Chapter 11

[AU1]

Claudin Family Proteins in *Caenorhabditis elegans*

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Abstract

In the last decade, the claudin family of integral membrane proteins has been identified as the major protein component of the tight junctions in all vertebrates. The claudin superfamily proteins also function to regulate channel activity, intercellular signaling, and cell morphology. Subsequently, claudin homologues have been identified in invertebrates, including *Drosophila* and *Caenorhabditis elegans*. Recent studies demonstrate that the *C. elegans* claudins, *clc-1* to *clc-5*, and similar proteins in the greater PMP22/EMP/claudin/calcium channel γ subunit family, including *nsy-1-nsy-4* and *vab-9*, while highly divergent at a sequence level from each other and from the vertebrate claudins, in some cases play roles similar to those traditionally assigned to their vertebrate homologues. These include regulating cell adhesion and passage of small molecules through the paracellular space. The claudin superfamily proteins also function to regulate channel activity, intercellular signaling, and cell morphology. Study of claudin superfamily proteins in *C. elegans* should continue to provide clues as to how core claudin protein function can be modified to serve various specific roles at regions of cell–cell contact in metazoans.

Key words: Claudin, Junctions, *Caenorhabditis elegans*, Epithelia, Morphogenesis, Actomyosin, Neuronal symmetry, VAB-9, CLC-1, NSY-4

1. Introduction: Claudins Regulate Barrier Functions in Vertebrate Epithelia

Epithelia function as regulated barriers in tissue and organ architecture, defining compartment boundaries within organisms and forming boundaries apposed to the external environment. The proper physiological state of epithelia depend on cellular contacts, or junctions, that mediate cell–cell adhesion, function as selective gates for the ingress or egress of specific ions and small molecules, and maintain unique membrane identities on apical and basal sides of the cellular layer, thus contributing to epithelial polarity. The tight junction is the most apical of cellular junctions in vertebrate epithelia. Freeze fracture TEM reveals one of the most striking features of tight junctions, a network of intertwined

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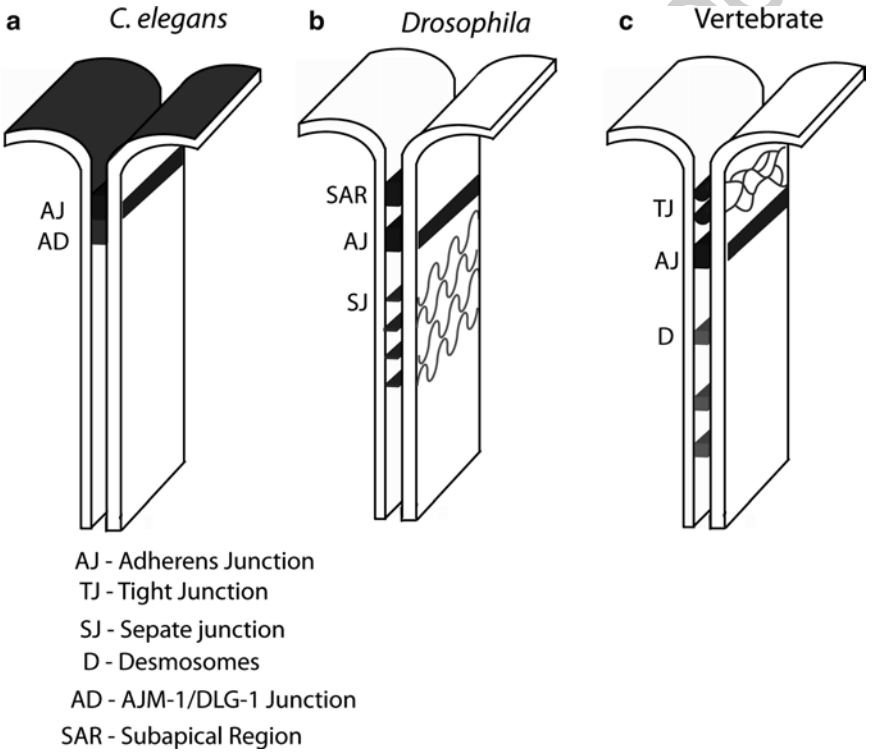
strands that occlude the intercellular or paracellular space at points of contact that often circumscribe cells. In this way, tight junction strands function as gaskets that separate and isolate the two sides of an epithelium (Fig. 1). The major protein component of the tight junction has been identified in the last decade as the claudin family of proteins. Claudins were originally identified in a search for the protein component of tight junction strands in vertebrate epithelia following a painstaking purification scheme (1, 2). Claudins 1 and 2 were shown to localize to tight junctions, to generate tight junction strands when expressed in fibroblasts, and to mediate homotypic and heterotypic cell–cell adhesion (1, 2). Consistent with these findings, loss of claudin activity results in paracellular barrier dysfunction (3–8). Overall tissue polarity does not appear to be heavily dependent on claudin activity,



[AU2] Fig. 1. Schematic of various cell junction arrangements from *C. elegans*, *Drosophila*, and vertebrates. Corresponding cell junction regions either in relative location, function, and/or molecular make-up are indicated with similar colors. The SAR-like region in *C. elegans,* the subapical region (SAR) in *Drosophila*, and tight junction in vertebrates, respectively, are indicated with *black shading*. *AJ* indicates the cadherin-based adherens junction. *SJ* identifies the septate junction in *Drosophila*, and *AD* identifies the AJM-1/ DLG-1 region in *C. elegans*. Vertebrates have no similar structure in epithelia, but share a molecularly similar barrier at the paranodal junction (see text). Vertebrate desmosomes are shown (eral views (*cutaways*) show the nature of the junctional structures in the membranes. Adherens junctions appear as solid bands in the membrane, Pleated S e junctions are characterized by regular wave-like strands, and tight junctions appear as irregular but connected anastomosing strands. For simplicity, only the composition of cell junction components in the paracellular space is shown.

since gross cell polarity is maintained following the loss of claudin function (6). The vertebrate claudin family has expanded over the years to include about 24 members all of which are predicted four-pass integral membrane proteins with cytoplasmic N and C termini, two extracellular loops, and one short intracellular loop (9–11).

Claudins have been shown to form oligomers in vitro, with hexamers being favored (12–14). This organization suggests that claudins, like gap junctions, might generate pores through the lipid bilayer, although there is no clear evidence at present that this is the case. Rather, claudins appear to establish charged pores *within* the paracellular space that regulate the traffic of charged ions across the epithelial barrier. Charged residues in the extracellular domain are responsible for regulating charge selectivity. As a result, claudins with different charged amino acids can, by virtue of their expression in overlapping and nonoverlapping patterns, define the paracellular charge selectivity of a wide variety of tissues (15). For example, in the vertebrate kidney, different claudins are expressed along the length of the nephron, suggesting that the differential expression correlates and may help generate that different charge selectivity among the distinct regions of the nephron (16). Supporting this hypothesis, claudin 16 maintains a negative charge required for the reuptake of Mg^{2+} and Ca^{2+} ions in the thick ascending part of Henle's loop in the nephron and loss of claudin 16 activity results in magnesium and calcium wasting (17). Similarly, in the longitudinal axis of the intestine as well as the crypt to villus axis of the intestine, the claudins are expressed in complex patterns (18). Charge selectivity of claudins is most dramatically illustrated by experiments in which the charge of the extracellular loops is reversed by swapping amino acids in this loop; in this case, the charge selectivity is reversed, as was demonstrated for claudins 10 and 15 (19, 20). In general, amino acid charge in extracellular loop 1 dramatically influences paracellular charge selectivity.

Claudins are also targets of viruses, and binding of viruses to extracellular claudin loops can disrupt epithelial barrier, open the paracellular space, and allow viral entry (21–23). The claudin C terminus functions in trafficking of the claudin protein, binding to junctional plaque proteins through PDZ domain binding motifs, or regulation of the paracellular space through phosphorylation (24–29). While the PDZ-binding peptide is found in most claudins, this domain is absent from many of the C termini in related protein families of both vertebrates and invertebrates, suggesting that there may be as many functions as there are unique C-terminal domains.

While much is now known about the function of vertebrate claudins, less is known about similar molecules in invertebrates. Since tight junction structures in invertebrates are uncommon,

claudin-like molecules in invertebrates will necessarily have functions distinct from those of their vertebrate relatives (30). This review will detail the known functions of this class of proteins in *Caenorhabditis elegans*.

2. Sequence Conservation Among Claudin Family Proteins

The sequence similarity of *C. elegans* and vertebrate claudin family proteins so low that it was proposed that sequence similarity was insufficient to establish homology or that claudins are present only in chordates (9, 31). Despite these initial assessments, claudin family proteins were ultimately identified in *C. elegans* by sequence homology searches and also through the identification of claudin-related proteins participating in diverse processes. Here we will review what is known about *vab-9*, *nsy-4*, *clc-1–5*, and related claudin superfamily genes. The comparison of *C. elegans* sequences with each other and with claudin family proteins from vertebrates indicates that the sequences are indeed highly divergent (Fig. 2). A similar observation was made when *Drosophila* claudins, including *Sinuous* and *Megatrachea*, were placed into a claudin family phylogenetic tree (32). A previous analysis of *Stargazin* indicated that there were at least three distinct clades within this greater tetraspan family: PMP22/EMP, claudin, and the gamma subunits of voltage-gated calcium channels (33). Generally, PMP22/EMP/MP/Claudin-related proteins belong to pfam00822 (15). Analysis of these protein groupings, in conjunction with VAB-9 and VAB-9 orthologs from *Drosophila* and vertebrates, indicates that a separate VAB-9 clade exists, which is distinct from other subgroups (Fig. 2). Previously PERP, a tetraspan protein required for desmosomal structure and epithelial integrity, was recognized as the closest ortholog to *TM4SF10* (or *TMEM47*) in the mouse (34, 35) (JSS and L. Attardi, unpublished observations). In our phylogenetic analysis, PERP associated with the VAB-9 clade (Fig. 2). Despite being closer to VAB-9 in homology, expression of mouse *PERP* in *C. elegans* epithelia is incapable of rescuing *vab-9* phenotypes, while similar expression of *TM4SF10* rescues *vab-9* (36) (JSS and L. Attardi, unpublished observations). Interestingly, NSY-4 clustered with *C. elegans* claudins, whereas some vertebrate gamma subunits clustered separately. Bootstrap analysis shows that the frequency of particular association nodes for *C. elegans* claudins are typically low, highlighting the diversity of this protein family.

Previously, a consistent motif was found in the first extracellular loop of the claudin superfamily of proteins. Very roughly, this consensus corresponds to W(8 or >X)GLWXXC(8-10X)C (Fig. 3).

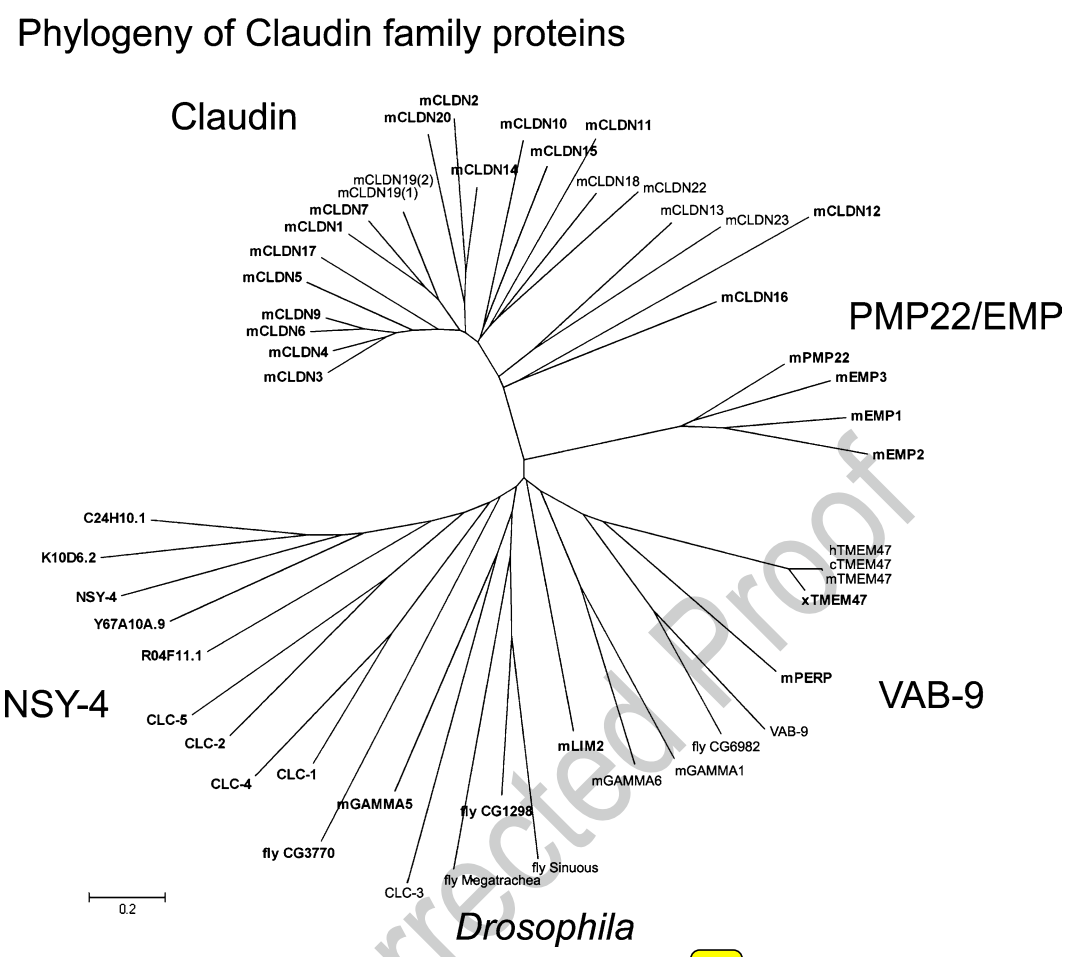
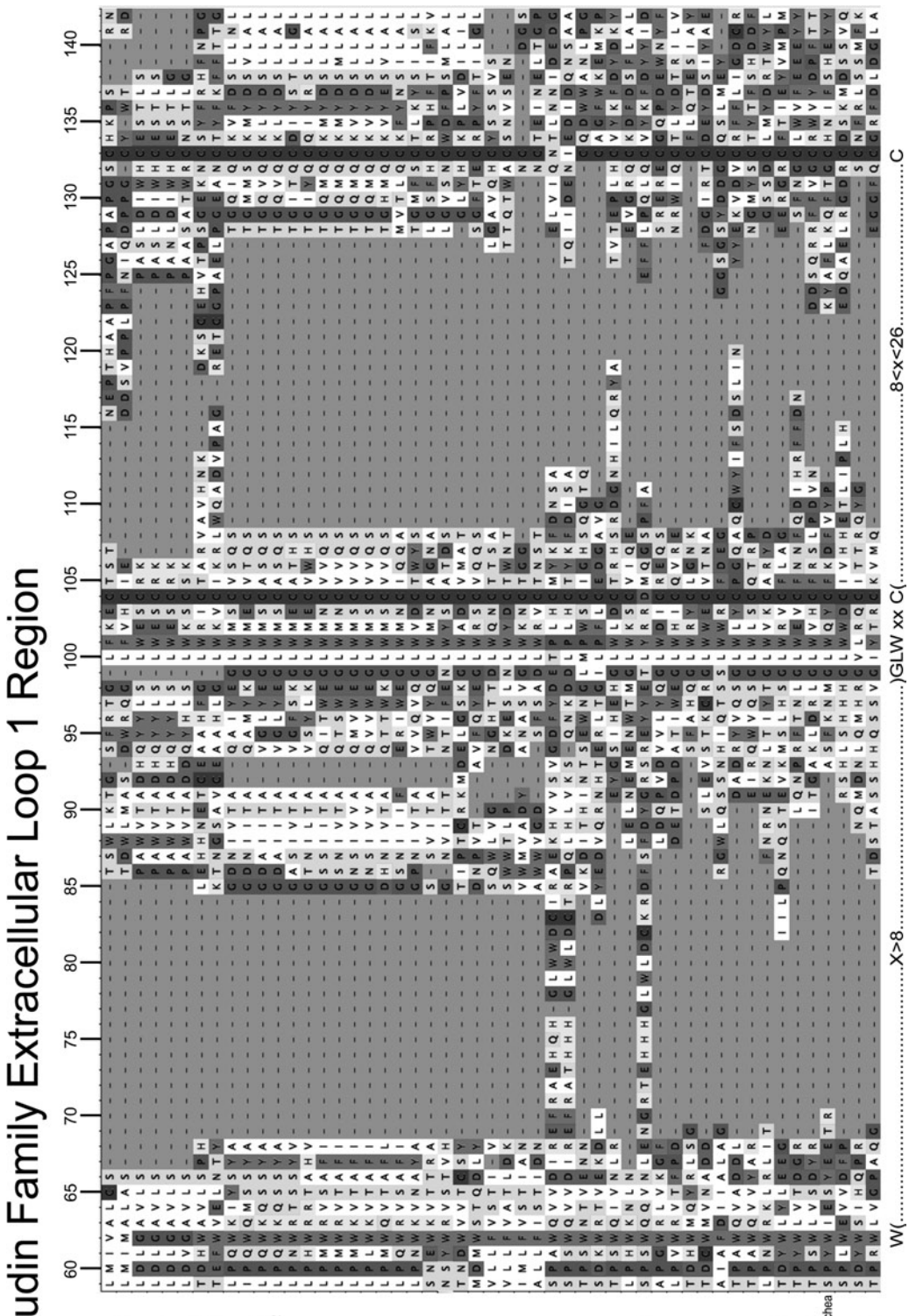


Fig. 2. Claudin family proteins loosely cluster in highly divergent clades. The Phylogenetic tree resulting from ClustalW2 analysis. Several different subgroup clades are observed. Phylogeny was constructed using the MEGA (Molecular Evolutionary Genetic Analysis) software program available at http://www.megasoftware.net (115). Units are in number of amino acid substitutions per site.

Direct sequence comparison shows that VAB-9 is not entirely conserved in this region, since VAB-9 lacks the highly conserved tryptophan in the GLW tripeptide. Nevertheless, the overall topology of VAB-9, including many of the residues in the motif, suggests that it may share structural features with other members of the claudin superfamily. Other proteins from *C. elegans* tend to lack one or more key residues but retain a broad similarity. Whether this similarity translates to a conserved structure will require functional tests. Since the claudin family has undergone extensive divergence, the study of claudin-like proteins in *C. elegans* and other invertebrates is likely to reveal both the very basic essential functions of the claudin family of proteins as well as how different members of this divergent family are adapted to unique functions.



3. Septate Versus Tight Junctions: Cell Junctions in *C. elegans*, *Drosophila*, and Vertebrates

While cadherin-based junctions appear to be present in epithelia all throughout the animal kingdom, the position and morphology of occluding junctions vary among metazoans. The most apparent distinction is the location and structure of cell junctions (Fig. 1). In vertebrates, the tight junction is apical to the adherens junction, while invertebrate junctions typically have no significant occluding junction apical to the adherens junction. Although some lower chordates and invertebrates have tight junction structures, most invertebrate epithelia have instead a different junctional structure, the septate junction, localized basal to the adherens junction (37–39). Septate junctions consist of at least two types: pleated septate junctions, which are characterized by ladder-like bridges, and smooth septate junctions, in which bridges are not detected using electron microscopy (30, 40). Pleated septate junctions are often distributed all along the paracellular space, from just basal to the adherens junction to the basolateral surface (41). The separation across the paracellular space at the septate junction is about 10–20 nm and there are no "kissing points," as in vertebrate tight junctions, where there is effectively no paracellular separation and opposing membranes appear to directly touch (42). The actual paracellular space-spanning component of the pleated septate junction is currently unknown, although it is likely to be a protein or protein–carbohydrate complex, much like other cell junctions. Despite the continuous pleats in septate junctions, freeze fracture TEM of insect and *Ascaris* epithelia typically reveals isolated contacting puncta, rather than the continuous strands of vertebrates tight junctions (43, 44). Despite the freeze fracture appearance of a

Fig. 3. Claudin family extracellular loop 1 region. A short stretch of homology is shown between all family members in the region of the first extracellular loop. The first loop includes a consensus W(x>8)GLWxxC(8-26x)C motif. The two cysteines may form an intramolecular disulfide bridge important for claudin extracellular structure. Aligned sequence file is displayed using Cinema v1.4.5, Advanced Interfaces Group. Accession numbers for proteins included in the alignments are: NSY-4 (NP_500189.4), K10D6.2(NP_505843), R04F11.1 (NP_506087), C24H10.1(NP_508863), Y67A10A.9(NP_502746.1), mGamma5 (voltage-dependent calcium channel gamma subunit 5) (NP_542375), mGamma1 (NP_031608), mGamma6 (NP_573446.1), mGamma3 (NP_062303), CLC-1(NP_509847), CLC-2(NP_509257), CLC-3 isoform a(NP_001024993), CLC-4 (NP_509800), CLC-5(NP_509258), VAB-9 (NP_495836), CG6982 (dVAB-9) (NP_001097876), mTMEM47(NP_620090), cTMEM47 (NP_001003045.1), hTm47 (NP_113630.1), xtmem47 (NP_001085134.1), mclaudin1 (NP_057883), mclaudin2 (NP_057884), mclaudin3 (NP_034032), mclaudin4 (NP_034033), mclaudin5 (NP_038833), mclaudin6 (NP_0247), mclaudin7 (NP_058583), mclaudin9 (NP_064689), mclaudin10 (a) (NP_076367), mclaudin11 (NP_032796), mclaudin12 (NP_075028), mclaudin13 (NP_065250), mclaudin14 (NP_001159398), mclaudin15 (NP_068365), mclaudin16 (NP_444471), claudin17 (NP_852467), mclaudin18(NP_062789), mclaudin19(1) (NP_001033679), Claudin19(2)(NP_694745), mclaudin20 (NP_001095030), mclaudin22 (NP_083659), mclaudin23 (NP_082274), fly_Sinuous (a) (NP_647971), fly_Megatrachea (NP_726742), fly_CG3770 (NP_611985.), fly_CG1298 (NP_610179), mEMP2 (NP_031955), mPMP22 (NP_032911), mEMP3(NP_001139818), mEMP1(NP_034258), mPERP(NP_071315), LIM2 (NP_808361).

more porous and incomplete barrier, the septate junction structure still fulfills the function of a barrier, since the loss of proteins that localize to the septate junction in *Drosophila*, including claudin-like proteins *Megatrachea*, and *Sinuous*, result in the disruption of junctional structure, epithelial cell adhesion, and the paracellular barrier gate function, based on Dextran-TRITC dye exclusion assays (32, 45–47).

More recently, molecular analysis of tight and septate junction components underscores the molecular differences between TJs and SJs, and suggests that the SJ may be more like the vertebrate lateral cell membrane than a vertebrate TJ. The vertebrate tight junction protein complexes containing PAR-3/PAR-6/aPKC/CDC-42 or CRB3/Pals1/PATJ have homologues in *Drosophila* that localize to the subapical region (also known as the apical marginal zone), a region *apical* to the adherens junction (48). In contrast, a distinct complex, including the MAGUKs Varicose and Dlg, the Erm protein Coracle, Neurexin IV, and other proteins localizes basal to the adherens junction at the SJ (46, 47, 49–52). Thus, although tight junctions and septate junctions share some common functions, they are not analogous structures at the structural and molecular levels.

Although there is a clear divergence in deployment of proteins to occluding junctions in vertebrates and invertebrates, recent evidence does suggest a conserved molecular complex in vertebrates that corresponds to the invertebrate SJ. Many of the protein components of the epithelial septate junction in flies, including the claudins *Sinuous* and *Megatrachea*, also localize to and are required for maintenance of the septate junction between glial and neuronal cells of the chordotonal sensory organ structure (46, 53–56). The chordotonal organ is analogous to the paranodal junction in vertebrates, and analogous cell junctions and similar proteins in both effectively establish the blood brain barrier (57, 58). For example, claudin-5 contributes to the blood brain barrier in the mouse brain (3, 4). The designation of analogous junctional structures from diverse organisms has been reexamined based on more recent and comprehensive structural, molecular, and functional studies of various cellular junctions. As more is learned about these complex structures, appreciation for their unique, specialized functions increases.

4. Function of Junctional Proteins in *C. elegans*: Variations on an Ecdysozoan Theme

Phylogenetically, nematodes are grouped with other ecdysozoans, including arthropods such as *Drosophila* (59). Unlike in *Drosophila*, however, in *C. elegans*, the epidermis contains a single discernable electron dense junctional region (60). Although the spatial distribution of specific molecular complexes within this single

electron density has not been performed, *C. elegans* epithelia contain many of the same proteins as in *Drosophila*, with similar spatial ordering. *C. elegans* possesses a single homologue of classical cadherins, HMR-1, and associated proteins HMP-2/β-catenin, HMP-1/α-catenin, and a divergent p120ctn, JAC-1 (61, 62). These proteins appear to localize the nematode equivalent of adherens junctions. Similarly, in *C. elegans*, the discs large homologue DLG-1 and its binding partner AJM-1 localize basal to the adherens junction. Costaining demonstrates that these cell junction proteins localize to different regions of the lateral cell membrane, with the adherens junction proteins being more apical (63–66). A third localization domain, apical to the adherens junction, which may extend from the junctions across the apical surface, contains of the familiar Par-3–Par-6–aPKC complex (Fig. 4) (63, 65–68). Other proteins, not part of the Par complex, such as

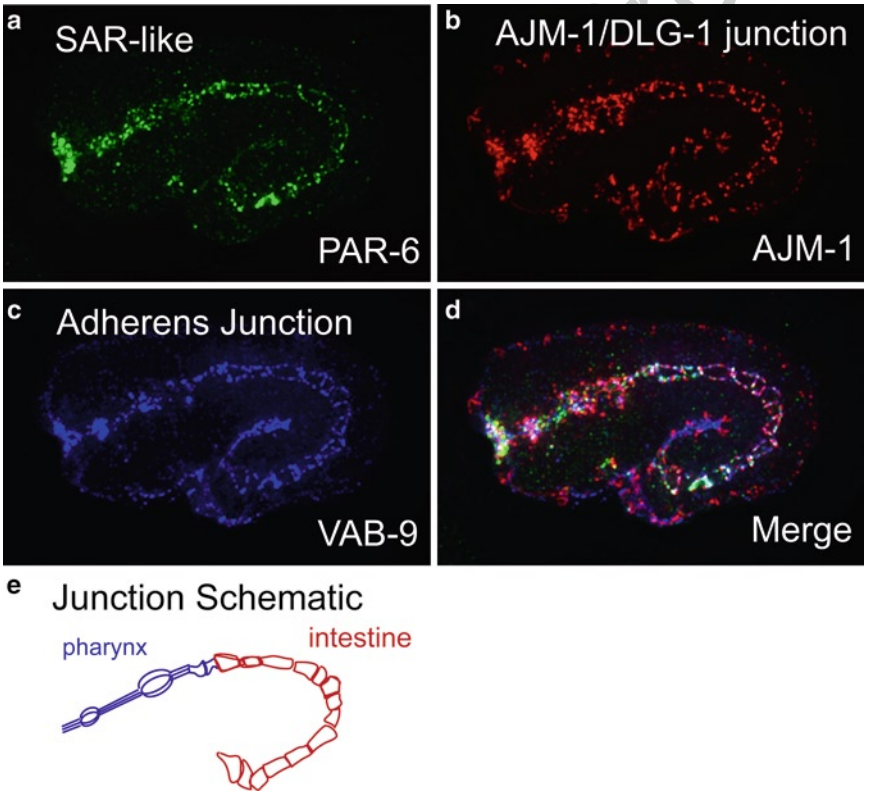


Fig. 4. Cell junctions in the embryonic alimentary system. Staining of the SAR-like region, the DLG-1/AJM-1 domain, and the adherens junction, with PAR-6 (**a**), AJM-1 (**b**), and VAB-9 (**c**) respectively, at pharyngeal and intestinal junctions, demarcating the apical–lateral regions of these cells, just below the apical surface that forms the lumen of the alimentary system. The staining of the markers only shows partial overlap (**d**, *white*), illustrating that the three junctional domains are all localized within a narrow lateral region, but remain largely distinct. At this stage, and in this preparation, most PAR-6 is closely associated with the cell junctions, and little is observed across the apical surface. (**e**) A schematic of cell junctions in the pharynx and intestine, determined by AJM-1 localization.

CHE-14 and EAT-20 (a crumbs homologue) are also localized across the apical surface (69, 70).

As in *Drosophila*, overall polarity of epithelial junctions is established and maintained by LET-413, the LAP (LRR and PDZ) domain containing protein homologue of *Drosophila* Scribble (71, 72). LET-413 localizes along the basolateral surface of epithelial cells, establishes global cell polarity and, therefore, is required for the localization of cellular junction components in epithelial cells and the structure of the intestinal terminal web, visualized by indirect immunofluorescence of cell junction components and by TEM of cell junctions (72, 73). Even though loss of *dlg-1* gene function can completely eliminate this cell junction as observed at the electron microscopy level, and cause the mislocalization of the AJM-1 protein, classical adherens junction proteins such as HMR-1 (cadherin), HMP-1 (α-catenin), and VAB-9 (TM4SF10) remain essentially localized, if not completely aggregated, in a narrow band. Similarly, loss of adherens junction proteins does not disrupt the localization of DLG-1 or AJM-1, suggesting that there are at least two independent junctional regions within or near the apical junction (63–67, 72). Furthermore, cell polarity and adhesion are only mildly disrupted in *ajm-1* and *dlg-1* RNAi animals, and these adhesion defects are enhanced by *vab-9* (67). Notably, the localization of apical proteins is not affected by the loss of adherens junction or DLG-1/AJM-1 domain proteins (66, 69). Loss of *dlg-1* can affect the localization of the *Drosophila* Crumbs homologue (CRB-1) protein, which is a component of a separate apical protein complex, but this protein is not required for polarity in *C. elegans* as is the case for *Drosophila Crumbs* (63). These findings indicate that there are at least three distinct apical cell junctional complexes in *C. elegans* epithelia that together contribute to cell adhesion and morphogenesis.

5. Spermathecal Junctions

Caenorhabditis elegans cellular junctions arising from the mesoderm, such as the cell junctions in the spermatheca of the somatic gonad, are distinct from the epidermal or intestinal epithelia. The spermatheca stores sperm in the hermaphrodite. During fertilization, the spermatheca expands dramatically to allow the passage of an oocyte from the syncytial gonad into the uterus (74–76). During this expansion, cell junctions must "unzip" on both sides of a stable cell junction so that the amount of cell membranes contributing to the expanded lumen is matched by an increase in the membranes comprising the basal membranes (77). Apical to the adherens junction is a junction with an appearance similar to a pleated septate junction, while basal is a junction with

a smooth septate junction appearance. Immunostaining of TEM spermathecal preparations with the MH27 antibody, which recognizes AJM-1, shows that there are at least three unique junctions, one decorated by MH27 (Fig. 5) (77) (David Hall, personal communication). Pleated SJ are characterized by ladder-like crossbridges between cells and are found apical (luminal) to the adherens junction. Based on MH27 immunogold staining (A), and localization of AJM-1::Cherry (G), AJM-1 expression appears to be strongest in the region of the stable (adherens) junction, while MEL-11::GFP localizes to the pleated septate

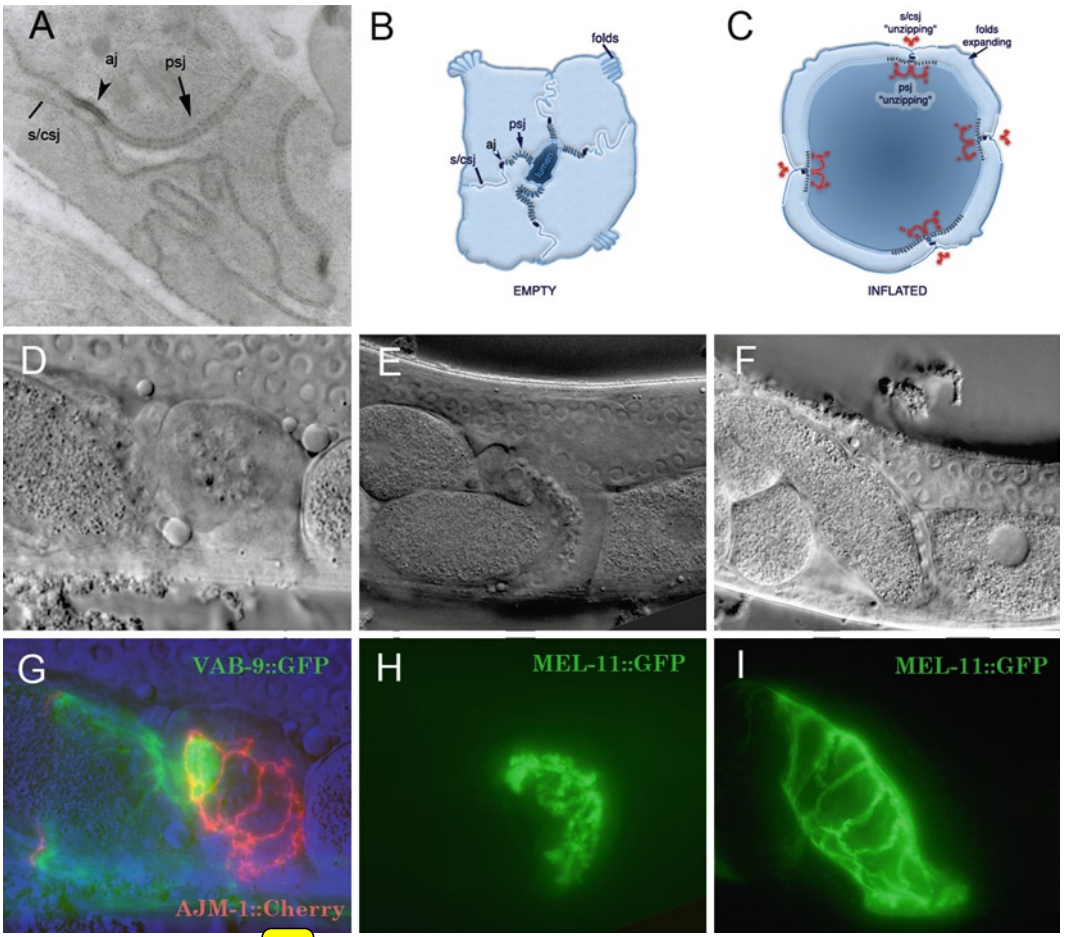


Fig. 5. Cell junctions in the Spermatheca. (**a**) Immuno-TEM pre ions of the spermatheca using anti-AJM-1 antibody MH27 demarcates three distinct junctional regions: the Pleated septate junction (pSJ), the Adherens junction (AJ), and the smooth septate junction (sSJ). In this preparation, MH27 only decorates the adherens junction (**b**, **c**). Representative drawings of the spermatheca either collapased (**b**) or expanded (**c**) to allow an embryo to pass through and be fertilized (**b** and **c** are reprinted, with permission, from *C. elegans* Wormatlas) (77). (**e**, **f**) Expression of MEL-11::GFP at the pSJ is shown, matching the stages in the drawings above; (**f**) and (**i**) are the matching DIC images for (**e**) and (**f**), respectively. (**d**) Expression of AJM-1::Cherry (*red*) and VAB-9::GFP (*green*) are shown for a stage matching (b). Note that strong AJM-1 expression at the AJ is more basal than MEL-11 at the equivalent stage. In these adults, VAB-9::GFP expression in the spermatheca is reduced and is largely restricted to the spermatheca-uterine valve.

junction (Fig. 5h, i). MEL-11 regulates actomyosin-mediated contraction during cytokinesis and embryonic elongation, and also is required in the spermatheca for fertility (78–80). Since actomyosin at cellular junctions regulates both apical constriction and the organization of cellular junctions, it is exciting to speculate that *mel-11* regulates actomyosin contraction required for unzipping and zipping of the pleated spermathecal junctions during fertilization. How similar are the spermathecal junctions to those of *Drosophila* pSJs based on the expression of claudins and other SJ proteins? Of the claudin-like proteins in *C. elegans*, both VAB-9 and CLC-1 are expressed in the spermatheca, with VAB-9 being expressed during the larval stages of development when junctions are forming. As in epidermal tissues, VAB-9 expression in the spermatheca does not overlap with AJM-1 (67) and (JSS, unpublished observation). However, in adults, during fertilization, VAB-9 is absent from spermathecal junctions and instead is present in the spermatheca-uterine valve (Fig. 5). Although both CLC-1 (and DLG-1) are expressed in the spermatheca, it is unknown whether they colocalize with AJM-1 in or near the adherens junction, as they do in the epidermis, or whether they associate with MEL-11 in the septate junction (63, 64, 66, 81). Based on localization data, at least one claudin-like protein, VAB-9, is likely to be involved in the "unzippering" of the septate junction. The data described below suggests that among the claudin family proteins, such a role may be unique to VAB-9, however further analysis of claudin family protein function in the spermatheca will be required. In any event, understanding the role of VAB-9 in the rapid, dynamic reorganization of cellular junctions should prove fascinating, particularly if regulation occurs through VAB-9 influence on nonmuscle myosin, as we suggest below.

6. VAB-9: A Claudin-Like Protein at Adherens Junctions

VAB-9 is an approximately 22 kDa protein with similarity to the PMP22/EMP/claudin/gas3 family of four pass membrane spanning proteins. VAB-9 is expressed in all *C. elegans* epithelia and colocalizes with the adherens junction proteins HMR-1 (cadherin) and HMP-1 and 2 (α- and β-catenin, respectively). HMR-1 is required for VAB-9 membrane localization, and HMP-1 is required to maintain uniform circumferential VAB-9 distribution about the adherens junction. *vab-9* mutants have defects in body morphology, likely due to defective filamentous actin organization in epidermal cells. Mutations in *vab-9* enhance the morphological defects of weak *hmp-1* loss of function and enhance cell adhesion defects in *ajm-1* and *dlg-1* animals. Thus, *vab-9* participates in the organization of F-actin at the adherens junction and, alongside with the AJM-1/DLG-1 complex, maintains proper epithelial adhesion.

Clues to the general functions of VAB-9 may come from the vertebrate ortholog, TM4SF10, previously known as BCMP1. *TM4SF10* is expressed strongly in the canine brain, and based on SAGE analysis, is expressed in human brain astrocytoma, ependymoma, and normal spinal cord. This finding, along with the genetic position of *TM4SF10* on the X-chromosome at p21.1, suggests an association with hereditary X-linked mental retardation loci; however, no disease-associated changes were detected in the *TM4SF10* locus in XLMR patients from 14 unrelated families (82, 83). Thus, mutations in *TM4SF10* are either rare among affected individuals or *TM4SF10* is involved in separate processes in the brain. In the developing mouse kidney, *TM4SF10* is expressed transiently in podocyte precursors, and expression diminishes as the cell junctions of these precursors transition from an occluding-type junction typical of columnar epithelia to the specialized adherens junctions of slit diaphragms (36). Slit diaphragms are organized around the transmembrane proteins of the nephrin family (84, 85). Nephrin and the related protein, Neph1, participate in homo- and heterotypic intercellular adhesion along interdigitated podocyte cell extensions (86, 87). Slit diaphragm development results from signaling through Nephrin family proteins cytoplasmic domains which in turn effect changes in the underlying actin cytoskeleton, cell process extension, and cell process interdigitation, ultimately generating the podocyte side of the filtration barrier in the glomerulus. Following phosphorylation of Nephrin and Neph1 by the Src family kinase Fyn, Nck, PI3K, and Grb-2 bind specific phosphotyrosines and signal to reorganize the actin cytoskeleton (88–95). Interestingly, our unpublished data (L. Bruggeman and JSS) indicates that *TM4SF10* expression regulates the activity of Fyn and Nephrin maturation in podocytes, suggesting that *TM4SF10* may regulate the ability of nephrin to control actin dynamics and cell process extension, possibly by influencing Fyn phosphorylation of Neprhin. Consistent with this hypothesis is the finding that overexpression of *TM4SF10* phenocopies inhibition of neurite outgrowth by RhoA overexpression following NGF stimulation of PC12 cells (96). Similarly, unpublished data from one of our laboratories (JSS) showed that the overexpression of *TM4SF10* in MDCK cells blocks Fyn-dependent cell process extension. These results indicate that *TM4SF10* may have a general role in maintaining a columnar epithelia phenotype indirectly by preventing the formation of actin-dependent cell extensions. To date, interactions between VAB-9 and homologues of Nephrin and Fyn in *C. elegans* have not been investigated, although FRK-1, a non-receptor FER-like kinase, is required for embryonic morphogenesis (97–99). It will be of future interest to explore the interactions between TM4SF10, Fyn, and Nephrin and determine whether such interactions are unique to vertebrate podocytes or whether they are conserved in all epithelial cell types that express these proteins.

Further insight into the function of *vab-9* comes from an analysis of the MAGUK protein ZOO-1. ZOO-1 is the *C. elegans* ortholog of the tight junction membrane-associated guanylate kinase (MAGUK) protein ZO-1 (100). ZOO-1 is a predicted 129 kDa protein with three PDZ motifs in the N terminus, an SH3 domain, a guanylate kinase domain (predicted to be inactive), and a C-terminal ZU-5 domain, unique to this class of MAGUKS and netrins (47). Several data indicate that *zoo-1* closely interacts with *vab-9*. First, ZOO-1 localizes to cell junctions in the epidermis and, as VAB-9 requires HMR-1 (cadherin), but not HMP-1 or HMP-2, for junctional localization. Second, *zoo-1* localization requires VAB-9. Third, similar to *vab-9* mutants, loss of *zoo-1* function enhances the morphogenetic phenotypes of a weak *hmp-1* loss of function allele. Fourth, loss of *zoo-1* and *vab-9* function affects the organization of filamentous actin in the epidermis of elongating embryos. Fifth, loss of *zoo-1* function does not enhance the phenotypes of *vab-9* mutants, suggesting that they act in a common pathway. Sixth, both *vab-9* and *zoo-1* interact with mutations in genes of the actomyosin contractile machinery. Loss of *zoo-1* activity enhances *mel-11* (myosin light chain phosphatase) mutants and suppresses *let-502* (ROCK) mutants, whereas *vab-9* mutations suppress *mel-11* alleles (JSS, unpublished data). Surprisingly, *zoo-1* mutants alone have virtually no effect on morphogenetic phenotypes, although very mild abnormalities of F-actin accumulation at junctions were noted (Lockwood). Together these results suggest a regulatory pathway in which HMR-1 regulates VAB-9 and *vab-9*, in turn, regulates a subset of epidermal morphogenetic processes; a subset of these processes are in turn mediated by *zoo-1* and *mel-11* in both dependent and independent pathways. Since our own unpublished data suggests that VAB-9 may be required for MEL-11 localization to the epidermis, it should be interesting to determine the nature of the interactions between these protein classes in regulating F-actin organization at epidermal cell junctions and within epidermal epithelia, particularly since ZO-1 has been shown to influence the paracellular barrier through the regulation of perijunctional actomyosin (101).

7. Claudin-Like Genes *clc-1* to *clc-5*

Claudin family proteins exist in all metazoans. In humans and mice, there are as many as 24 different claudins. To date, five claudin-like proteins have been identified in *C. elegans* by virtue of sequence identity to mammalian claudins or to identified *C. elegans* claudins (81). Not surprisingly, sequence homology was relatively low, with CLC-1, CLC-2, and CLC-3 sharing 25%,

23%, and 26% identity with claudin-6, 5, and 4, respectively. CLC-4 shares just 36% identity with CLC-1. CLC-5 is the paralog of CLC-2, probably resulting from a gene duplication, being located just 3′ of *clc-2* in the genome. Despite this location, CLC-2 and CLC-5 are only 31% identical. Interestingly, BLAST searches identify similarities between several *C. elegans* claudins and the *nsy* family, suggesting possible overlap in function between the adhesive/charge/paracellular barrier properties of classic claudins and the channel regulatory properties of the gamma subunits of the voltage-gated calcium channels (102). Phylogenetic analysis also suggests a close association between *nsy-4*-like genes and the *clc* genes. Of the five putative *C. elegans* claudins, two have been further analyzed to determine the extent of their function as classical claudins. GFP fusion proteins were constructed for both CLC-1 and CLC-2. CLC-1 is localized at the apical region of the lateral cell membranes in all four regions of the *C. elegans* pharynx, the procorpus, metacorpus, isthmus, and terminal bulb. In the isthmus region, six cellular junctions connect three pharyngeal myoepithelial and three marginal cells. Freeze substitution electron microscopy reveals a thick subapical junction – as a classic adherens junction – and a thinner apical region with a more narrow paracellular space. It is possible that CLC-1 and the cell junction protein AJM-1 localize to this region and adherens junction proteins localize to the thicker basal junction. In the epidermis and intestine, AJM-1 and DLG-1 localize basal to the adherens junction. It may be possible that in the pharynx, the localization of the two junctions is reversed; however, that has not been demonstrated. It remains for immuno-TEM with different diameter gold particles linked to secondary antibodies or PALM microscopy of fluorescently labeled/tagged proteins to resolve this issue. An alternative possibility is that the narrow apical region discovered by freeze substitution is the locale for one or more CLC proteins and this may define a separate lateral region more typical of tight junctions in mammals, rather than the septate junctions of invertebrates. CLC-1 expression was also reported in the vulva, spermatheca, and pore cell of the excretory system, but was not assigned to a specific junctional complex. CLC-2::GFP shows expression in the hypodermal seam cells of adults. Following RNAi of *clc-1* and *clc-2*, a 10,000 MW TRITC-dextran is able to infiltrate to the interior of the RNAi worms in the pharynx and body cavity for *clc-1* and into the body cavity for *clc-2*, suggesting that both these claudins maintain epithelial barriers. *clc-1* and *clc-2* expression only acc[illegible] for a subset of epithelial tissues with cell junctions in nematodes. Further studies should indicate whether th[illegible]*c-3–5* are expressed in epithelia lacking *clc*[illegible] and *cl*[illegible]nd whether inactivation of these claudins in other tissues hav[illegible]milar effects on the epithelial barrier function of such tissues or whether there are alternative

phenotypes. It will be interesting to see whether all *C. elegans* claudins are localized to junctional regions with similar TEM appearances, since in vertebrates, specific claudins are associated with specific cell junction types. It will also be interesting to determine whether the CLC proteins can generate tight junction strands when expressed in L cells, as mammalian claudins. Expression of VAB-9 in L cells does not result in the formation of tight junction strands (JSS unpublished data).

8. *nsy-4* and Neuronal Cell Fate Specification

nsy-4 (*Neuronal SYmmetry* gene 4) was identified for its role in specifying the distinct fate of one AWC olfactory neuron; specifically, *nsy-4* is required for one AWC neuron to express the G protein-coupled odorant receptor gene *str-2* and detects the odor 2-butanone (102). Without *nsy-4* activity, both AWC neurons fail to express *str-2* and detect the odor 2,3-pentadione (102). These alternate fates, based on the expression of *str-2*, are referred to as AWC^{on} and AWC^{off}, respectively. In *C. elegans*, NSY-4 is most similar to uncharacterized genes K10D6.2 and C24H10.1 as well as claudin genes *clc-1 to clc-5* and R04F11.1. Outside *C. elegans*, NSY-4 is most similar to *Drosophila Stargazin*, TARPS, and gamma subunits of voltage-gated calcium channels. Surprisingly, *nsy-4* rescue by human claudin-1 expression was weak, but was stronger than stargazin/γ2 expression, while rescue by human γ7 channel was complicated by additional phenotypes. NSY-4 is thought to function as a gamma channel based on the genetic data, which indicate that *nsy-4* represses the function of calcium channel genes *unc-2* and *unc-36*. Together these findings suggest that NSY-4 may have additional functions in AWC specification beyond regulation of gamma channels. Additional functions may involve claudin-like adhesive functions. Genetic and expression studies demonstrate that NSY-4 levels correlate with the AWC^{on} fate: *nsy-4* expression in a single AWC is sufficient for the AWC^{on} fate, while overexpression of *nsy-4* in both AWC cells results in an increased frequency of animals with AWC^{on} fates in both AWC cells. NSY-4 regulates the choice of cell fate by influencing the activity of a signaling pathway consisting of calcium channels encoded by *unc-2* and *unc-36*, CAMKII encoded by *unc-43*, a Toll-interleukin 1 repeat protein encoded by *tir-1*, a MAPKKK encoded by *nsy-1*, a MAPKK encoded by *sek-1*, and a homeodomain protein encoded by *nsy-7* (103–107). The innexin protein, NSY-5, acts in parallel with NSY-4 to regulate the activity of this pathway, so that the over-expression of *nsy-4* can partially compensate for the loss of *nsy-5* and vice versa (108). While both proteins are localized at cell

membranes (NSY-4 broadly and NSY-5 in puncta at junctions), neither protein affects the other's localization. Results from *nsy-5* mosaic analysis and targeted expression using cell-type-specific promoters indicate that transient *nsy-5* gap junction networks are involved in specifying cell fate and that the expression of AWC^{on} fates involves signaling across the midline as well as feedback inhibition of AWC^{on} fates from surrounding cells (108). Similarly, *nsy-4* is proposed to regulate communication between AWC cells in contacting axons across the midline.

Normally, the determination of which cell will express the AWC^{on} fate is stochastic, suggesting that some small fluctuation in activity upstream or parallel to NSY-4 tips the choice of signaling resulting in *str-2* expression in favor of one cell over another. Feedback signaling to reinforce this decision resulting in one, and only one, cell expressing the AWC^{on} fate is supported by genetic mosaic experiments. Such experiments show that the overexpression of NSY-4 in one AWC cell suppresses the expression of the AWC^{on} fate in the neighboring wild-type AWC cell. This signaling is reminiscent of the familiar stochastic cell fate specification of anchor cell and ventral uterine fates, which involves lateral signaling via the Delta-Notch ligand receptor system encoded by *lag-1/2* and *lin-12*, respectively (109–114). During the AC/VU decision, the LAG ligand signals through the LIN-12 receptor to specify anchor cell and ventral uterine fates and by analogy during AWC specification, it seems likely that NSY-4 is one half of a signaling pair and that a currently unidentified protein may function as an NSY-4 ligand/receptor. Since claudin family members are known to form homotypic and heterotypic pairs and since the *nsy-4* homolog *Stargazin* has been shown to mediate both homotypic and heterotypic cell adhesion when expressed in L cells, we speculate that NSY-4 interacts with NSY-4 and/or an unidentified claudin superfamily protein on neighboring AWC (and perhaps other) opposing cell membranes (33). Binding to NSY-4 then influences *nsy-4*-dependent signaling by refining forward and feedback signaling. Thus, NSY-4-related proteins, claudin family members, or VAB-9 may participate in heterotypic interactions with NSY-4 and mediate axon interaction and communication, participating in the specification of AWC cell fate and possibly the fates of other neurons. It is interesting to note that VAB-9 is expressed in the nerve ring, although the specific cells have not been identified (67). The expression pattern of other *nsy-4*-related genes has not yet been determined; while *clc-1* and *clc-2* are not expressed in the nerve ring, the expression patterns of *clc-3*, *clc-4*, and *clc-5* are currently unknown. Given the combinatorial possibilities, multiple distinct axonal signaling events could be mediated by these proteins. Specific cell–cell interactions mediated by distinct claudin pairs may activate multiple signaling events within the same or among closely related cells.

9. When Is a Claudin a Claudin?

The superfamily of proteins that fall into the general category of similarity to claudins has grown, begging the question of what exactly are the essential properties of a claudin, as opposed to a similar, but fundamentally distinct four-pass integral membrane protein. Most claudins generate a charge-selective pore in the paracellular space that regulates electrical properties of a cell or groups of cells due to specific charge permeability. Most claudins mediate adhesion and this adhesion can be homotypic or heterotypic between various claudin types. The formation of paired strands within the membrane by claudins reduces the paracellular space to zero and the number and arrangement of the strands correlates with the tightness of the epithelium. Most claudins appear able to traffic to cell membranes and generate paired strands in the absence of other cell adhesion molecules, but require targeting proteins to designate the proper membrane location. As other transmembrane proteins, claudins transduce extracellular information, such as the binding of ligands, small molecules, and viruses, to the nucleus to change gene expression or to transduce signals to junctional proteins (including themselves) to modulate the nature of the paracellular space. Claudin superfamily proteins are likely to have at least a majority of these properties; however, due to the various organisms, organs, tissues, and developmental and physiological systems in which claudin-like proteins have been shown to function, along with the dramatic divergence of the family, it is highly likely that the functional repertoire of claudin-like proteins will continue to expand. The claudin family proteins in *C. elegans* described in this review support this hypothesis, since they are involved in diverse roles such as maintaining tissue integrity, epithelial morphogenesis, regulating cell junction dynamics through filamentous actin, signal transduction, and cell fate specification. Our understanding of the roles for all the members of this family in *C. elegans* is incomplete, suggesting that future research will reveal novel functions.

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