas. 1991. Complex cellular and subcellular regulation of Notch expression during embryonic and imaginal development of *Drosophila*: implications for Notch function. *J. Cell Biol.* 113:657–669.

Fehon, R.G., I.A. Dawson, and S. Artavanis-Tsakonas. 1994. A *Drosophila* homologue of membrane-skeleton protein 4.1 is associated with septate junctions and is encoded by the *coracle* gene. *Development.* 120:545–557.

Feng, Y., L. Huynh, K. Takeyasu, and D.M. Fambrough. 1997. The *Drosophila* Na,K-ATPase α-subunit gene: gene structure, promoter function and analysis of a cold-sensitive recessive-lethal mutation. *Genes Funct.* 1:99–117.

Firth, J.A. 2002. Endothelial barriers: from hypothetical pores to membrane proteins. *J. Anat.* 200:541–548.

FlyBase. 1996. FlyBase: the *Drosophila* database. *Nucleic Acids Res.* 24:53–56.

Garcia-Alonso, L., S. Romani, and F. Jimenez. 2000. The EGF and FGF receptors mediate neuroglian function to control growth cone decisions during sensory axon guidance in *Drosophila*. *Neuron.* 28:741–752.

Gerbi, A., S. Sennoune, S. Pierre, J. Sampol, D. Raccah, P. Vague, and J.M. Maixent. 1999. Localization of Na,K-ATPase α/β isoforms in rat sciatic nerves: effect of diabetes and fish oil treatment. *J. Neurochem.* 73:719–726.

Gilbert, M., J. Smith, A.J. Roskams, and V.J. Auld. 2001. Neuroligin 3 is a vertebrate gliotactin expressed in the olfactory ensheathing glia, a growth-promoting class of macroglia. *Glia.* 34:151–164.

Huber, J.D., R.D. Egleton, and T.P. Davis. 2001. Molecular physiology and pathophysiology of tight junctions in the blood-brain barrier. *Trends Neurosci.* 24:719–725.

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

Lawrence, P.A., R. Bodmer, and J.P. Vincent. 1995. Segmental patterning of heart precursors in *Drosophila*. *Development.* 121:4303–4308.

Lebovitz, R.M., K. Takeyasu, and D.M. Fambrough. 1989. Molecular characterization and expression of the (Na+/K+)-ATPase α-subunit in *Drosophila melanogaster*. *EMBO J.* 8:193–202.

Lopina, O.D. 2001. Interaction of Na,K-ATPase catalytic subunit with cellular proteins and other endogenous regulators. *Biochemistry.* 66:1122–1131.

Marfatia, S.M., R.A. Lue, D. Branton, and A.H. Chishti. 1994. In vitro binding studies suggest a membrane-associated complex between erythroid p55, protein 4.1, and glycophorin C. *J. Biol. Chem.* 269:8631–8634.

Marfatia, S.M., R.A. Leu, D. Branton, and A.H. Chishti. 1995. Identification of the protein 4.1 binding interface on glycophorin C and p55, a homologue of the *Drosophila* discs-large tumor suppressor protein. *J. Biol. Chem.* 270:715–719.

Menegoz, M., P. Gaspar, M. Le Bert, T. Galvez, F. Burgaya, C. Palfrey, P. Ezan, F. Arnos, and J.A. Girault. 1997. Paranodin, a glycoprotein of neuronal paranodal membranes. *Neuron.* 19:319–331.

Morin, X., R. Daneman, M. Zavortink, and W. Chia. 2001. A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in *Drosophila*. *Proc. Natl. Acad. Sci. USA.* 98:15050–15055.

Nelson, W.J., and P.J. Veshnock. 1987. Ankyrin binding to (Na+/K+)ATPase and implications for the organization of membrane domains in polarized cells. *Nature.* 328:533–536.

Oda, H., T. Uemura, Y. Harada, Y. Iwai, and M. Takeichi. 1994. A *Drosophila* homolog of cadherin associated with armadillo and essential for embryonic cell-cell adhesion. *Dev. Biol.* 165:716–726.

Pagliusi, S.R., M. Schachner, P.H. Seeburg, and B.D. Shivers. 1990. The adhesion molecule on glia (AMOG) is widely expressed by astrocytes in developing and adult mouse brain. *Eur. J. Neurosci.* 2:471–480.

Patel, N.H., P.M. Snow, and C.S. Goodman. 1987. Characterization and cloning

of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. *Cell.* 48:975–988.

Prokop, A., M. Landgraf, E. Rushton, K. Broadie, and M. Bate. 1996. Presynaptic development at the *Drosophila* neuromuscular junction: assembly and localization of presynaptic active zones. *Neuron.* 17:617–626.

Rajasekaran, S.A., L.G. Palmer, S.Y. Moon, A. Peralta Soler, G.L. Apodaca, J.F. Harper, Y. Zheng, and A.K. Rajasekaran. 2001a. Na,K-ATPase activity is required for formation of tight junctions, desmosomes, and induction of polarity in epithelial cells. *Mol. Biol. Cell.* 12:3717–3732.

Rajasekaran, S.A., L.G. Palmer, K. Quan, J.F. Harper, W.J. Ball, Jr., N.H. Bander, A.P. Soler, and A.K. Rajasekaran. 2001b. Na,K-ATPase β-subunit is required for epithelial polarization, suppression of invasion, and cell motility. *Mol. Biol. Cell.* 12:279–295.

Salzer, J.L. 1997. Clustering sodium channels at the node of Ranvier: close encounters of the axon-glia kind. *Neuron.* 18:843–846.

Spradling, A.C., D. Stern, A. Beaton, E.J. Rhem, T. Laverty, N. Mozden, S. Misra, and G.M. Rubin. 1999. The Berkeley Drosophila Genome Project gene disruption project: single P-element insertions mutating 25% of vital *Drosophila* genes. *Genetics.* 153:135–177.

Sun, B., and P.M. Salvaterra. 1995a. Characterization of nervana, a *Drosophila* melanogaster neuron-specific glycoprotein antigen recognized by anti-horseradish peroxidase antibodies. *J. Neurochem.* 65:434–443.

Sun, B., and P.M. Salvaterra. 1995b. Two *Drosophila* nervous system antigens, Nervana 1 and 2, are homologous to the β subunit of Na^+,K^+ ATPase. *Proc. Natl. Acad. Sci. USA.* 92:5396–5400.

Sun, B., W. Wang, and P.M. Salvaterra. 1998. Functional analysis and tissue-specific expression of *Drosophila* Na^+,K^+ ATPase subunits. *J. Neurochem.* 71: 142–151.

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

Tepass, U., G. Tanentzapf, R. Ward, and R. Fehon. 2001. Epithelial cell polarity and cell junctions in *Drosophila*. *Annu. Rev. Genet.* 35:747–784.

Tsukita, S., M. Furuse, and M. Itoh. 2001. Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell Biol.* 2:285–293.

Ward, R.E., R.S. Lamb, and R.G. Fehon. 1998. A conserved functional domain of *Drosophila coracle* is required for localization at the septate junction and has membrane-organizing activity. *J. Cell Biol.* 140:1463–1473.

Ward, R.E., L. Schweizer, R.S. Lamb, and R.G. Fehon. 2001. The protein 4.1, ezrin, radixin, moesin (FERM) domain of *Drosophila* Coracle, a cytoplasmic component of the septate junction, provides functions essential for embryonic development and imaginal cell proliferation. *Genetics.* 159:219–228.

Woods, D.F., C. Hough, D. Peel, G. Callaini, and P.J. Bryant. 1996. Dlg protein is required for junction structure, cell polarity, and proliferation control in *Drosophila* epithelia. *J. Cell Biol.* 134:1469–1482.

Xu, P., B. Sun, and P.M. Salvaterra. 1999. Organization and transcriptional regulation of *Drosophila* Na(+), K(+)- ATPase β subunit genes: Nrv1 and Nrv2. *Gene.* 236:303–313.