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NEURAL GPI-ANCHORED CELL ADHESION MOLECULES

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1. ABSTRACT

When first identified, neural cell adhesion molecules (neural CAMs) were thought to act simply by providing cell surfaces with differential adhesion properties. In the decades following the identification of the first neural CAMs, it has been realized that these proteins are actually involved in very complex processes such as axon guidance, neuronal migration, neurite outgrowth and fasciculation, target selection, synapse formation, plasticity and more recently, the maintenance of the integrity of myelinated fibers. In this review we will summarize work relating to glycosylphosphatidylinositol-anchored CAMs (GPI-CAMs) and will highlight expression/function issues, protein interactions and the role of the GPI in signaling.

2. INTRODUCTION

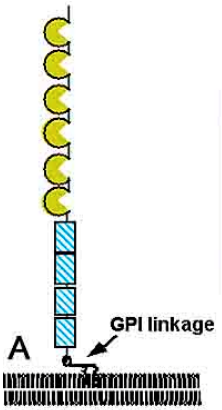
Neural CAMs of the immunoglobulin superfamily (IgSF) have been known for a long time to regulate adhesion, neurite outgrowth and migration (1- 4). However, understanding how CAM proteins regulate these intricate functions in the nervous system has lagged behind studies on other adhesion proteins such as integrins and cadherins. The IgSF represents one of the largest superfamilies in the human, fly and worm genomes (5-7). Its members are characterized by the presence of a variable number of immunoglobulin (Ig) -like modules bearing homology usually to the C2 region of Ig molecules as defined by Williams and Barclay (8). Other members consist of both Ig and fibronectin type III (FNIII)-related repeats. This latter group of Ig/FNIII-like proteins consists of glycosylphosphatidylinositol (GPI)-anchored and transmembrane CAMs. In addition, there are several GPI-CAMs containing exclusively Ig domains (IgLON subfamily, Thy-1).

In this review we will summarize current knowledge on the GPI-linked neural CAMs, in particular as it relates to their expression, structure/function and signaling potential. We will also focus on the major advances in this field in the past few years as they relate to GPI-CAM interactions with their receptors and the role of the GPI anchor in signaling.

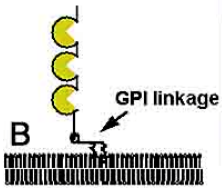
3. SUBGROUPS OF GPI-LINKED CAMS-MAJOR SIMILARITIES AND DIFFERENCES

3.1. Structural Issues

The two major subgroups of GPI-linked CAMs are the TAG-1/contactin and the IgLON families. Apart from those, we will briefly discuss two other GPI-anchored CAMs, Thy-1 and NCAM-120, which do not fall into these two gene families, but are related molecules.



human	rodent	avian
CNTN1	F3/contactin	F11
CNTN2	TAG-1	axonin-1
CNTN3	BIG-1	
CNTN4	BIG-2	
CNTN5	NB-2	FAR-2
CNTN6	NB-3	



human	rodent	avian
LAMP	LAMP	AvGrp50
OBCAM	OBCAM	ChOBCAM (GP55A)
	Neurotrimin	CEPU-1 (CEPU-Se)
	Kilon	Neurotractin

Figure 1. A. The structure of GPI-CAMs of the immunoglobulin superfamily. The hemicircles represent the Ig domains and the rectangles the fibronectin type III repeats. B. The structure of the IgLON proteins comprising of three Ig repeats. The names of the different proteins and species are presented on the tables on the right.

The TAG-1/contactin family comprises of TAG-1, its chick orthologue axonin-1, F3/contactin and its chick orthologue F11, BIG-1, BIG-2, NB-2 and its putative chick orthologue FAR-2 and NB-3 (9-17). The members of this subfamily are grouped together due to their sequence homology and due to shared structural features, i.e. same number of Ig (six) and fibronectin (four) modules in addition to the GPI anchor (Figure 1A).

It has been estimated (7) that 765 human genes contain Ig domains, meaning that the IgSF is one of the largest protein superfamilies in the human genome (5). All members of the TAG-1/axonin-1 subfamily consist of six Ig modules in their extracellular N-terminal part followed by four FNIII repeats (Figure 1). Colinear domains show the highest degree of similarity.

The crystal structure of the first four Ig domains of TAG-1/axonin-1 and FAR-2 indicates that these domains are compact and U-shaped due to contacts between domains 1 and 4 and domains 2 and 3 (17-18). In the crystals, the axonin-1 $_{Ig1\text{-}4}$ molecules are arranged in an anti-parallel orientation, suggesting that axonin-1 mediated cell-cell adhesion involves a zipper-like string of molecules from apposed membranes (18). Crystal structures of other IgSF members are not available, except for the two N-terminal, extracellular domains of NCAM, which provide the structural basis for the homophilic interaction. The molecular arrangement of the two-domain structure forms a cross-shaped, antiparallel dimer, which is implicated in the trans-cellular recognition mediated by NCAM (19).

The IgLON family comprises of LAMP, OBCAM, neurotrimin/CEPU-1, AvGP50, and Kilon/neurotractin (20- 29). These proteins are characterized by the presence of three Ig domains and a GPI-anchor (Figure 1B).

Both subfamilies exhibit a restricted distribution in the nervous system and they represent CAMs expressed by neurons during early phases of their differentiation. Their members display homophilic adhesive properties (with the notable exception of F3/contactin) and they all regulate the outgrowth of neurites, positively or negatively. Details on the expression patterns, functions and interactions of these proteins follow in the sections below.

4. THE TAG-1/CONTACTIN SUBFAMILY

4.1. Expression meets function

4.1.1. During development

The members of the TAG-1 subfamily have been extensively studied in terms of their expression patterns and of their functional roles during the construction of the nervous system (30-33, 14-16). A good example has been the TAG-1/axonin-1 CAM, which is expressed with a very complicated and dynamic pattern in developing fiber tracts, as well as migrating neurons. In general, its axonal expression is associated with the initial phase of outgrowth. However, the protein is also detected on cell bodies. Sites of expression of TAG-1 include commissural fibers and motor neurons in the spinal cord, the dorsal root ganglia (DRG), the retinal ganglion cells (RGC), the inner part of the external granule cell layer (EGLb) in the cerebellum, the corticofugal fibers and tangentially migrating neurons forming some of the precerebellar nuclei (30-36). In most of these areas, the function of TAG-1/axonin-1 has been studied with blocking antibodies or the use of soluble protein (endogenously purified or in the form of TAG-1-Fc chimera). A recent TAG-1 deficient mouse has been described, but not yet characterized in terms of potential developmental defects (see section 2.3).

In the spinal cord, perturbation of axonin-1 function *in vivo* or *in vitro* results in pathfinding errors of commissural axons (37-39). Nociceptive fibers (projecting from the DRG and targeting to layers in the dorsal horn) require axonin-1 for pathfinding (40). In the cortex, TAG-1 mediates early stages of the migration of GABAergic interneurons which occurs upon the TAG-1-labeled corticofugal fibers (35; Figure 2). If the function of TAG-1 is blocked in cortical slice cultures, a decreased number of GABAergic neurons migrate towards the cortex. In the caudal medulla, TAG-1-expressing tangentially-migrating cells originate from the rhombic lip and move towards the ventral midline, cross it and proceed contralaterally to form the lateral reticular (LRN) and external cuneatus nuclei (ECN). If TAG-1 function is blocked, the migration is perturbed (36, Figure 2). Other functional experiments, most of them based on assays *in vitro*, implicate TAG-1 as a potent neuritogenic substrate (for DRG neurons) and as a strong mediator of adhesion (12, 41-42, see below), processes that may underlie other activities such as the ability to migrate or extend neurites. In summary,

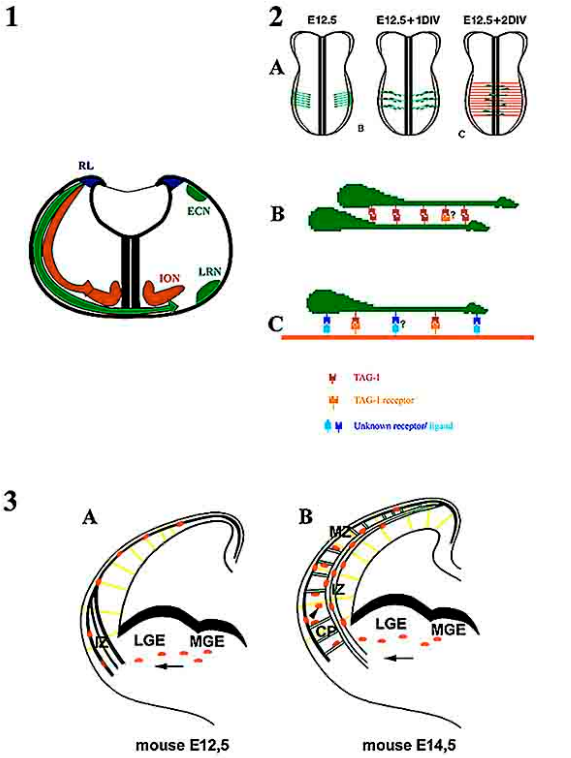


Figure 2. Top panel. 1. A schematic representation of a transverse section through the caudal hindbrain of an E13.5 mouse embryo. The olivary migrations that give rise to the ipsilateral inferior olive (ION) are shown in red while the neurons of the superficial stream forming the contralateral lateral reticular (LRN) and external cuneatus (ECN) nuclei and expressing TAG-1 are shown in green. The origin of the migratory cells, the rhombic lip (RL), is shown in blue and the midline floor plate by double black lines. 2. A schematic representation of an open-book preparation of hindbrain explants. TAG-1 expressing, superficially migrating cells are shown in green, tangentially oriented axons are shown in red. Laterally, the cells migrate in contact with each other, extending long leading processes. Medially, close to the floor plate (lines in black), the cells migrate by following pre-existing axonal fibers (neurophilic migration). DIV, days *in vitro*. 3. Cells migrating in contact with each other exhibit trans homophilic TAG-1/TAG-1 interactions and possibly TAG-1 heterophilic interactions as well (the TAG-1 receptor if there is one in this case, is unknown). 4. During neurophilic migration, there may be trans heterophilic interaction between TAG-1 on the migrating cells and leading processes and a yet unknown TAG-1 receptor on the axons. Lower panel. 1. A proposed mechanism for the migration of cortical interneurons from the medial ganglionic eminence (MGE). The TAG-1 immunopositive corticofugal fibers are shown in green, migrating interneurons in red, radial glial fibers in yellow and the ventricular zone of both MGE and the lateral ganglionic eminence (LGE) in blue. MGE cells use the TAG-1 immunopositive axons in the marginal zone (MZ) and intermediate zone (IZ) to migrate into the neocortex (A) and then reach their positions in the cortical plate (CP) by using the radially arranged bundles of efferent axons or radial glial fibers (B).

functional studies mainly from *in vitro* or *ex vivo* assays point to a role of TAG-1 in axon pathfinding, neuronal migration (in particular neurophilic migration), neurite outgrowth and adhesion.

A close homologue of TAG-1 is the GPI- linked neural CAM F3/contactin protein or F11 in chick (9-11). In most cases, its expression peaks between the first and second postnatal week following that of TAG-1, with which it shares ~50% amino acid identity. However, both molecules exhibit different expression patterns (93). F3/F11/contactin has been mainly studied in the cerebellum. Postnatally, F3/contactin is expressed in the EGL and is particularly enriched on the premigratory granule cells in the inner portion of the EGL, as well as the molecular layer, where the axons of granule cells (parallel fibers) extend (44-45). There are conflicting reports about F3/F11/contactin expression on Purkinje cells (45-47). It is also expressed by other cerebellar cell types, such as Golgi cells (44). At later postnatal stages, F3/contactin is also found in the white matter tracts. In summary, F3/contactin is prominently expressed throughout postnatal cerebellar development and is found concentrated on extending parallel fibers, glomeruli and on afferent mossy and/or climbing fibers in the white matter. In the chick spinal cord, F11, the chick orthologue of F3/contactin, is expressed by motor neurons, DRG axons and proprioceptive neurons. These neurons, which establish connections with ventrally located motor neurons, depend on F11 in order to grow towards their target layers in the spinal cord (see also the interactions section below; 40).

A number of *in vitro* assays suggest an involvement of F3/contactin in adhesion and neurite outgrowth, as well as in fasciculation and repulsive processes (46, 48-51). Recently it has been proposed that F3 may regulate cell proliferation. Transgenic mice expressing F3/contactin from the human TAG-1 promoter display a developmentally regulated cerebellar phenotype in which the size of the cerebellum is reduced during the first two postnatal weeks but subsequently recovers (52). Granule cell number is reduced and their precursor proliferation is decreased. In the same developmental time window the molecular layer is reduced and Purkinje cell dendrites fail to elaborate normally (52). The authors suggest that F3/contactin affects granule cell proliferation in addition to the known effects on neurite growth and fasciculation. A similar role for N-CAM, a transmembrane CAM, has been postulated, but this assumption awaits further corroboration (53).

4.1.2 In the adult

Although GPI-CAM molecules have been studied mainly in the developing nervous system, it has become evident in recent years that they play important roles in the adult nervous system, as well. Low levels of TAG-1 mRNA are detected in some neurons of the adult mouse brain, specifically in olfactory mitral cells, hippocampal pyramidal neurons and cerebellar granule cells (33; our unpublished data). In the fish visual pathway, TAG-1 is restricted to the nasal RGCs (54). Re-expression of TAG-1

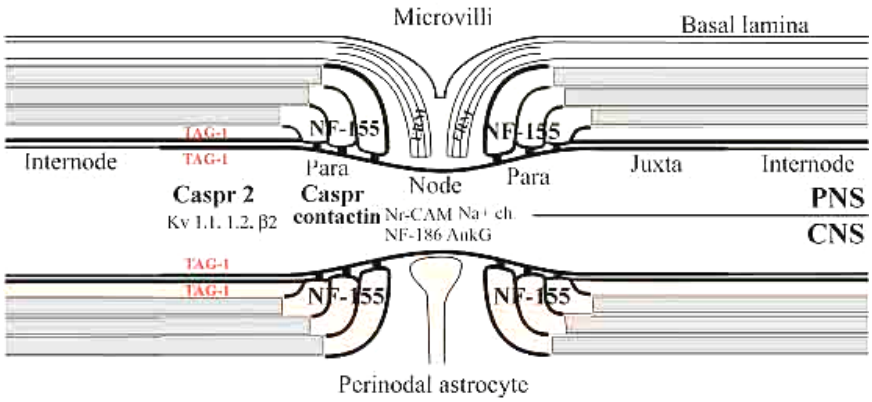


Figure 3. A schematic depiction of the subdomains of myelinated fibers. The molecular organization of the nodal, paranodal and juxtaparanodal regions are shown (adapted from 140).

is observed *in vivo* in axotomized adult RGCs that is dependent upon target contact (54).

Results from our group show that TAG-1 expression persists in the rodent adult spinal cord and DRG. In the PNS, TAG-1 is expressed by DRG neurons and in Schwann cells (see paragraph below), particularly those associated to peripherally projecting DRG neurites. During peripheral nerve regeneration induced by a sciatic nerve lesion, TAG-1 is not upregulated, but is decreased in DRG neurons. Instead, TAG-1 might be upregulated in Schwann cells at the lesion site since enhanced expression is detected locally. A similar upregulation of TAG-1 is observed in non-neuronal cells invading the spinal cord after a kainic acid-induced lesion. Overall, this data may suggest a putative involvement of TAG-1 in the structural plasticity of adult nervous system (M. Traka, D. Karagogeos and F. Nothias, unpublished data).

TAG-1 is not only expressed by neurons but also by myelinating and ensheathing Schwann cells, but also by oligodendrocytes (55). In mature myelinated fibers, TAG-1 is localized to the juxtaparanodal region, along with the Shaker-type Kv1.1 potassium channels and Caspr2, a member of the neurexin family (Figure 3; 55). Thus, the adult expression profile of TAG-1 differs to a large extent from its embryonic.

F3/contactin has been reported as a selective neuronal component of the paranode that regulates junctional attachment between axon and glial membranes. Specifically, it is found on the axolemma of adult peripheral and central myelinated fibers where it has been shown to interact with paranodin/Caspr, another neurexin superfamily member and neurofascin-155 (NF-155) (Figure 3; 56-59). Ablation of contactin in mutant mice (see also section below) disrupts junctional attachment at the paranode and reduces nerve conduction velocity 3-fold probably due to the altered localization of potassium channels (60). The mutation impedes intracellular transport and surface expression of Caspr (60). Previous *in vitro* studies showed that F3/contactin is required for the surface transport of Caspr (61). Ablation of F3/contactin *in vivo* also leaves NF155 on apposing paranodal myelin disengaged (60). Thus, F3/contactin is a crucial part of the machinery which controls junctional attachment at the paranode, and ultimately influences the physiology of myelinated nerves.

The phenotypes of F3/contactin-deficient and TAG-1-deficient mice indicate that the molecular organization of paranodes, as well as juxtaparanodes may depend on the cis association of neurexin-type transmembrane proteins (paranodin/Caspr, Caspr2) with GPI-CAMs of the IgSF F3/contactin and the TAG-1 families. In addition, a trans association with another IgSF member (neurofascin in the paranodes, possibly TAG-1 in the juxtaparanodes, see Figure 3) may be required. However, there are important differences underlying this intriguing conservation of multimolecular complexes: in the paranodes, their association may lead to the formation of septate junctions, while in the juxtaparanodes it does not.

F3/F11/contactin is also present in the adult retina and cerebellum, mainly on fibers (9, 11). F3/contactin is present in the adult brain at synaptic sites and may be implicated both at pre-synaptic sites to regulate neurosecretion in the hypothalamo-neurohypophysial system and at post-synaptic sites (62-64). Contactin is also implicated in a selective form of hippocampal synaptic plasticity. Long-term depression is impaired in the hippocampus of contactin-deficient mice (65).

4.2. Related molecules

Two other members of the TAG-1/F3/contactin subfamily are BIG-1 and BIG-2 (14-15). BIG-1 is strongly expressed in adult brain and is restricted to subsets of neurons, such as Purkinje cells in the cerebellum, granule cells in the dentate gyrus and neurons in the superficial layers of the cerebral cortex. BIG-2 is 65.2% identical to BIG-1 at the amino acid level (15), while it shows only 20-28% identity at the amino acid level with other neural CAMs. As in the case for BIG-1, BIG-2, mRNA is also at the highest in the adult brain. The BIG-1, BIG-2, TAG-1 and F3/contactin expression patterns in the adult brain have a significant overlap, but also exhibit differences. Both

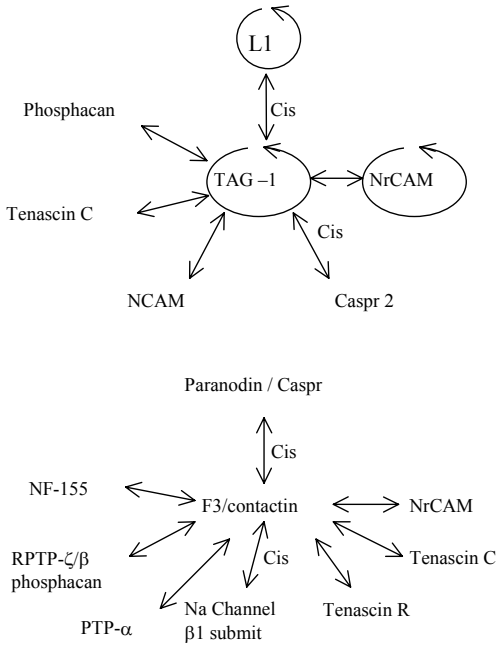


Figure 4. The interactions of TAG-1 and F3/contactin are shown. The circle with the arrow depicts homophilic binding.

BIG-1 and BIG-2 are potent neurite outgrowth promoting proteins (14-15).

The newest members of this subfamily are the adhesion molecules NB-2 and NB-3 (16). The amino acid sequence of NB-2 and NB-3 are 51% similar. Both molecules are neural specific, with NB-2 being expressed in the cerebellum and cerebrum and NB-3 in the cerebellum, cerebrum and spinal cord. NB-3 mRNA expression reaches a maximum at P7 in the cerebrum, while in the cerebellum it increases into adulthood (66).

Recently, a novel member of the IgSF, which is closely related to F3/ F11/contactin, has been identified in chicken and may represent the avian orthologue of NB-2. The unique expression pattern of this molecule, called FAR-2, suggests that it contributes to the somatotopic maps of cerebellar afferents. FAR-2-positive Purkinje cell clusters alternate with FAR-2-negative cell clusters and the protein is expressed by different subpopulations of climbing fibers in the developing cerebellum (17).

Finally, the NCAM-120 isoform, a splice variant of the NCAM gene, is the only NCAM isoform with a GPI anchor (67). During biosynthesis, NCAM-120 is transported to the cell surface, where it is slowly released into the extracellular milieu (68). NCAM-120 is expressed by astrocytes and muscle cells and is expressed later in development than the other NCAM isoforms (69, 70).

4.3. GPI-CAM deficient mice

TAG-1 deficient animals have been generated and preliminary analysis of their phenotype does not point out to major alterations in neural structures (71). The mice grow and reproduce normally, but they have not been examined in behavioral or motor tests. An upregulation of adenosine A1 receptors and increased susceptibility to convulsant stimuli has been reported in these mutants (71). However, a detailed analysis is still missing, especially in regions where a particular function has been assigned to TAG-1. Our analysis of the TAG-1 deficient phenotype in the region of the node of Ranvier (Traka, Goutebroze, Girault, Karagogeos, unpublished) shows that the mice have normal nodal and paranodal regions, as well as normal myelin sheaths. Electrophysiological studies of sciatic nerves also found no abnormalities. However, the juxtaparanodes are molecularly altered (see also section above), making this the only mouse model so far with a defined defect in this region. Thus, TAG-1 is essential for the correct molecular organization of juxtaparanodes and controls the localization of potassium channels and Caspr2 in the juxtaparanodal axolemma.

Contactin-deficient mice display several neurological defects including severe cerebellar ataxia, uncontrolled movements and hindlimb weakness (45). They survive only until postnatal day 18. An analysis of their cerebella reveals defects in granule cell axon guidance and in dendritic projections from granule and Golgi cells. These results indicate a critical role for F3/contactin in mediating interactions between guiding populations of granule cell axons and in supporting dendritic growth of cerebellar interneurons (45). The hindlimb weakness is attributed to the disruption of the junctional attachment at the paranode (see section above) due to the absence of F3/contactin (60).

4.4. A jungle of interactions

As it is evident from the previous section, IgCAMs participate in a variety of complex processes such as adhesion, neurite extension, axon pathfinding and migration. The mechanisms of actions underlying these processes necessitate an impressive number of interactions among IgCAMs and other receptors as well. The level of complexity increases even more, if one takes into account that IgCAMs are able to interact both in the same plane of the membrane (cis) and between opposing membranes (trans). Figure 4 shows a summary of molecular interactions, which involve the GPI-linked neural CAMs of the TAG-1 subgroup. For the best-studied examples, TAG-1/axonin-1 and F3/contactin/F11, it is obvious that more or less the same players (with some exceptions) are involved in a variety of responses.

TAG-1/axonin-1 binds to itself (homophilic binding); in the case of TAG-1, this occurs in trans via the FNIII repeats (72) and in the case of axonin-1, it has been shown that two regions of the molecule (one located in the N-terminal Ig domains and the other in third and fourth FN domains) are involved. Furthermore, the crystal structure of the first 4 Ig domains of axonin-1, as well as distance measurements are consistent with a cis-assisted trans-binding mechanism, which mediates axonin-1-based, homophilic cell-cell contacts (73).

Heterophilic binding of TAG-1/axonin-1 has been shown to take place with a number of other

transmembrane members of the IgSF, namely L1, NrCAM, NCAM, other adhesion molecules, such as tenascin C, phosphacan and the neurexin protein Caspr2 (74-76, 42, Traka, Goutebroze, Girault, Karagogeos, unpublished). All these partners recognize each other either in a cis or trans configuration.

The first examples of cis-interactions have been provided through the study of TAG-1 and axonin-1 with L1 and NgCAM (77-80). A cis TAG-1/L1 interaction recruits the membrane skeleton protein ankyrin in areas of cell contact and this cis-interaction is a result of homophilic binding between TAG-1 molecules *in vitro* (79). This TAG-1-dependent recruitment of the membrane skeleton provides an example of how GPI-linked CAMs are able to transduce signals to the cytoplasm. Cis-binding of axonin-1 and NgCAM (the chick orthologue of L1) has been observed in cultures of primary sensory neurons and cell lines (77). Furthermore, substrate-associated axonin-1 mediates neurite-extension via NgCAM (74). The four N-terminal domains of axonin-1 have are necessary for NgCAM binding (81). Nevertheless, axonin-1 is a neurite outgrowth-promoting substratum in the absence of the NgCAM binding site (81). Similar studies, utilizing different assays *in vitro* have been performed with TAG-1. Although blocking L1 in cultures of primary sensory neurons severely diminished the TAG-1-mediated outgrowth, the requirement for L1 does not seem to be absolute, since DRG from L1-deficient animals extend neurites normally on TAG-1 (82, 42).

Purified axonin-1 and NrCAM interact with each other (83). Binding experiments using chimeric-Fc proteins also showed that the Ig domains of TAG-1 are able to bind to NrCAM and L1, but not to F3/contactin (42). Similar to the interaction with NgCAM, the first four Ig domains of axonin-1 are essential for binding to NrCAM (80-81). Axonin-1 has been implicated as the receptor on sensory neurons, which mediates NrCAM-elicited neurite outgrowth by a trans interaction (84). However, in the reverse assay, where TAG-1, instead of NrCAM, is the neuritogenic substrate, blocking NrCAM function with antibodies does not result in a reduction of neurite lengths. This indicates that TAG-1-elicited neurite outgrowth is independent of NrCAM (42). Other experiments also suggest a direct interaction of axonin-1 with NrCAM, which does not result in neurite growth, but in the guidance of commissural axons (see below, 80). This hypothesis postulates that guidance and neurite growth are distinct functions, which are mediated by different combinations of IgCAMs.

Axonin-1 has been shown to exist in two distinct conformations: an extended conformation capable of forming a cis heterodimer with NgCAM (the chick orthologue of L1) and a horseshoe-like conformation capable of forming the trans link to NrCAM (81). A functional assay for studying these interactions is the stripe assay, in which commissural axons are given the choice to grow on alternating stripes, either containing a mixture of NrCAM and NgCAM or NgCAM alone (80). The growth cones prefer to extend on the NrCAM substrate and this effect is blocked by antibodies to axonin-1. *In vivo*, this situation is equivalent to the choice of axonin-1-positive commissural axons to cross the NrCAM-positive floor plate. When antibodies against axonin-1 or NrCAM are injected *in vivo*, commissural axons commit pathfinding errors suggesting that these two IgCAMs interact at the level of the floor plate (37).

A recent study of the molecular interactions in myelinated adult fibers revealed a new binding partner for TAG-1. TAG-1 and the neurexin protein Caspr2, which were already known to co-localize in the juxtaparanodal regions, were found to associate directly in brain tissues and to form a cis-complex in transfected cells (Traka, Goutebroze, Girault, Karagogeos, unpublished). In TAG-1 -/- mice (see also relevant section) no enrichment of Caspr2 and potassium channels is evident at the juxtaparanodes, indicating a targeting failure of juxtaparanodal components in the absence of TAG-1 protein (Traka, Goutebroze, Girault, Karagogeos, unpublished). The trans-homophilic interaction between glial and axonal TAG-1 appears to precede the cis-interaction between Caspr2 and TAG-1 in the axolemma (Figure 3). The role of the TAG-1/Caspr2 complex in this region could be to maintain/stabilize potassium channels by “locking” them in the juxtaparanodal axolemma.

Unlike most IgCAMs, the other member of this subgroup, F3/contactin/F11, does not bind to itself. Its adhesive properties have been mapped to the FNIII domains (85). The molecule's Ig and FN repeats provide binding sites for a variety of proteins, such as other IgCAMs, tenascins, receptor tyrosine phosphatase beta and Paranodin/Caspr (86-87, 51, 88-91, 56-57, 49, 92, 93-94). F3/contactin expressing cell lines inhibit outgrowth and induce fasciculation of cerebellar granule cell neurites. This effect is mediated by the Ig domains of F3, which bind to NrCAM on the growth cones of granule cells (95-96). The interaction of F11 and NrCAM in the chick spinal cord is important for the axon guidance of proprioceptive sensory neurons (42).

A functional interaction between TAG-1 and F3/contactin has also been shown to modulate neurite outgrowth and fasciculation of cerebellar granule cells (49). When TAG-1 and F3/contactin are co-expressed the inhibitory effect of F3 is blocked (49). How TAG-1 and F3 interact, is not known, but a simple trans-binding is excluded by bead-binding assays (42).

F3/contactin/F11 also mediates the repulsive activity of the oligodendrocyte-derived tenascin-R (51). The Ig domains of F3 and the epidermal growth factor-like (EGF) repeats of tenascin-R are implicated in this interaction. A neuronal cis complex consisting of F3/contactin and NrCAM binds to RPTP-beta/phosphacan, which is expressed by glial cells and presumably plays a role in bidirectional signaling to modulate neuron-glial interactions (97).

More recently, the role of F3/contactin in the establishment of septate-like junctions in the paranodes of

myelinated peripheral nerves has been elucidated (60). The neuronal F3/contactin-paranodin/Caspr complex interacts with glial neurofascin-155 and is likely to form the core structure of paranodal junctions. These junctions are severely disrupted in both F3/contactin and Caspr mutant mice (60, 98-99). In addition to this role, F3/contactin has been shown to associate with sodium channels, which are expressed in the nodal gaps of the nodes of Ranvier (100). The functional relevance of this association may lie in the F3/contactin-mediated increase of sodium channel expression (ibid).

With the exception of FAR-2, the molecular interactions of other members in this subgroup have not been investigated in detail (17). FAR-2 binds weakly to tenascin-R and L1/NgCAM, but not to NrCAM. Thus its binding profile is more similar to F3/F11/contactin than to TAG-1/axonin-1 (ibid).

4.5. Outside-in signaling: the case of GPI-CAMs

Apart from the trans and cis interactions involving their extracellular domains, GPI-CAMs are also linked to intracellular proteins. Recent studies explore the mechanism(s) by which GPI-CAMs are targeted to lipid rafts, as well as their interaction with gangliosides and intracellular signaling molecules, including the Fyn and Src tyrosine protein kinases.

Lipids rafts are membrane microdomains and are also called detergent insoluble glycosphingolipid-enriched domains (DIGs). These microdomains contain cholesterol and also (remove) gangliosides (sialic acid-containing glycosphingolipids or GSLs), which are found in the outer leaflet of the plasma membrane and are thought to be important for neural development. Because a variety of signaling molecules, such as members of the Src family of kinases, are associated with them, these GSL microdomains appear to be involved in signal transduction. The association of ganglioside GD3 with TAG-1 has recently been established (101-102). These data support the hypothesis that TAG-1 transduces signals via Lyn to p80, since antibody-mediated cross-linking of TAG-1 induces Lyn activation and phosphorylation of p80, a presumed target protein of Lyn, which is present in the lipid rafts of cerebellar membranes (101). Recently, a direct interaction of TAG-1 with gangliosides in sphingolipid-enriched membrane domains from cerebellar neurons has also been demonstrated (103).

Work with oligodendrocytes in culture has shown that GPI-anchored proteins from mature oligodendrocytes and myelin can be isolated as complexes, which are associated with glycosphingolipids and cholesterol (104). The plasma membrane microdomains mentioned above contain the GPI-CAMs NCAM 120 and F3/contactin, as well as Fyn and Lyn kinases (105). Compartmentalization of oligodendrocyte GPI-anchored proteins (i.e. F3/contactin) into lipid rafts is a prerequisite for their association with Fyn (ibid).

One possible mechanism for activating intracellular events via GPI-linked CAMs involves their cis-interaction with L1 family members. L1 and NCAM family members are palmitoylated and are also targeted to lipid rafts. However it seems that GPI-linked CAMs can trigger selective signaling pathways, which are distinct from the ones induced by their transmembrane-associated partner. Clustering of axonin-1 with NgCAM induced the formation of cell-cell contacts, which were correlated with a reduction of the axonin-1-associated fyn activity and an increased phosphorylation of NgCAM by the associated casein kinase II-related activity. Thus, axonin-1 and NgCAM trigger distinctive intracellular signals during *in vitro* differentiation depending on their state of association (106).

The example of F3/contactin associating with paranodin/Caspr (see above) and being necessary for sorting paranodin/Caspr to the plasma membrane and lipid rafts is an instructive one and may be common to other GPI-CAMs. Paranodin/Caspr in turn associates with the submembrane spectrin-actin cytoskeleton. It is possible that complexes of more than two proteins are necessary to trigger intracellular signaling via GPI-CAMs.

4.6. GPI and released CAMs

GPI-CAMs exist in a membrane-bound and in a released isoform. In the case of TAG-1, it was shown that the surface form is developmentally regulated in primary neuronal cell cultures, whereas the released form persisted (107). The soluble isoforms are spontaneously released from the neuronal cell surface. In the case of axonin-1, it has been suggested that an endogenous glycosyl phosphatidylinositol specific phospholipase D may be responsible for its release from DRG neurons (108). In fact, the released isoforms of TAG-1/axonin-1 and F3/contactin are functional, as they are able to promote neurite outgrowth (48, our unpublished data). Cerebrospinal fluid and vitreous fluid are natural sources of F3 and axonin-1 protein, respectively (48, 109). As they may bind to the membrane-bound isoforms or to their receptors, the presence of these spontaneously released forms *in vivo* may have important consequences for the regulation of GPI-CAM function.

5. THE IgLON SUBFAMILY

5.1. Expression patterns

Members of this subfamily of GPI-linked CAMs have 3 Ig-like domains (Figure 1B) and represent the earliest GPI-anchored proteins, which are expressed by neurons in a restricted manner. The best-studied members of this subfamily are the limbic system-associated protein (LAMP), the neurotrimin/CEPU-1 (Ntm/CEPU-1) and the opiod-binding cell adhesion molecule (OBCAM).

LAMP is an early marker of cortical and subcortical limbic regions that can promote outgrowth of limbic axons (20). LAMP may be a selective guidance cue for limbic axons (110). Ntm is expressed largely complementary to LAMP, with the highest expression in the sensorimotor cortex (111). In general, its expression pattern suggests a role in the development of thalamocortical and pontocerebellar projections.

CEPU-1 was identified by PCR and is expressed in the developing chick nervous system. Subsequently, it was found to be identical with Ntm. During chick development, Ntm/CEPU-1 is strongly expressed on Purkinje somata, dendrites and axons (21). It is also expressed in the ventral spinal cord (motor neurons), dorsal roots and DRG (24). In early embryonic chick brain, CEPU-1 is initially expressed broadly in the forebrain, midbrain and anterior hindbrain and its expression is subsequently narrowed down to a ring-shaped domain at the mid-hindbrain boundary. In addition, it is expressed in the dorsal aspect of rhombomere 4 and its emigrating neural crest cells (29).

A secreted isoform of CEPU-1 termed CEPU-Se has been identified as an alternatively spliced molecule (28). CEPU-Se is co-localized with CEPU-1 in the retina, cerebellum and DRG neurons. In the cerebellum, CEPU-1/CEPU-Se is expressed mainly on granule cells and in the molecular layer (28).

Of the three IgLON members, OBCAM has a more restricted distribution with the highest expression in the cortical plate and hippocampus (111). AvGp50 is another member of the IgLON subfamily, which is expressed in the chick nervous system (23). AvGp50 shares a 90% homology with LAMP. Therefore, AvGp50 is probably the avian orthologue of LAMP. It is predominantly expressed on the surface of axons, in particular Purkinje and granule cell axons of the cerebellum.

Neurotractin has been identified by PCR and is a new member of this subfamily (25). Two isoforms of neurotractin have been identified, the more abundant long (L) form and the short (S) form. In the chick nervous system, their expression pattern is restricted to subsets of developing commissural and longitudinal axon tracts. Expression of neurotractin increases during development and persists in the adult brain (25). The neurotractin orthologue Kilon is expressed in the rodent cerebral cortex and hippocampus, especially on dendrites and somata of pyramidal neurons (26).

5.2. Interactions involving IgLON proteins

LAMP engages in homophilic, as well as in heterophilic interactions (112-113). It inhibits sensory neurite outgrowth by a heterophilic mechanism (Gil *et al.*, 2002). Its presumed avian orthologue, AvGp50o, does not appear to regulate of neurite outgrowth. Thus it this protein appears to act at later developmental stages (23).

Ntm/CEPU-1 forms non-covalent homodimers in the plane of the membrane, promotes adhesion by a homophilic mechanism and regulates neurite outgrowth (22). In contrast to LAMP, it promotes sensory neurite outgrowth via a heterophilic mechanism. Both membrane and soluble Ntm/CEPU-1 isoforms inhibit the outgrowth of sympathetic neurons (22). Recently, it has been shown that both Ntm/CEPU-1 and LAMP interact in a homophilic and heterophilic manner (113). Another recent report favors the hypothesis that IgLON proteins play a role in cell adhesion, rather than axon guidance (114). OBCAM also can bind homophilically and LAMP, OBCAM and CEPU-1 interact heterophilically with each other (27). It therefore has been hypothesized that IgLON activity will depend on the specific combination of IgLONs, which are expressed by each neuron.

CEPU-Se interacts with CEPU-1 and other IgLON members in a divalent form and does not promote neurite outgrowth from sensory neurons. This is in contrast to the other results showing promotion of neurite outgrowth from DRG neurons with Ntm-Fc (i.e. soluble Ntm) (22). However, the effects of IgLONs on neurite outgrowth may vary depending on experimental design and analysis, which in this particular case was different. It has been hypothesized that CEPU-Se may either prevent the dimerization or clustering of membrane-bound IgLONs or that they inhibit the recruitment of putative receptors in DIGs (28).

Neurotractin promotes neurite extension of telencephalic neurons, where it is more abundantly expressed (25). Neurotractin-L interacts with CEPU-1 and more weakly with LAMP. No homophilic binding or binding to other IgSF members has been observed for neurotractin (25). However, as revealed by antibody perturbation experiments, these interactions are not required in the neurotractin-L-mediated neurite outgrowth of telencephalic neurons (25).

5.3. Related molecules

Thy-1 is the smallest member of the IgSF, consisting of a single Ig domain only and anchored to the membrane via GPI. It is expressed in several tissues, mainly in cells of the T-cell lineage and in neurons (115-116), where it is an abundant cell-surface glycoprotein. In neurons, it represents 2-7% of the total surface protein and has been implicated in cell adhesion and the regulation of neurite outgrowth (117). Thy-1 deficient mice exhibit an impairment in long-term potentiation, which does not affect spatial learning (118). The absence of Thy-1 does not affect normal development or the maintenance of axon pathways, functional synaptic connections, regeneration and plasticity in the CNS (119). An astrocytic binding site has been described for Thy-1 present on neurons in the past (117). Recently, integrin b_3 has been identified as a ligand of Thy-1 on astrocytes. Binding of Thy-1 to integrin b_3 triggers tyrosine phosphorylation of focal adhesion proteins, thus promoting formation of focal contact sites. This argues for a role of Thy-1 in bi-directional signaling between neurons and astrocytes (120). Despite these recent advances, the full spectrum of Thy-1 functions remains elusive.

6. GPI-CAMS IN TWO INVERTEBRATE MODEL SYSTEMS, *DROSOPHILA MELANOGASTER* AND *CAENORHABDITIS ELEGANS*

Shortly after the release of the worm, fruit fly and human genomes, several reports and reviews have appeared commenting on the extent of conservations and novelties among functionally and/or structurally related gene families. Several gene families encoding IgSF members can

be identified in both the Drosophila melanogaster and the Chaenorhabditis elegans genome (121-122). According to these studies, among the estimated ~500 molecules, which contain typical CAM domains (corresponding to roughly 4% of all genes!), there are about 150 protein with immunoglobulin protein domains in the fly genome. Only 70 IgSF members are predicted in the worm genome. These proteins come in different flavors, containing only Ig domains or Ig domains in combination with other 'modules', like the FNIII domain. These proteins associate with the plasma membrane either by a GPI-linker or by a transmembrane segment, or they are secreted. Rather few GPI-linked IgSF members of the CAM subgroup have been functionally characterized. These include Drosophila Wrapper, Klingon, D-Contactin, and Fasciclin II (FasII). In the worm, no detailed analysis of a GPI-IgCAM has been reported so far and, with one exception (see below), no obvious phenotypes have been identified for GPI-IgCAMs in several large-scale RNAi screens.

6.1. *Drosophila melanogaster*

FasII is the fly orthologue of N-CAM, with which it shares a conserved protein domain arrangement (5 Ig domains and 2 FNIII domains) and 23% identity at the amino acid level. Its gene encodes several isoforms, one of which gives rise to a GPI-linked FasII protein (FasII/GPI) (123). *In vitro* FasII mediates homophilic cell aggregation (123). On the basis of several loss-of-function genetic analyses, it has been implicated in several processes in the developing and adult fly nervous system. These range from the control of axon fasciculation in embryonic longitudinal fascicles (123, 124) and motoraxons (125), to the control of proneural gene expression (126), to the establishment of synapses at the neuromuscular junction (NMJ), the modulation of synaptic plasticity and to learning and memory (127-131). Importantly, all these studies relate functions of the transmembrane FasII isoform(s) (FasII/TM/PEST+ and FasII/TM/PEST-, 123). For example, all rescue experiments of the mutant FasII phenotypes have been conducted by re-expressing the transmembrane isoform FasII/TM/PEST- in a FasII mutant genetic backgound. Therefore these experiments do not address the question whether FasII/GPI also contributes to these functions or has distinctive functions of its own. Zito *et al.* (132) showed that a stretch of amino acids in the C-terminal cytoplasmic domain of FasII is necessary and sufficient to drive the synaptic localization of a heterologous protein. They postulated a direct interaction with the PDZ-containing protein Disc-large. This suggests that FasII/GPI has a different (more widespread?) localization on the cell membrane. However it is not known whether its expression is spatially and temporally regulated and how this might modulate FasII function.

The Drosophila Wrapper, F3/Contactin, and Klingon proteins only associate with the plasma membrane via their GPI-moiety. They all contain Ig and FNIII domains. In the Drosophila embryo, contralateral axons cross the midline in two bundles in each segment, forming the anterior and posterior commissures. Wrapper is expressed by a subset of midline glial cells, which normally contact and ensheath the commissural axons. An electron microscope analysis showed that these glial cells differentiate and migrate normally in a wrapper mutant background, but fail to contact the commissural axons and die. As a consequence of this loss of glial cells, the anterior and posterior axonal commissures do not separate properly (133).

Drosophila F3/Contactin (DCont) (CG1084) has been recently cloned (134). It consists of 6 Ig domains and 4 FNIII domains, and has a C-terminal sequence, which suggests the protein being GPI-linked. No TAG-1/axonin orthologue has been identified in the Drosophila genome. Therefore, Dcont might be derived from a common TAG1/Contactin ancestor and evolved before the divergence of vertebrate and invertebrate lineages (but see below).

DCont co-localizes with NeurexinIV (a component of septate junctions, which is related to vertebrate NCP1/ Caspr/ Paranodin) in epithelial cells, and its apically-restricted expression is dependent on NrxIV function. Dcont also co-localizes with Neuroglian (Nrg, the single Drosophila member of the L1 family), which is also a component of septate junctions in insect epithelial cells. In addition, Dcont is co-expressed with NrxIV and Nrg by glial cells in the peripheral nervous system. The three molecules appear to interact physically in a tripartite complex, which mediates cell-cell adhesion in epithelia. Embryos, which are deficient for DCONT, exhibit an abnormal distribution of NRX IV and NRG along the basolateral membrane. These data, together with what is already known for the vertebrate counterparts, suggest that DCont may play an important role in the biogenesis of septate junctions.

Drosophila Klingon protein consists of 3 Ig and 1 FNIII domain plus a GPI-moiety and is involved in the development of photoreceptor cells (in particular R7 and R8) in the fly eye (135). It mediates homophilic adhesion in the S2 cell aggregation assay (135). Therefore, it has been postulated that Klingon mediates cell-cell contacts in the Drosophila eye and that the loss of these contacts induces cell death in the mutant background. This observation is reminiscent of the loss of glial cells in wrapper mutants.

6.2. *Caenorhabditis elegans*

GPI-linked IgSF proteins in the worm include TAG-1/axonin-1 (C33F10.5/6), Klingon (C53B7.1) and Wrapper (F41D9.3). A FasII/N-CAM orthologue has also been predicted (F02G3.1), but it is yet unclear, whether it also encodes a GPI-linked isoform (122). Classical mutant have not been identified for any of the corresponding genes, nor have their functions been analyzed by RNA-interference or by the expression of dominant-negative alleles.

All of them have been 'knocked-out' in a least one of the large-scale genomic functional studies of the worm genome by RNAi (136-138). However, in all these studies only a limited subset of all possible phenotypes was analyzed, specifically sterility, embryonic lethality and size and in some cases also grossly abnormal morphology and crawling behavior. Therefore, more subtle or tissue specific defects were probably missed. In addition, the RNAi

approach is known not to work well for the genes, which are expressed in the nervous system. Therefore, all phenotypes linked to GPI-anchored proteins with specific neural expression patterns were probably not identified (139).

C33F10.5 / 6 encodes the predicted worm orthologue of TAG-1/axonin-1 (122). In their report, Teichmann and Chothia actually predict a transmembrane domain for C. elegans TAG-1. Therefore, further experimental analysis is necessary to ascertain, whether its gene product is a GPI-linked IgCAM. Neither its expression pattern nor its functions are known. In their large-scale screen, Maeda *et al.* (137) performed RNAi against the C33F10.5 sequence (clone yk379e12) and reported a complex phenotype, which includes small body size, sterility and abnormal gonad morphology. In contrast, Kamath *et al.* (138) did not report any phenotype for this gene. These data show no correlation with the known functions of vertebrate TAG-1. A more detailed analysis of C. elegans TAG-1 should clarify these contradictions and open issues.

At this point, it is worth noting that LAD-1, the worm orthologue of L1/Nrg, has been cloned and extensively characterized (131). In their beautiful study, Chen and colleagues show that LAD-1 is expressed in multiple tissues, including in the epidermis and some axons. It appears to accumulate at the sites of cell-cell contacts, where ankyrin is enriched. These findings agree well with the results obtained for its vertebrate counterparts. In addition to its interaction with ankyrin, LAD-1 is also phosphorylated in an FGFR-dependent manner at a conserved cytoplasmic tyrosine residue. Interestingly, phosphorylated LAD-1 does not co-localize with ankyrin at sites of axon-body wall contact and cell-cell contacts in epithelia, which are mechanically stressed. Although an RNAi approach did not yielded any results for this gene, Chen and colleagues inhibited LAD-1 function by driving expression of a dominant-negative form of the protein. The resulting phenotypes are pleiotropic and include strong germline and early embryonic phenotypes, uncoordinated movement and gonadal morphogenesis defects. Given the reported abnormal gonad morphology in TAG-1 RNAi experiments, the last phenotype could be particularly interesting with respect to a possible functional interaction between LAD-1 and C. elegans TAG-1 (137).

C. elegans Klingon appears to be encoded by the predicted C53B7.1 gene (122). In their large scale RNAi screen, Kamath *et al.* (138) do not report any phenotype for this gene and no further detailed analysis of C53B7.1 has been reported so far.

C. elegans wrapper is encoded by the F41D9.3 sequence and has been also named wrk-1 (wrapper kilon rega) (122, and Wormbase). As for the C53B7.1 gene, Kamath *et al.* (138) do not report any RNAi phenotype and no other molecular or functional analysis of this gene has been published.

The C. elegans representative of the N-CAM/FasII gene family is encoded by the predicted gene F02G3.1. Again, no expression or functional study has been published for this gene and Kamath *et al.* (138) do not report a phenotype for it.

In the nervous system, the emerging theme for Drosophila GPI-IgCAMs is that they contribute to cell-cell contacts (and to a large extent axo-glia interactions), which are essential for the survival of and the proper patterning and/or functioning of neuronal cells. Due to the lack of corroborating functional analyses of the corresponding C. elegans genes, it is still unknown, whether this will hold true also in nematodes. In Drosophila and C. elegans, most IgSF gene families are usually represented by one, single gene. This obviously eliminates the molecular and functional redundancy, which complicates their functional analysis in vertebrate species, and will facilitate the future analysis of their *in vivo* functions by genetic loss-of-function approaches.

So far, no extensive biochemical studies are available on how these proteins integrate into cellular signaling networks and how they interact with the cellular cytoskeleton. Certainly, the constant exchange of information with vertebrate model systems will also increasingly benefit our understanding of the biological roles of GPI-CAMs in invertebrate organisms.

7. PERSPECTIVES

The understanding of the mechanisms, by which IgCAMs function, has recently made significant progress. The analysis the expression patterns of CAMs have provided useful and novel insights into their biological functions (e.g. F3/contactin and TAG-1 in myelinating fibers are involved in axon-glial interactions). Structural and biochemical studies have contributed to the analysis of their multiple molecular interactions. Specifically revealing are the differences in ligand-receptor interactions during development, when compared with the situation in adults. At the same time, multiple functional aspects of TAG-1 and F3/contactin are conserved. For example, many CAM binding partners act in cis configuration and one has to appreciate the importance of lipid anchors for segregating CAM-containing signaling complexes into DIGs. We still need a better understanding, how the internal signaling machinery is turned on. Furthermore, an analysis of the Drosophila and C. elegans GPI-CAM orthologues will enable us to use additional experimental approaches, which are unique to these organisms. This will nicely supplement the wealth of information, which already exists for the vertebrate GPI-CAMs.

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