Immunoglobulin superfamily receptors: cis-interactions, intracellular adapters and alternative splicing regulate adhesion

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The immunoglobulin domain is a module found in vertebrates and invertebrates. Its ability to form linear rods when deployed in series, combined with its propensity to bind specifically to other proteins has made it ideal for building cell surface receptors and cell adhesion molecules. These features have resulted in the incorporation of immunoglobulin domains into many hundreds of cell surface molecules. Recently three major advances have been made in understanding immunoglobulin receptors. One is the recognition that their intracellular binding partners are likely to link to multiple cell surface molecules, allowing cross-talk or oligomeric complex formation. A second, but related phenomenon, is their participation in cis-interactions on the extracellular surface that regulate signaling or adhesion. The third is the dramatic ability to form dozens to thousands of different isoforms via alternative splicing. Although antibodies may have been the first example of immunoglobulin-domain-containing proteins using cis-interactions to form receptor like molecules, and the grandest instance of diversity production from limited genetic material, these are clearly old ideas in this superfamily.

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Abbreviations

AJ	adherens junction
CAM	cell adhesion molecule
CST	corticospinal tract
DCC	deleted in colorectal carcinoma
DSCAM	Down syndrome cell adhesion molecule
Ena	Enabled
FN3	fibronectin type III-like domain
Ig	immunoglobulin
IgSF	Ig superfamily
IgCAM	cell adhesion molecule of the IgSF
JAM	junctional adhesion molecule
MAGUK	membrane-associated guanylate kinase
Mena	mammalian Enabled
PDZ	PSD95, DLG, ZO-1
Robo-1	roundabout-1
RYK	related to tyrosine kinase
TJ	tight junction
Y2H	yeast two hybrid cloning method

Introduction

The completion of the human genome has provided the answer to the question of how many members there are of the immunoglobulin superfamily (IgSF) (final score: humans 765, flies 140 and worms 64, see below [1,2]). More importantly, it will allow cell biologists to get on with the sticky business of studying cell adhesion. Over the past 12–18 months some of the most interesting news about the IgSF has concerned alternative splicing: cis-interactions in the plane of the membrane and interactions with intracellular targets. These stories bear directly on how adhesion is controlled and what cellular processes it influences.

Using criteria defined by the InterPro database (http://www.ebi.ac.uk/interpro), it has been estimated that 765 human genes contain Ig domains, which means that the IgSF represents one of the largest protein superfamilies in the human genome [1••]. Furthermore, Ig domains have a high tendency to be present in large modular multidomain proteins, which is illustrated by the observation that Ig domains occur along with more than 60 different other domains (as defined by the Pfam database http://www.sanger.ac.uk/Software/Pfam/index.shtml). The human genome encodes five times more IgSF members than the *Drosophila* genome and 12 times more than the *Caenorhabditis elegans* genome. This is due, in part, to the invention of the immune system. However, it may also be caused by the greater complexity of developmental processes in which IgSF members are involved in vertebrates [1••,2••]. It is worth remembering, however, that the rich alternative splicing of cell adhesion molecules (CAMs), such as Down syndrome cell adhesion molecule (DSCAM) (see below), neurofascin and protocadherins, dramatically increases the number of available cell–cell recognition molecules, as the isoforms from those molecules probably exceed the number of genes in the insect or vertebrate genome!

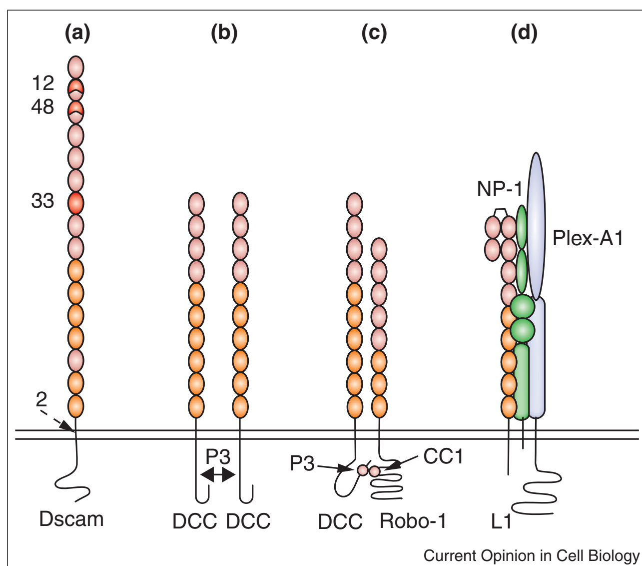
Ig proteins as axon guidance receptors

During nervous system development, outgrowing axons are guided by specialized subcellular structures, called growth cones, which have distinct classes of surface receptors for the molecular cues in their environment. In the past year, new functional principles have emerged for several guidance receptors of the IgSF, in particular DSCAM, DCC (deleted in colorectal carcinoma), Robo-1 (roundabout-1) and L1.

Drosophila DSCAM comes in multiple variants

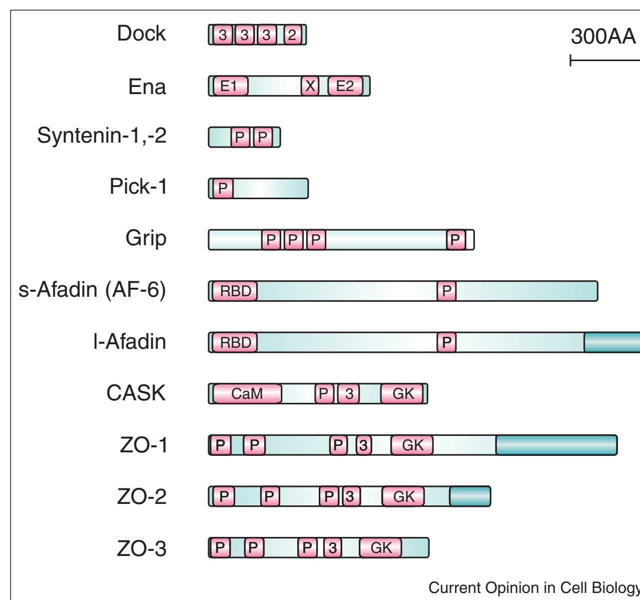
Alternative pre-mRNA splicing generates molecular diversity in many systems, including the nervous system. Among CAMs, there are high numbers of isoforms for neurexins (more than 1000) and for neurofascin (more than 50) [3,4]. Recently, a novel *Drosophila* homologue of DSCAM has been described (Figure 1a), which shows striking sequence diversity [5••]. Four regions of the protein are coded for by alternative exons, which could give rise to more than 38,000

Figure 1



Axon guidance receptors of the IgSF: alternative splicing and cis-interactions. (**a**) Dscam is an axon guidance receptor in *Drosophila* [5••] containing Ig domains (red ellipses) and FN3 domains (brown ellipses). Molecular diversity is generated by alternative splicing (number of variants is given) of half and complete Ig domains (dark red areas) and of the transmembrane domain. (**b**) DCC forms multimers via interactions of the cytoplasmic domains, which mediates chemoattraction of growth cones [12]. (**c**) DCC also interacts with Robo-1 via conserved elements in the cytoplasmic regions (P3, CC1), which modulates DCC function [13••]. (**d**) Neuropilin-1 (green) interacts directly with Plexin-A1 (blue) and also with L1 [62••]. Although formation of a ternary complex has not yet been demonstrated directly, it is consistent with functional data. Domain arrangement of L1 is suggested on the basis of aminoproximal similarity with axonin-1 [66].

Figure 2



Modular intracellular adapter proteins. Red boxes represent conserved domains, abbreviated as follows: 2, SH2 domain; 3, SH3 domain; E1, Ena/VASP-homology domain 1; E2, Ena/VASP homology domain 2; X, proline-rich region; P, PDZ domain; RBD, ras binding domain; CaM, CaM kinase domain, GK, Guanylate kinase domain. Dark green boxes represent actin-binding sites [6,21••,26,36,45,50,51•,53,54]. Only single isoforms of ZO-proteins are depicted.

distinct isoforms! cDNA analyses revealed that a considerable proportion of those forms are expressed, suggesting that they are part of a combinatorial cell surface code that may specify subpopulations of neurons. Indeed, Dscam contributes to axon guidance of specific photoreceptor axons. Dscam's cytoplasmic domain interacts with Dreadlocks (Dock), an adapter protein that consists exclusively of SH2 and SH3 domains (Figure 2). Dock binds directly to Pak (p21-activated kinase), a conserved regulator of the actin cytoskeleton [6]. Thus, a signal transduction pathway is emerging that could link Dscam to remodeling of the actin cytoskeleton in growth cones.

Dscam has two counterparts in vertebrates, DSCAM and a protein termed KIAA1132 [7•,8]. Dock has several vertebrate relatives, including two forms of the proto-oncogene protein Nck [9]. It remains to be established whether the Dscam–Dock pathway exists in vertebrates and whether Nck or Nck-related proteins interact with vertebrate DSCAM-related receptors. In humans, DSCAM has been genetically linked to nervous system defects [8] and congenital heart disease [10]. It will be interesting to see how DSCAM homophilic binding [7•] influences intracellular signaling or if heterophilic binding partners for DSCAM exist.

Receptor multimerization contributes to DCC's signaling

In bilaterally symmetrical animals, both sides of the nervous system have to communicate with each other, using specific types of axons that cross the midline of the nervous system to project to target regions in the contralateral side. Several attractive and repellent molecules have been implicated in this process. DCC is an IgSF receptor for the laminin-related attractive midline guidance cue netrin [11]. In a recent study, DCC signaling was analyzed using biochemical approaches in combination with quantification of growth cone responses of transfected neurons [12]. Netrin binding to DCC was found to induce multimerization of the receptor (Figure 1b), which was mediated by a conserved sequence element in the cytoplasmic domain, termed P3. To assess if multimerization contributes to receptor function of DCC, modified variants lacking P3 were tested in growth cones of transfected neurons. Expression of a P3-deleted form of DCC leads to lack of attraction by netrin-1, which strongly suggests that multimerization of DCC plays a role in signal transduction.

DCC forms a receptor complex with Robo-1

After commissural axons have crossed the nervous system midline, their growth cones express DCC and the distantly related Robo-1, a receptor for the midline repellent slit, a modular ECM protein [11]. Thus, those growth cones seem to recognize two contradictory midline signals, attraction by

netrin-1 and repulsion by slit. A recent study showed that if those growth cones were exposed to netrin-1 and slit-2 simultaneously, the attractive effect of netrin-1 was completely neutralized [13••]. Furthermore, biochemical analyses and yeast two-hybrid (Y2H) experiments showed that both receptors heterodimerize via conserved sequence elements in their cytoplasmic domains (Figure 1c), namely CC1 (conserved cytoplasmic region-1) in Robo-1 and P3 in DCC (which is also involved in DCC multimerization). Formation of the DCC–Robo-1 complex is crucial for the neutralizing effect of slit-2, suggesting that integration of contradictory axon guidance signals may take place directly at the growth cone membrane.

Linking Robo-1 to the downstream target Ena

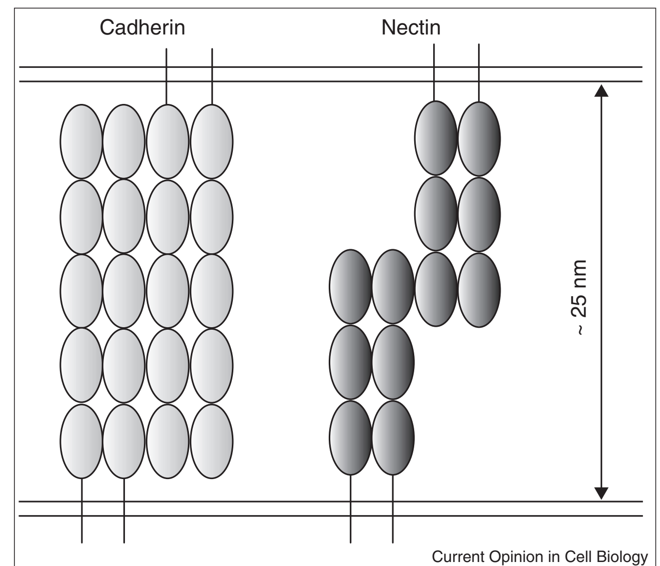
Robo-1, and the related subgroup members Robo-2 and Robo-3, are differentially expressed in a combinatorial manner by subpopulations of longitudinal axons in *Drosophila* [14•]. Recently, a series of complementary gain-of-function and loss-of-function experiments strongly suggested that this combinatorial expression of Robo-related receptors contributes to pathway selection by longitudinal axons [15••,16••]. Considerable progress has also been achieved in understanding the intracellular interactions of Robo-1. Its cytoplasmic region contains a conserved sequence element, called CC2 (conserved cytoplasmic region-2), which is a candidate binding site for Ena-related proteins [17]. These intracellular phosphoproteins (Figure 2), which include *Drosophila* Ena (Enabled) and a mammalian relative, termed Mena (mammalian Enabled), have been implicated in cell motility and actin dynamics. Ena mutations are linked to axon guidance in *Drosophila* [18], and Mena knockout mice have defects in the corpus callosum and hippocampal commissure [19].

A recent study now demonstrates that Ena does in fact directly bind to Robo-1 and that the CC2 region is crucial for this interaction [20••]. These results, which implicate Ena in Robo-1-dependent growth cone repulsion, are supported by analyses of Mena function in a fibroblast model system, which reveal a negative role for Ena-related proteins in cell motility [21••]. However, genetic analyses also demonstrated that Robo-1 binds to Ena as well as the tyrosine kinase Abl and that they play opposing roles in Robo-1's signal transduction pathway [20••]. Furthermore, additional functions of Abl and Ena-related proteins in axon guidance have been reported, including interactions of Abl and Ena with the protein tyrosine phosphatase Dlar [18], interaction of Abl with the guanine nucleotide exchange factor Trio [22] and binding of the Ena-related protein EVL to the transmembrane semaphorin Sema6A-1 [23•].

IgCAMs team up with PDZ adapter proteins

Recently, yeast two-hybrid (Y2H) approaches have led to the identification of intracellular binding partners for several Ig proteins, including, nectins, junctional adhesion molecules (JAMs) and neurofascin. These interactions are discussed in the following paragraphs.

Figure 3



Model of trans interactions of cadherins and nectins at AJs, on the basis of surface force measurements [33] and biochemical analyses [31•]. Ig-like domains (black ellipses) and cadherin-like domains (grey ellipses) have similar sizes.

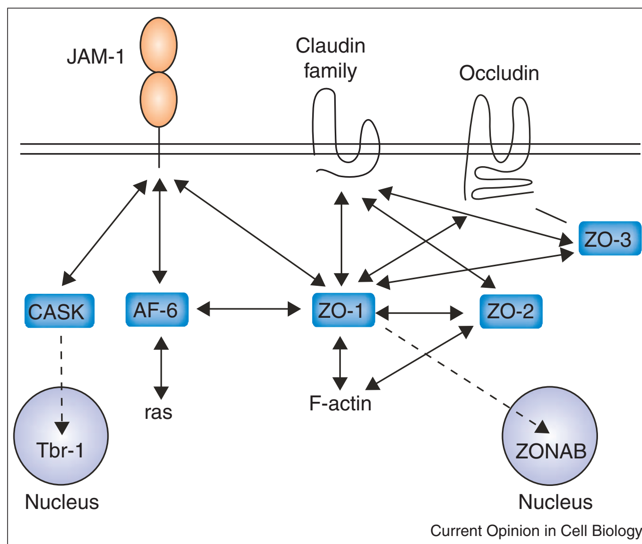
Afadin, a common denominator of adherens junctions and tight junctions

Apical junction complexes of epithelial and endothelial cells consist of adherens junctions (AJ), which form adhesion belts, and tight junctions (TJ), which control paracellular permeability. Recently, an intracellular adapter protein, termed afadin, emerged as a player at both types of junctions [24–26]. It contains a ras-binding domain, suggesting that small GTPases may regulate AJs and TJs, and a PDZ (PSD95/DLG/ZO-1) domain, which interacts with several transmembrane proteins (see below). PDZ domains, which are 80–90 amino acids long, have been identified in over 90 proteins, mostly associated with the cytoplasmic face of the cell membrane. Two splicing variants of afadin have been identified (Figure 2), namely L-afadin, which binds to actin filaments, and S-afadin (originally described as AF-6), which does not [26]. The function of afadin at cell junctions seems to be crucial, as AF-6 deficiency leads to embryonic lethality in mice [24,25].

Nectins are afadin-binding IgCAMs at adherens junctions

It is generally thought that the cadherin–catenin system is the primary adhesive component of AJs. However, recent studies suggest that the nectin family of IgCAMs (cell adhesion molecules of the IgSF) also represents an important component of AJs [27]. Nectins form a subgroup of the IgSF with three Ig domains, and incidentally serve as receptors for αherpesvirus [28]. Nectin-1 (also termed PRR1, PVRL1, HveC and HigR), nectin-2 (PRR2, PVRL2 and HveB) and nectin-3 (PRR3) all bind afadin [28,30,31•]. Nectins form cis-homodimers that undergo homophilic and heterophilic trans-interactions with each other [31•] to mediate cell–cell adhesion (Figure 3).

Figure 4



Adhesion proteins at the tight junction. Intracellular interactions of JAM-1 and other tight junction proteins in epithelial cells are shown [43••,44,47••]. Doubleheaded arrows indicate direct molecular interactions, and dashed arrows indicate an influence on nuclear gene transcription [45,46••]. AF-6 has a larger isoform, termed l-afadin, which contains an F-actin-binding site and which interacts with the src-homology-domain-containing protein ponsin/SH3P12 [26,67].

Nectin-1 is recruited to cadherin-based AJs by the binding of its carboxy-terminus to the PDZ domain of afadin [27]. Although afadin seems to interact with α-catenin, it remains to be established whether additional proteins also contribute a molecular link to the cadherin–catenin system [32]. Interestingly, the cooperation of nectins with cadherins in AJs supports a model of homophilic cadherin interaction via fully interdigitated, antiparallel extracellular domains (Figure 3; [33]) rather than via interactions restricted to the amino-terminal domains of cadherins.

Orofacial development and pathway convergence on afadin

Truncation of the cytoplasmic region of nectin-1 causes an autosomal recessive human hereditary disease, namely cleft lip/palate-ectodermal dysplasia. This disease led Suzuki *et al.* to suggest that binding of nectin-1 to afadin plays a role in embryonic development [34••]. Similar developmental defects have also been observed in mice deficient for the receptor 'related to tyrosine kinases' (RYK) or in mice simultaneously deficient for EphB2 and EphB3 kinases [35••]. Interestingly, both RYK and the two Eph kinases bind afadin [36,37], and the Eph kinases phosphorylate RYK [35••]. Therefore, nectin-1, RYK and the Eph kinases appear to cooperate in specific histogenetic processes during orofacial development. It remains to be established whether those interactions are restricted to adherens junctions.

Two emerging links from tight junctions to the nucleus

Junctional adhesion molecule-1 (JAM-1) is the prototype of a subgroup of epithelial and endothelial IgCAMs with two Ig-like domains [38] that also includes JAM-2 [39] and VE-JAM [40]. Cell culture experiments and *in vivo* studies indicate that this tight junction transmembrane protein plays a role in trans-endothelial migration of leukocytes [41]. JAMs bind homophilically [42] and also bind to three distinct adapter proteins containing PDZ domains (Figure 2); AF-6, ZO-1 and CASK (calcium/calmodulin-dependent serine protein kinase) [43••,44]. Interactions with AF-6 and ZO-1 provide molecular links from JAM-1 to other tight junction components [45] and also to the actin cytoskeleton (Figure 4). Interestingly, CASK has been reported to interact with a T-box transcription factor [44,46••], whereas ZO-1 interacts with a Y-box transcription factor [47••]. Thus, two feedback mechanisms from tight junctions to the transcriptional machinery in epithelial cells have now been defined.

Intracellular interactions suggest functional differences among L1 family members

Neurofascin is a member of the L1 subgroup of neural IgCAMs that has been implicated in neuron–neuron and neuron–glia interactions during nervous system histogenesis. Proteins of the L1 subgroup, which also includes L1/NgCAM, NrCAM and CHL1, share a highly conserved cytoplasmic region with phosphorylation sites and interaction motifs for the membrane skeleton protein ankyrin-G [48,49]. The carboxyl termini, however, differ among L1 family members, suggesting that distinct additional intercellular binding partners may exist. A recent study used Y2H screening to identify a novel neurofascin-binding protein, termed syntenin-1 [50,51•]. This adapter protein contains two PDZ domains (Figure 2) and the carboxyl terminus of neurofascin interacts with the second domain. Binding to other L1 family members could not be demonstrated, suggesting that syntenin-1 may play a role in functions that are specific for neurofascin. As syntenin also interacts with other neural receptors [36,37,52–54], which interact with additional PDZ-domain-containing proteins (Figure 2), neurofascin emerges as a player in a network of cross-talking receptors (Figure 5). As mentioned above, one of the network members, CASK, binds a T-box transcription factor providing a link to the regulation of neuronal differentiation [46••].

L1 cis-interactions – new twists and turns

L1 binds homophilically in trans and also has several distinct heterophilic binding partners, such as the neural cell recognition molecules TAG-1/axonin-1 and F3/F11/contactin, the ECM protein neurocan, and integrins [55,56••,57]. In the past year several new ideas about L1 function have emerged that may have general implications for IgCAMs, especially those with fibronectin-like (FN) domains. The first idea concerns L1 multimerization in cis. The L1 FN3 domain has long been known to be the site of proteolytic cleavage of L1 *in vivo* [58,59••], and indeed other L1 family members and even phosphotyrosine phosphatases with related structures show similar cleavage in their FN domains. Montgomery and colleagues [56••]

showed that a dibasic region in the third FN domain binds to integrins and also mediates trimerization of this domain. Treatment with plasmin dissolved the FN3 trimers and also inhibited integrin-based adhesion to the FN3 domain. Montgomery and colleagues proposed that L1 trans binding might alter L1 conformation, permitting FN3 trimerization and enabling integrin binding.

Support for the idea of trimerization of L1 came from studies by Hall *et al.* [60] who showed that artificially trimerized L1, containing all six Ig and all five FN domains, was significantly better at promoting attachment and neurite growth of PC12 cells than monomers of L1. Stallcup [61] reported that the FN3 domain is essential for L1-dependent neurite growth. The function of FN3 in L1 homophilic binding has not been tested directly, so it is difficult to know whether the role of FN3 in CNS axon growth is principally in trans binding or in multimerization, or both. Nonetheless, the independent data linking L1 trimerization, integrin binding, proteolytic cleavage and neurite growth to FN3 argues that this is a critical domain for L1 function.

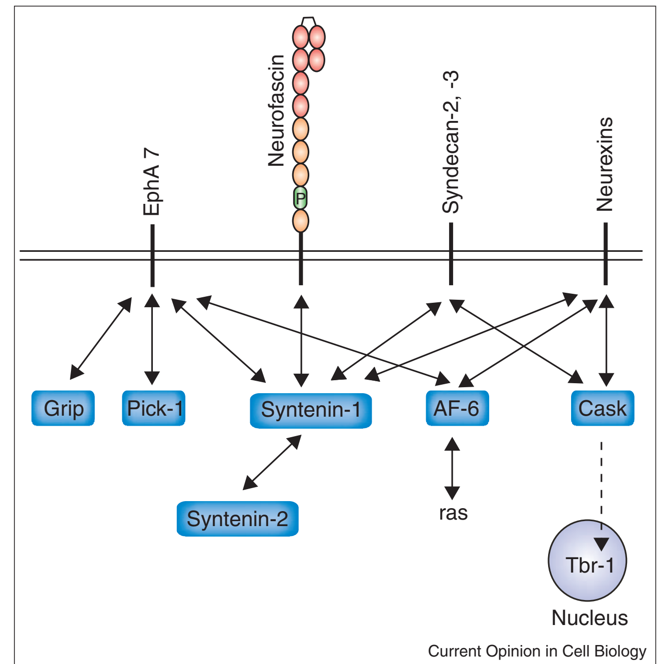
L1 gained a new cis-interaction partner in 2000, and it came from an unexpected direction [62••]. A search for molecules involved in the crossing of corticospinal tract (CST) axons from one side of the brainstem to another implicated semaphorin 3A (Sema3A), a repulsive guidance factor. It was previously known that L1 knockout mice are defective in CST crossing. Neurons from these mice failed to collapse in response to Sema3A, and biochemical experiments showed association of L1 and the semaphorin receptor, neuropilin. The physical interaction between neuropilin and L1 and the altered behavior of neurons from L1 knock-out mice in functional assays *in vitro* argue that L1 serves as a co-receptor along with neuropilin for Sema3A signal transduction (Figure 1d).

The regulation of L1 shedding by the ADAM class metalloproteases [59••] appears to be part of a theme, as similar proteases were implicated in axon guidance. Netrin-1 chemoattraction is mediated by DCC (see above), and this is potentiated by metalloprotease inhibitors [63••]. Also, contact-mediated repulsion via an Eph receptor and ephrin-A2 requires Kuzbanian, an ADAM class metalloprotease [64••]. This begs the question: do ADAM class proteases provide a mechanism for global regulation of fasciculation and defasciculation?

Clathrin-based regulation of adhesion

Adhesion through CAMs can be altered in a variety of ways, including transcriptional regulation, cytoskeletal interactions, conformational changes and proteolytic cleavage. In the immune system, it is known that regulated externalization of CAMs can rapidly increase adhesion. It has now been shown that L1-mediated adhesion can be rapidly altered by clathrin-mediated internalization [65•]. If clathrin-mediated endocytosis is blocked, L1-based adhesion increases significantly in 5–10 minutes. Any mechanism, such as phosphorylation or protein–protein interactions, that inhibits L1 interactions with clathrin adaptors could prevent L1 internalization and thereby stabilize cell–cell contacts. This mechanism for regulating adhesion is likely to apply to numerous CAMs.

Figure 5



Common intracellular interactions of neurofascin and other neural receptors. Doubleheaded arrows indicate direct molecular interactions [36,37,50,51•,53,54], a dashed arrow indicates an influence on nuclear gene transcription [46••]. Neurofascin occurs in multiple isoforms, and only a single form is depicted [3]. AF-6 has a larger isoform, termed l-afadin, which contains an F-actin-binding site and which interacts with the src-homology-domain-containing protein ponsin/SH3P12 [26,67]. Domain arrangement of neurofascin is proposed on the basis of aminoproximal similarity with axonin-1 [66]. P, proline/alanine/serine-rich domain. The color coding is the same as Figure 1.

Conclusions

CAMs, such as NCAM and N-cadherin, were originally identified when investigators began studying cell and tissue sorting events in early development. At that time it was imagined that cell adhesion was primarily regulated by expression levels of CAMs. Subsequent studies suggested that CAM interactions with the cytoskeleton stabilized or enhanced adhesion. Next, links between CAMs and second messenger systems were uncovered. It should come as no surprise that many of the intracellular proteins that interact with CAMs are the kind of molecules that link them to the cytoskeleton or promote cis-interactions with other cell surface molecules. These interactions could increase adhesion by enhancing avidity or decrease adhesion by disassembling contact sites. However, the function of IgSF members as mutual cis-regulators (e.g. Robo and DCC) and co-receptors (e.g. L1 and neuropilin) opens a

new and important research arena. Exploring the functions of IgSF proteins will require 'lateral thinking'. Old prejudices about CAM function will have to be set aside so that novel functions can be adequately investigated.

Update

Recent work has shown that L1 family members have a horseshoe shape, with the first two Ig domains folded back along the third and fourth Ig domains [68••]. In addition, a third member of the NCAM family, named PCAM, has been identified [69••]. PCAM is a good candidate for serving a compensatory role in NCAM knockout animals. Finally, a novel member of the F11/F3/contactin subgroup, called FAR-2, was recently characterized [70••]. The authors, Plagge *et al.*, suggest that it may contribute to map formation within this brain region.

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- •• of outstanding interest

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