

Mini review

THE INTERACTION BETWEEN L1-TYPE PROTEINS AND ANKYRINS - A MASTER SWITCH FOR L1-TYPE CAM FUNCTION #

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Abstract: L1-type cell adhesion molecules (CAMs) are important mediators of neural differentiation, including axonal outgrowth and pathfinding and also of synapse formation and maintenance. In addition, their interactions with cytoskeletal components are highly conserved and regulated. How these different aspects of CAM functionality relate to each other is not well understood. Based on results from our and other laboratories we propose that ankyrin-binding to L1-type CAMs provides a master switch. The interaction with ankyrins directs L1-type adhesive proteins into different functional contexts, either ankyrin-independent functions, such as neurite outgrowth and axonal pathfinding or into ankyrin-dependent functions, such as L1's role at axon initial segments (AIS), paranodal regions, synapses and in dendrites.

Key words: Cell adhesion, Ankyrins, Membrane skeleton, Tyrosine phosphorylation, Neurite outgrowth, Neuromuscular junction, Synapse, Synaptogenesis, *Drosophila*

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Abbreviations used: AIS – axon initial segments; CAM – cell adhesion molecule; EGFR – epidermal growth factor receptor; FGFR – fibroblast growth factor receptor; GF – giant fiber; GPI – glycosyl phosphoinositol; NMJ – neuromuscular junction; RTK – receptor tyrosine kinase; SAP – synapse-associated proteins; TTMn - tergotrochanteral motoneuron; VUM – ventral unpaired median

INTRODUCTION

One of the major connections in non-erythroid cells between the actin-spectrin membrane skeleton and the plasma membrane is provided by the interaction between ankyrin linker proteins and the cytoplasmic domains of L1-type CAMs. L1-type proteins form a functionally and structurally conserved family of type 1 transmembrane proteins, which have been identified in the genomes of a wide range of species, from nematodes to humans [1]. Whereas invertebrate species usually contain only one L1-type gene, such as the *Drosophila neuroglian* gene, most vertebrate genomes encode four L1-type proteins, named L1-CAM, CHL1, neurofascin and NrCAM. They share a common extracellular protein domain structure, typically consisting of six aminoterminal immunoglobulin domains that are followed by 5 fibronectin type III domains (Fig. 1). The size of their cytoplasmic domains varies from 85 to 148 amino acid residues and contains several highly conserved protein sequence motifs. L1-type proteins are highly expressed in neuronal and glial cells. However, they also have important functions in other tissues, e.g., for epithelial stability in *Drosophila* by

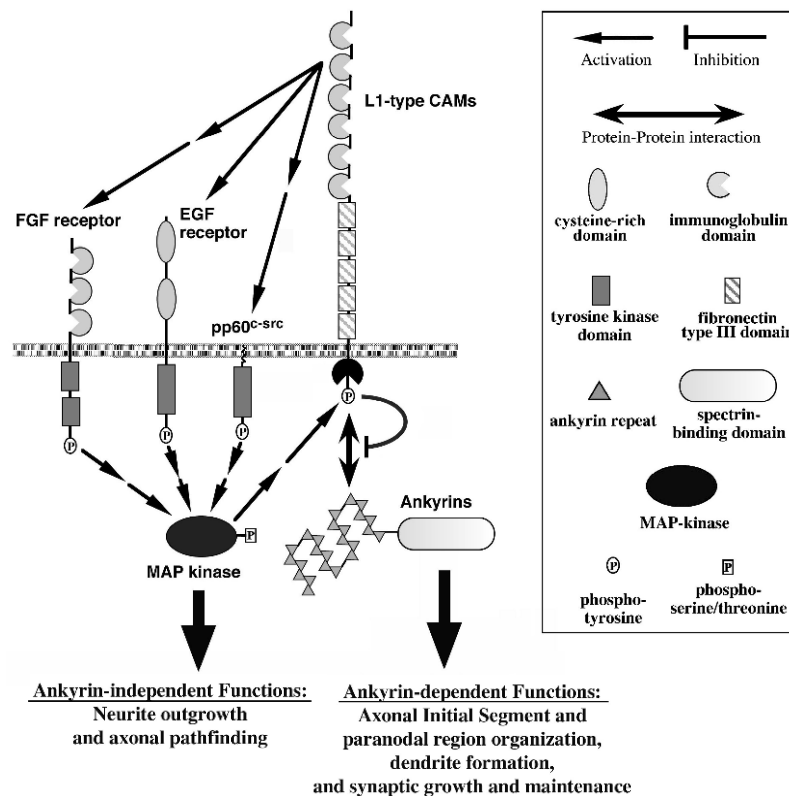


Fig. 1. Model, how ankyrin-binding relates to different L1-type CAM functions.

serving as essential components of septate junctions [2]. L1-type proteins normally perform these functions using their homophilic adhesive abilities. In addition, they also interact with a plethora of extracellular heterophilic binding partners, including other CAMs, Integrins, proteoglycans and RTKs.

It has been known for a considerable time that neuronally-expressed L1-type proteins promote neurite outgrowth and axonal pathfinding during nervous system development [3]. This important activity is in part mediated by the adhesion-dependent activation of neuronally-expressed RTKs, especially FGFR and EGFR. A relatively newly-identified functional role for L1-type CAMs is their involvement in the formation and maintenance of synaptic contacts in arthropod and chordate nervous systems [4-6]. They appear to be essential for the presynaptic organization of specific synapses in *Drosophila*, chicken and mice. L1-type proteins are also known for their ability to bind to members of the ankyrin family, which serve as cytoskeletal linker proteins. Almost all L1-type family members contain a highly conserved ankyrin-binding motif in their cytoplasmic domain [7] and this interaction is regulated at several levels.

In this mini-review, we will discuss two major aspects of L1-type function in the nervous system, neurite outgrowth and axonal pathfinding, as well as synaptogenesis and synapse maintenance. Based on the available published data, we will propose a hypothesis, how several functional aspects of L1-type proteins may be differentially regulated by the binding of ankyrin to the L1 cytoplasmic domain. As this interaction itself is subject to highly conserved regulatory processes, it may serve as a master switch for directing L1-type CAMs into different functional tasks.

THE REGULATION OF ANKYRIN-BINDING TO L1-TYPE CAMS

In 1993 the Bennett laboratory published the first report that a member of the L1 family has a specific binding activity for ankyrin proteins [8]. Using an ankyrin affinity chromatography approach they isolated rat neurofascin as an ankyrin-binding protein from a total protein homogenate of a rat brain. This result was extended by the demonstration that other vertebrate members of the L1-type family also have an ankyrin-binding activity [9] and that the *Drosophila* member of the L1-type family, neuroglian, interacts with the *Drosophila* ankyrin1 protein [10]. By expressing neuroglian in *Drosophila* S2 cells, Dubreuil *et al.* showed that in response to neuroglian protein that is engaged in a homophilic interaction ankyrin1 protein is selectively recruited to cell contact sites [10]. Similarly, human L1-CAM protein is also able to recruit *Drosophila* ankyrin1 protein to cell contact sites and can bind *Drosophila* ankyrin1 in response to cis-interactions with homophilic human TAG-1 adhesion complexes [11]. This adhesion-dependent interaction between neuroglian/L1-CAM and *Drosophila* ankyrin1 was subsequently mapped to a 36 amino acid carboxyterminal region of the neuroglian¹⁶⁷ cytoplasmic domain [12]. The amino acid sequence of this ankyrin-binding site is highly conserved throughout the

L1-type gene family, suggesting that all known L1-type family members are probably able to interact with ankyrin proteins (Fig. 2). It includes two tyrosine residues that are found in the sequences of almost all L1-type proteins (a LADY and an FIGQY motif). The second conserved tyrosine residue is a phosphorylation target of a still unidentified tyrosine kinase [13]. Because fusion proteins containing all 24 aminoterminal *Drosophila* ankyrin1 and ankyrin2 repeats interact with the cytoplasmic neuroglian domain in a yeast two-hybrid assay, the dephosphorylated state appears to be the biological active form to bind ankyrins [14]. In contrast, phosphorylation of the FIGQY motif results in the complete inhibition of ankyrin-binding [15]. In its phosphorylated state, the FIGQY motif becomes the binding partner for the microtubule-associated

Consensus	-SD-DSLADYG--VDVQFNEDGSFIGQY-GKK I: II:::I: :::::IIIIII:I :::
Human L1-CAM	-SD-DSLADYGGSVDVQFNEDGSFIGQYSGKKE
Human NrCAM	-SD-DSLVDYGGVNGQFNEDGSFIGQYSGKKE
Human CHL1	-SA-DSLVEYEGGDHGLFSEEDGSFIGAYAGSKE
Human Neurofascin	-SD-DSLVDYGGEGGQFNEDGSFIGQY-TVKK
Mouse L1-CAM	-SD-DSLADYGGSVDVQFNEDGSFIGQYSGKKE
Chicken Ng-CAM	-SD-DSLADYGGSVDVQFNEDGSFIGQY---RG
Zebrafish L1.1	-SSNDLADYGDSVDIQFNEDGSFIGQYSGRRD
<i>Drosophila</i> Nrg ¹⁶⁷	-SDTDSMAEYGDG-DTGMNEDGSFIGQY-GRKGL ^{COOH}
Leech Tractin	-SETDSLGEYEDPDPKFNEDGSFIGQYGGKKK
<i>C. elegans</i> L1-type	-SETDSMAQYGDTPDGFVTFEDGSFIGQYVVPQKS
	I P

Fig. 2. Core sequences of the ankyrin-binding regions in the cytoplasmic domains of several L1-type CAMs. The P symbol indicates the conserved tyrosine residue that can be optionally phosphorylated and when modified abolishes ankyrin-binding.

doublecortin protein instead [16]. Antibodies that are specific for the phosphorylated form of the FIGQY peptide specifically recognize a subset of neuroglian protein at epidermal muscle attachment sites, developing NMJs and septate junctions in epithelia of *Drosophila* embryos and L1-type proteins in vertebrates at paranodal regions, NMJs and areas of neuronal migration and axon extension [17]. Several publications report that EGFR, FGFR and MAP kinase activation indirectly result in the phosphorylation of the FIGQY motif, placing the responsible tyrosine kinase downstream of the MAP kinase signaling pathway [13, 18-20]. A mutated form of the *Drosophila* neuroglian protein, in which the FIGQY motif had been changed to FIGQF and thereby preventing its phosphorylation, mediates a significantly lower level of homophilic adhesion, when expressed in S2 cells [12]. In a yeast two-hybrid assay the same mutant protein also exhibits an approximately 50% reduction in its ability to bind *Drosophila* ankyrins, indicating that this conserved cytoplasmic tyrosine residue has a twofold importance for the ability of L1-type proteins to interact with ankyrins. First, when phosphorylated it is a potent inhibitor of ankyrin-binding and, secondly, when dephosphorylated it serves as an important residue for ankyrin-binding itself. In addition, these results indicate that ankyrin-binding is

regulated by at least two mechanisms, first by the adhesive activity of the L1-type protein and also by the phosphorylation state of the FIGQY motif.

THE ANKYRIN2 PROTEIN, A BINDING PARTNER OF THE NEUROGLIAN CYTOPLASMIC DOMAIN IN *Drosophila* NEURONS

Genes encoding the principal components of the membrane skeleton, like actin, spectrins, band 4.1 and ankyrins, can be found throughout the metazoan kingdom [21]. Although initially identified and characterized in erythrocytes, these genes significantly predate the evolutionary appearance of red and white blood cells in vertebrates. Vertebrate genomes usually encode 3 paralogous ankyrin genes/proteins, designated *ankyrin1*, 2 and 3 or ankyrinR/B and G [22]. In contrast, the nematode *C. elegans* genome contains only a single gene encoding an ankyrin-type protein (*unc-44*) [23]. An independent gene duplication event in the arthropod lineage has resulted in two genes encoding ankyrin-type proteins in the fruit fly *Drosophila melanogaster*, which are identified as *Drosophila ankyrin1* and 2 [14, 24]. The *Drosophila ankyrin1* gene encodes only a single canonical ankyrin protein, containing 24 ankyrin protein repeats and a single spectrin binding domain [24]. In contrast, the *Drosophila ankyrin2* gene gives rise to a multitude of small, medium, large and extra large protein products, ranging from 1159 to 11640 amino acid residues [25, 26]. Also the expression pattern of the two *Drosophila ankyrin* loci differs dramatically. The *ankyrin1* gene appears to be ubiquitously expressed. In contrast, expression of the *Drosophila ankyrin2* gene is completely restricted to neuronal cells throughout the life cycle of the fly [14, 26]. As neuroglial is co-expressed with ankyrin2 in most neurons, it probably constitutes the major membrane-associated binding partner for the different ankyrin2 isoforms. However, neuroglial is not expressed by all neurons and therefore other ankyrin2-binding membrane proteins must exist, e.g., in ventral unpaired median (VUM) neurons along the midline of the embryonic ventral cord [14].

All *Drosophila* neurons appear to express multiple different ankyrin2 isoforms, which are specifically localized to different neuronal subcellular domains. The smallest *Drosophila* ankyrin2 isoform remains restricted to neuronal cell bodies, whereas the longer protein isoforms are localized to dendrites, axons and synaptic contacts [25-27]. A differential subcellular localization of ankyrin protein isoforms has also been reported for other invertebrate and some vertebrate ankyrins [28, 29]. With the exception of synaptically-localized ankyrins, it remains currently unknown whether these different ankyrin protein forms are involved in specific functions at their respective locations.

There is only limited genetic information about the biological role of ankyrins in the *Drosophila* nervous system. The cellular localization of ankyrin proteins is undisturbed in *neuroglial* null mutants and vice versa [14, 26]. Rather other membrane skeleton proteins like spectrins are essential for the proper subcellular localization of specific ankyrin2 isoforms, such as to NMJs [25, 27]. However,

neuroglian expression affects the stability of ankyrin2 protein in *Drosophila* neurons. When comparing *neuroglian* null mutants with wild type embryos, immunostaining for ankyrin2 protein is significantly lower in mutant cells [14]. Although the nervous system of fruit flies with mutations affecting or abolishing the longer ankyrin2 protein isoforms is somewhat less organized and causes a lethal phenotype, overall axonal growth and pathfinding appears to be normal [25-27]. This indicates that neuroglian's interaction with ankyrin proteins is not essential for this L1-type function.

NEURITE OUTGROWTH AND AXONAL PATHFINDING, ANKYRIN-INDEPENDENT FUNCTIONS OF L1-TYPE CAMS

The ability of L1-type proteins to induce neurite outgrowth has been extensively studied *in vitro* [3]. In addition, the *in vivo* analysis of mutant L1-type proteins in *Drosophila*, mice and humans uncovered a wide range of neurological dysfunctions, including underdeveloped axonal tracts, axon guidance defects, mental retardation, spastic paraplegia and others. All these studies point to an important role of L1-type proteins during the extension and pathfinding of many axons during nervous system development. These effects are mediated by neuronally-expressed RTKs and/or non-receptor tyrosine kinases. The L1-dependent activation of these signaling molecules induces intracellular signaling pathways, specifically MAP kinase and/or phospholipase C activities, which ultimately result in the localized release of calcium from intracellular stores and the activation of a calmodulin-dependent signaling cascade. Co-transfection experiments in *Drosophila* S2 cells demonstrated that homophilic, L1-CAM-mediated cell adhesion induces human EGFR tyrosine kinase activity [30]. These *in vitro* findings have been confirmed *in vivo* using a genetic approach in *Drosophila* [31, 32]. The overexpression of L1-type CAMs induces a gain-of-function phenotype in the peripheral nervous system of *Drosophila* [30]. Sensory neurons in developing wings respond with robust axonal growth and a profound axonal pathfinding defect. This phenotype can be rescued by a genetic reduction of *Drosophila* EGFR and FGFR expression levels [32]. In addition, this L1-type gain-of-function phenotype is phenocopied by the expression of a constitutively active form of the EGFR in the developing wing [32]. Neuroglian loss-of-function phenotypes not only can be rescued by expressing transgenic L1-type molecules, but also by constitutively active versions of RTKs in the affected neurons [31]. Most importantly, an artificial GPI-anchored isoform of *Drosophila* neuroglian, which lacks the intracellular ankyrin-binding domain, induces the same RTK-mediated gain-of-function phenotype in developing wings [30]. These *in vitro* and *in vivo* experiments indicate that L1-mediated neurite outgrowth and axon guidance does not involve an interaction between the cytoplasmic L1-type domain and neuronally-expressed ankyrins.

This conclusion is also supported by *in vitro* studies using vertebrate primary neuronal and ND-7 neuroblastoma cells [19, 33]. Although ankyrinB binding to the L1-CAM cytoplasmic domain appears to be important for neurite initiation, it is not involved in coupling L1-CAM to actin flow in growth cones or in L1-induced neurite elongation [33]. Rather ankyrin binding results in a stationary behavior of L1-CAM molecules in the neuronal plasma membrane and interferes with the actin-dependent retrograde movement of L1-CAM [19]. In addition, inhibiting the interaction between ankyrins and L1-CAM promotes axonal growth of mouse cerebellar granule neurons in culture.

NEUROGLIAN'S ROLE IN *Drosophila* SYNAPTOGENESIS: ANKYRIN-BINDING REQUIRED

First reports that L1-type proteins are involved in the formation and maintenance of synaptic contacts were published fairly recently. Ango *et al.* demonstrated that the vertebrate L1-type CAM, neurofascin, is involved in directing GABAergic innervation to AIS of cerebellar Purkinje cells [5]. The ability of basket interneurons to target and establish these "pinceau synapses" depends on the interaction between the 186 kDa isoform of neurofascin with ankyrinG. A role for L1-CAM at synapses of the nicotinic pathway in chick ciliary ganglia was demonstrated by Triana-Baltzer *et al.* [6]. In this system L1-CAM is needed pre- and postsynaptically for the positioning of presynaptic structures and for facilitating nicotinic synaptic signal transduction. Two independent publications reported an interaction of NrCAM with the synapse-associated proteins SAP90/PSD95 and SAP97, as well as SAP102, which colocalizes with NrCAM in cerebellar granule cells [34, 35]. However, the potential functional significance of these interactions for synaptogenesis remains unknown.

In *Drosophila*, neuroglial's synaptic function was uncovered by a point mutation in the second immunoglobulin domains of the neuroglial polypeptide [4]. The giant fibers (GF) in the adult central nervous system of flies homozygous for the missense mutation in the *nrg*⁸⁴⁹ allele only occasionally show guidance defects, but in most cases reach their target, the tergotrochanteral motoneuron (TTMn) in the second thoracic neuromere. However, the synaptic connection between these two neuronal cells, the GF and the TTMn, is underdeveloped and lacks normal electrophysiological activity in these mutant animals. An ultrastructural analysis of *nrg*⁸⁴⁹ mutant presynaptic termini revealed significantly lower densities of mitochondria, synaptic vesicles and microtubules. Pre- or postsynaptic expression of wild type *Drosophila* neuroglial results in a significant increase of GFs with normal synapse structure and function. However, a complete rescue was only observed when the *Drosophila* neuroglial or human L1-CAM protein was expressed pre- and postsynaptically at the same time. Most importantly, neither the artificial GPI-anchored nor the Y1234F mutant neuroglial form is able to rescue the GF synaptic phenotype. On the contrary, when expressed in a genetic wild type background, both neuroglial^{GPI}

and neuroglian^{Y1234F} results in a disruptive phenotype, which affects the GF synapse function. As the *nrg*^{8,49} mutation also reduces the level of neuroglian Y1234 tyrosine phosphorylation, these results only demonstrate that the FIGQY tyrosine residue is important for neuroglian's synaptic function. However, they do not answer the question, whether this function involves the binding of ankyrin to the neuroglian cytoplasmic domain or not.

Evidence for the importance of the membrane skeleton during synapse maintenance was recently provided by two publications demonstrating the requirement for synaptic ankyrin2 protein at *Drosophila* NMJs [25, 27]. Both publications report that several point mutations and a transposon insertion in the *ankyrin2* gene, which only affect the larger ankyrin2 protein isoforms, destabilize NMJs in *Drosophila* larvae. Two additional larger ankyrin2 isoforms were identified, which are localized to the presynaptic membrane. One of these isoforms contains a carboxyterminal microtubule-binding activity. Accordingly, in mutant larvae the presynaptic microtubular network is disassembled, resulting in the disruption of general presynaptic organization and synaptic activity. However, it is currently unknown, whether neuroglian participates in the stabilization and maintenance of *Drosophila* NMJs. Nevertheless, it appears likely that neuroglian provides the plasma membrane target for ankyrin2 binding at the NMJ. Therefore, the published data suggest an important role for neuroglian in the organization of microtubules at the presynaptic terminus, as well as for the stability of a number of different *Drosophila* synapses in general. This function may also extend to the postsynaptic side, as the non-neuronal neuroglian¹⁶⁷ isoform is expressed by larval muscles [36] and because ankyrin1 has been identified as a component of the postsynaptic spectrin-based membrane skeleton at NMJs [37].

This newly discovered function of the lone *Drosophila* member of the L1-type family is not the only neuroglian function that involves and requires an interaction with one of the two *Drosophila* ankyrins. Yamamoto *et al.* recently identified a role for neuroglian and ankyrin2 during dendritic branch formation in embryonic peripheral sensory neurons [38]. Neuronally-expressed neuroglian interacts with peripheral glial cells and thereby prevents abnormal axonal sprouting. This function also requires the expression of *Drosophila* ankyrin2 in these neuronal cells, thus providing a second function for neuroglian in the *Drosophila* nervous system, which depends on its interaction with ankyrins.

Several publications using vertebrate model systems suggest that L1-type proteins have additional ankyrin-dependent functions. A co-localization of L1-type proteins (specifically L1-CAM, neurofascin and NrCAM) with ankyrinB and ankyrinG has been reported in myelinated nerves at AIS and at paranodal regions of nodes of Ranvier [39-41]. As axons don't become properly myelinated in invertebrate species, these structures can't be studied in the *Drosophila* nervous system. In vertebrate neurons, AIS have a barrier function separating the plasma membrane of the neuronal cell body from the axonal plasma membrane domain [42]. In cultured hippocampal neurons, L1-CAM and

neurofascin accumulate together with ankyrinG at AIS, where they exhibit slow diffusion and a low detergent extractability [43]. This suggests that these L1-type CAMs interact with ankyrinG and the membrane skeleton at AIS. In contrast, membrane mobility and detergent extractability for both L1-CAM and neurofascin are considerably higher in distal axon regions of cultured hippocampal neurons [43]. This does not exclude that L1-ankyrin interactions have some additional roles in axons, as Bennett and Lambert reported that L1-CAM and ankyrinB both mediate axon fasciculation and stabilize axon bundles in premyelinated axons of the optic nerve [39]. Otsuka *et al.* reported that mutations in the *C. elegans* ankyrin gene *unc-44* induce an axonal guidance phenotype [23]. However, as L1-type proteins are not the only membrane-associated ankyrin binding partners, it is currently unknown, whether this axonal pathfinding phenotype is mediated by an interaction with the nematode L1-type protein LAD-1 or by a different *unc-44* ankyrin ligand. All these reports indicate that L1-type proteins have other ankyrin-dependent functions than synapse formation and maintenance. However, more research is needed to determine how the interactions between L1-type proteins and ankyrins influence these L1 functions and how they are regulated, especially at AIS and at the paranodal regions of nodes of Ranvier.

ANKYRIN-DEPENDENT AND -INDEPENDENT FUNCTIONS – A MODEL FOR THE REGULATION OF L1-TYPE PROTEINS

Based on these aforementioned results we propose that ankyrin-binding to L1-type CAMs provides a master switch, which guides L1-type adhesive proteins into different functional contexts, either ankyrin-independent functions, such as neurite outgrowth and axonal pathfinding or into ankyrin-dependent functions, such as L1-type's role at the synapse or in dendrites (Fig. 1). The ability of L1-type cytoplasmic domains to interact with ankyrin proteins is regulated first by extracellular adhesive interactions and secondly by the phosphorylation of a conserved tyrosine residue. Although the identity of the tyrosine kinase, which targets the L1-type cytoplasmic domain, is currently unknown, Whittard *et al.* showed that this kinase is activated by MAP kinase signaling [18]. As MAP kinase activity is positively regulated by various receptor and non-receptor plasma membrane tyrosine kinases, such as EGFR and FGFR, and as the activity of these RTKs is stimulated by L1-type-dependent adhesion, this suggests the existence of a positive feedback-loop (Fig. 1). We postulate that this feedback-loop maintains conditions favorable to axonal growth and pathfinding. Once the growth cone reaches its target, this loop is interrupted by a so far unknown mechanism, e.g., by a tyrosine-specific phosphatase, which removes the phosphate group from the FIGQY motif. This would allow the binding of ankyrin proteins to the L1-type cytoplasmic domain and redirect the L1-type protein to engage in ankyrin-dependent functions, such as the organization and maintenance of synaptic contacts or the participation in

the formation of the dendritic tree. The binding of other proteins, for example doublecortin [16], [44], shootin1 [45], synapse-associated proteins SAP102 [34] and SAP90/PSD95 and SAP97 [35] and the AP-2 adapter [46], to L1-type cytoplasmic domains indicates that other switches for regulating L1-type function might exist. So far, the interaction between ankyrins and L1-type cytoplasmic domains provides the most convincing example for connecting a link with a specific family of cytoskeletal proteins to profound changes in the functional engagement of L1-type CAMs.

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