

RESEARCH ARTICLE

Functional phylogenetic analysis of LGI proteins identifies an interaction motif crucial for myelination

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ABSTRACT

The cellular interactions that drive the formation and maintenance of the insulating myelin sheath around axons are only partially understood. Leucine-rich glioma-inactivated (LGI) proteins play important roles in nervous system development and mutations in their genes have been associated with epilepsy and amyelination. Their function involves interactions with ADAM22 and ADAM23 cell surface receptors, possibly in apposing membranes, thus attenuating cellular interactions. LGI4-ADAM22 interactions are required for axonal sorting and myelination in the developing peripheral nervous system (PNS). Functional analysis revealed that, despite their high homology and affinity for ADAM22, LGI proteins are functionally distinct. To dissect the key residues in LGI proteins required for coordinating axonal sorting and myelination in the developing PNS, we adopted a phylogenetic and computational approach and demonstrate that the mechanism of action of LGI4 depends on a cluster of three amino acids on the outer surface of the LGI4 protein, thus providing a structural basis for the mechanistic differences in LGI protein function in nervous system development and evolution.

KEY WORDS: Evolution and development, Myelination, Leucine-rich glioma-inactivated, ADAM23, Schwann cell, Mouse

INTRODUCTION

Dynamic cell-cell interactions determine key morphological and functional properties of the nervous system during ontogeny and adult life. The formation and dynamics of the neuronal synapse and the elaboration of the myelin sheath that surrounds the majority of axons during the postnatal development of the vertebrate nervous system probably illustrate this most profoundly. However, relatively little is known about the molecular mechanisms that coordinate such interactions.

One class of molecules that plays an important role in cellular interactions in nervous system development and function is the leucine-rich glioma-inactivated (LGI) protein family (reviewed by Kegel et al., 2013). LGI proteins are secreted glycoproteins that consist of a leucine-rich repeat (LRR) domain and a so-called epilepsy-associated or epitempin (EPTP) domain (Gu et al., 2002). Both protein domains are generally involved in protein-protein interactions. LGI genes and proteins have been associated with a

wide variety of neurological disorders. *LGII* mutations are found in patients with autosomal dominant lateral temporal lobe epilepsy (ADLTE; OMIM 600512) characterized by auditory auras (Kalachikov et al., 2002; Morante-Redolat et al., 2002), and auto-antibodies against LGI1 have been found in sera from patients with limbic encephalitis and associated neurological diseases (Irani et al., 2010; Lai et al., 2010; Ohkawa et al., 2013). More recently, a mutation in *Lgi2* was found associated with a juvenile form of remitting epilepsy in an Italian breed of water dogs (Seppala et al., 2011). The fourth member of this protein family, LGI4, has been associated with childhood convulsions and absence epilepsy (Gu et al., 2004; Ishii et al., 2010). Moreover, targeted and spontaneous (claw paw mice; *Lgi4^{clp}*) mutations in *Lgi4* in mice cause severe congenital hypomyelination of the developing peripheral nervous system (PNS) and premature death (Bermingham et al., 2006; Henry et al., 1991; Nishino et al., 2010; Ozkaynak et al., 2010).

Genetic and biochemical evidence suggests that the mechanism of action of LGI proteins involves binding to a subset of cell surface receptors belonging to the ADAM (a disintegrin and metalloproteinase) family, i.e. ADAM11, ADAM22 and ADAM23 (Kegel et al., 2013; Novak, 2004; Seals and Courtneidge, 2003). In particular, LGI1 and LGI4 bind to cell surface expressed ADAM22 and ADAM23, and both proteins co-precipitate from brain lysates with ADAM22 or ADAM23 antibodies (Fukata et al., 2006; Ogawa et al., 2010; Ozkaynak et al., 2010; Sagane et al., 2008). It has also been suggested that LGI1 contributes to trans-synaptic interactions and synaptic strength in hippocampal neurons through binding postsynaptic ADAM22 and presynaptic ADAM23 (Fukata et al., 2010; Yokoi et al., 2012).

LGI4 function has been primarily analyzed in the context of the developing PNS where it is secreted from Schwann cells and binds to ADAM22 in the axonal membrane (Nishino et al., 2010; Ozkaynak et al., 2010). The interaction between LGI4 and ADAM22 is required for timely axonal sorting and myelination (Bermingham et al., 2006; Henry et al., 1991; Sagane et al., 2005), developmental processes that involve extensive interactions between the axonal and Schwann cell membranes. ADAM23 is expressed in the PNS and in particular in Schwann cells (Dhaunchak et al., 2010), but its role in axonal sorting and myelination has not been explored. The high degree of homology between LGI1 and LGI4 and their high affinity for ADAM22 and ADAM23 suggests that a common mechanism might underlie their respective functions in the CNS and PNS.

In this study, we test this hypothesis and show that LGI4 functions in the PNS through a distinct mechanism that does not require ADAM23. We further show that the function of LGI4 is unique in that none of the other LGI family members is able to elicit myelination. Here, we have adopted a general integrated approach that includes phylogenetic, computational and functional analysis, to identify interaction motifs in LGI proteins required for coordinating myelination in the developing PNS. We demonstrate

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that the unique function of LGI4 depends on small set of amino acids that present a novel interaction surface in these proteins. The data presented here have implications for our understanding of LGI protein function in development and evolution.

RESULTS

ADAM23 is expressed in the PNS but is not required for axonal sorting and myelination

Adam23 is widely expressed in the CNS and in neurons and Schwann cells (SCs) of the PNS (Dhaunchak et al., 2010; Goldsmith et al., 2004; Ozkaynak et al., 2010; Sagane et al., 1999). Indeed, an ADAM23-specific antibody detects a 70 kD protein in extracts from mouse postnatal day (P) 12 sciatic nerve (Fig. 1A; MSN) and in membrane extracts of cultured rat Schwann cells (Fig. 1A, RSC). This band corresponds to the glycosylated, mature form of ADAM23 (Goldsmith et al., 2004).

To test whether ADAM23 is mechanistically involved in LGI4-ADAM22-mediated interactions in PNS development, we generated a mouse line with an *Adam23* floxed allele (*Adam23^{Lox}*; Fig. 1B). A germline-expressing Cre mouse was crossed with *Adam23^{Lox/Lox}* mice and fully deleted (*Adam23^{Δ1/Δ1}*) mice were examined. *Adam23^{Δ1/Δ1}* were born at expected frequencies, but all animals developed a severe tremor in the second week of postnatal life and died by P15, in accordance with previous reports on gene-trap *Adam23* mutant mice (Mitchell et al., 2001; Owuor et al., 2009). Western blot analysis of sciatic nerves dissected from P12 mice revealed the presence of the ADAM23 protein in wild-type, but not in *Adam23^{Δ1/Δ1}* mice (Fig. 1C). Levels of the major myelin proteins MPZ and MBP were unaffected in *Adam23^{Δ1/Δ1}* sciatic nerves, indicating that there is myelin formation. Normal paranodal junctions were formed in the absence of ADAM23 as judged by staining for CASPR (CNTNAP1 –

Mouse Genome Informatics), a major cell adhesion molecule involved in paranodal junction formation and stability (Fig. 1D; reviewed by Salzer et al., 2008). Interestingly, ADAM23 protein accumulates in the juxtapanodal domain, abutting the paranodal junction (Fig. 1D, WT), the same site at which shaker-type potassium channels, ADAM22, CASPR2 (CNTNAP2) and TAG-1 (CNTN2) accumulate (Ogawa et al., 2010; Rasband, 2011). Microscopic examination of semi-thin transverse sections of sciatic nerves of *Adam23^{Δ1/Δ1}* mice did not reveal any abnormalities, in marked contrast with the severe hypomyelinating phenotype observed in the sciatic nerve of *Adam22* null mice of the same age (Fig. 1E). Thus, it is unlikely that ADAM23 is mechanistically involved in LGI4-ADAM22 interactions that govern myelin formation in the developing peripheral nerve.

LGI4, but not LGI1, 2 or 3, facilitates myelination in the PNS

The fact that LGI4-ADAM22 interactions in the PNS do not require ADAM23 for myelination to occur in the PNS suggests that LGI4-ADAM22 interactions are mechanistically distinct from the LGI1-ADAM22/ADAM23 interactions involved in excitatory synapse maturation and strength in the CNS. This difference cannot be explained by affinity differences of LGI1 and LGI4 for the ADAM22 and ADAM23 receptors, as both proteins bind avidly to both receptors (Owuor et al., 2009; Sagane et al., 2008). To explore potential functional differences between these LGI proteins, we performed genetic complementation experiments in primary sensory neuron-SC cultures derived from dorsal root ganglia (DRG) of *Lgi4^{clp/clp}* and *Lgi4^{clp/+}* (referred to as *clp/clp* and *clp/+*, respectively, in the following text) mouse embryos at embryonic day (E) 13. Control *clp/+* cultures exhibit robust myelination, whereas *clp/clp* cultures do not myelinate (Birmingham et al., 2006). Cultures were retrovirally transduced with different *Lgi* cDNA

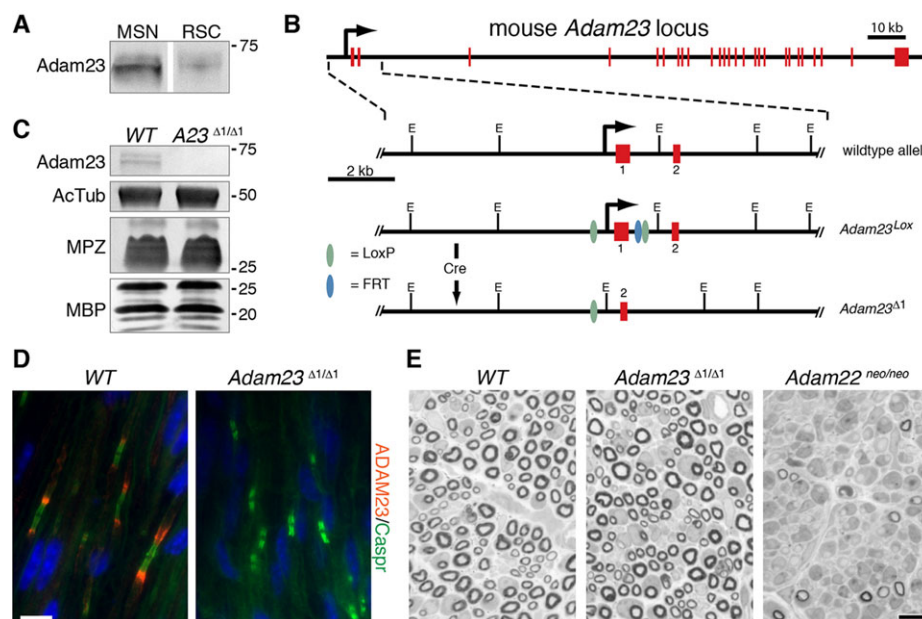


Fig. 1. *Adam23* is expressed in the peripheral nervous system but is not required for axonal sorting and myelination. (A) Western blot analysis of membrane proteins extracted from P12 mouse sciatic nerve (MSN) or cultured rat Schwann cells (RSC) reveals that ADAM23 is expressed as a 70-kD protein. (B) The mouse *Adam23* locus spans over 250 kb of genomic DNA with <25 exons. The structures of the wild-type, conditional and null alleles of *Adam23* are depicted. (C) Western blot analysis of P12 sciatic nerve extracts from wild-type and *Adam23* null (*A23^{Δ1/Δ1}*) mice demonstrates the specificity of the ADAM23 antibody. Expression levels of the major myelin proteins, myelin protein zero (MPZ) and myelin basic protein (MBP) and acetylated alpha tubulin, which is mainly axonal, are not altered in *Adam23* null nerves. (D) Longitudinal sections of cryopreserved P12 nerves immunolabeled with antibodies against ADAM23 (red) and CASPR (green). (E) Transverse semi-thin section of sciatic nerves at P12, isolated from wild type, *Adam23* null (*Adam23^{Δ1/Δ1}*) and *Adam22* null (*Adam22^{neo/neo}*) stained with PPD. The number and thickness of myelin rings around axons in *Adam23* null animals does not differ from wild-type animals, whereas only few axons are thinly myelinated in *Adam22* null mice at this stage of development. Scale bars: 10 μ m.

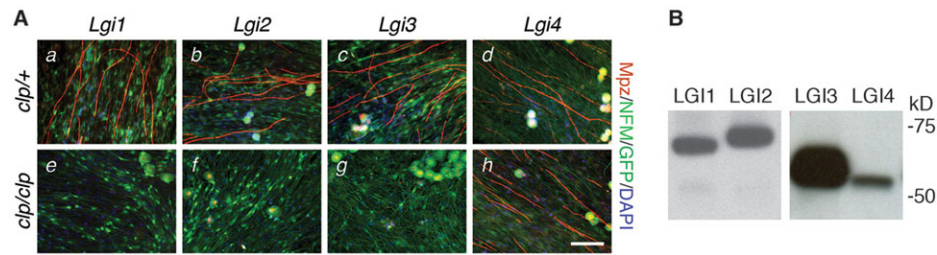


Fig. 2. *Lgi4* is the only *Lgi* member to genetically complement myelination in *clp/clp* cultures. (A) Retroviral transduction of *Lgi1-4* in *clp/clp* cultures restores myelination. *Clp/+* and *clp/clp* cultures were infected with retrovirus containing *Lgi1-4* and maintained in myelinating conditions for 19 days and then stained for MPZ and neurofilament (NFM). Infected Schwann cells express GFP as the retroviral construct carries an IRES-GFP cassette. *Clp/+* cultures express normal levels of MPZ (a-d) whereas *Lgi1-3*-infected *clp/clp* cultures show no MPZ expression (e-g). Numerous myelin segments are evident in *Lgi4* infected *clp/clp* cultures (h). More than six cultures of each genotype were infected per construct. Scale bar: 100 μ m. (B) LGI proteins are secreted and can be recovered from the tissue culture supernatant of *clp/clp* cultures. LGI proteins were purified from tissue culture supernatant three days after infection. Proteins were detected by western blot using a V5 antibody.

expression constructs (*Lgi1*, 2, 3 and 4). Transduction of *clp/clp* cultures with *Lgi4* restores myelination, as shown by expression of MPZ, the major protein in compact myelin (Fig. 2Ad). This is in line with our previous observations (Bermingham et al., 2006). By contrast, none of the other *Lgi* family members (*Lgi1*, 2 or 3) restored myelination in *clp/clp* cultures (Fig. 2Ae-g) despite high-level LGI protein expression, as shown by western blot (Fig. 2B).

The PNS-specific function of LGI4 requires the LRR domain but crucially depends on its EPTP domain

To establish whether the LGI4-specific myelination function is a property of the LRR domain or the EPTP domain, we generated and tested domain deletion constructs (Fig. 3A). Previously, it was shown that LGI1 binds ADAM22 through its EPTP domain (Fukata et al., 2006). We found that this was also true for LGI4 (supplementary material Fig. S1A,B). However, no myelination was found in *clp/clp* cultures when the LGI4^{EPTP} domain or the LGI4^{LRR} domain was expressed (Fig. 2Be,f), indicating that both domains are required for LGI4 function. It is possible that LGI4 is tethered to the axonal membrane through its EPTP-dependent binding to ADAM22, whereas the LRR domain engages in other specific interactions.

We next examined whether the LRR domain of LGI4 contributes unique properties. We exchanged the LRR and EPTP domains of LGI3 and LGI4 to generate LGI^{L4E3} and LGI^{L3E4} (Fig. 3A). Similar domain swapping constructs were created by exchanging the LRR and EPTP domains of LGI1 and LGI4 (supplementary material Fig. S1C). These chimeric proteins are efficiently expressed and secreted, and bind to cell surface-expressed ADAM22 (supplementary material Fig. S1D,E and Fig. S3). Transduction of these expression constructs into *clp/clp* cultures revealed that the LRR domains of LGI3 (Fig. 3C) and LGI1 (supplementary material Fig. S1D,E) are interchangeable with the LGI4 LRR domain, thus demonstrating that the unique myelin-promoting function of LGI4 depends on its EPTP domain.

We further delineated the LGI4 EPTP functional domain by creating and testing an additional chimeric construct in which only the last four repeat motifs of the LGI4 EPTP domain were retained [LGI3^{E4(4-7)}]. This chimeric protein fully restores myelination in *clp/clp* cultures (Fig. 3Cf), further structurally localizing LGI4 function to the carboxyl-terminal half of the EPTP domain.

Functional analysis identifies the zebrafish homolog of mammalian *Lgi4*

We reasoned that the protein domain responsible for LGI4 specificity must be conserved in other vertebrates. Taking advantage of the more than 800 million years of independent evolutionary history between

modern day bony fishes and mammals, we sought to identify specific amino acids within the LGI4 protein that contribute to its myelin-promoting function. Comparative genomics has so far failed to identify a clear *Lgi4* homolog among the five *Lgi* genes in the genomes of zebrafish and other teleost fish, leading some authors to suggest that *Lgi4* is most closely related to an ancestral *Lgi1* gene (Gu et al., 2005). On the basis of a much larger dataset, others suggested it is most closely related to an ancestral *Lgi3* gene (Leonardi et al., 2011). To shed more light on the ancestry of the *Lgi4* gene and to identify the functional homolog of LGI4 in the zebrafish genome, we cloned the five zebrafish *Lgi* cDNAs and investigated which of the five *Lgi* proteins can restore myelination in *clp/clp* cultures. Immunoprecipitation experiments showed that zebrafish *Lgi1a* and *Lgi1b* directly bind the ectodomain of mouse ADAM22 (supplementary material Fig. S2A) and ADAM23 (supplementary material Fig. S2B). By contrast, zebrafish *Lgi2a*, *Lgi2b* and *Lgi3* did not bind (supplementary material Fig. S2) and expression of *Lgi2a*, *Lgi2b* and *Lgi3* in *clp/clp* cultures did not restore myelination (Fig. 4). However, both *Lgi1a* and *Lgi1b* did restore myelination in *clp/clp* cultures, strongly suggesting that they are the functional homologs of LGI4 in zebrafish. These data are in line with the suggestion that the *Lgi4* gene arose in tetrapods from an ancestral *Lgi1* gene and rapidly acquired an essential function in the nervous system. By contrast, these functions remained associated with the duplicated *Lgi1* genes in teleost fish (Gu et al., 2005) and with the single *Lgi1* gene in lobe finned fish, such as the coelacanth (supplementary material Fig. S4).

Phylogenetic comparison and structural modeling identifies a three amino acid cluster on the surface of the EPTP domain

The distant evolutionary relationship between LGI4 and *Lgi1a/b* provides us with a saturated mutation analysis of LGI4 function in the PNS. Selective pressure will have retained those amino acid residues crucial for the myelination-promoting activity of LGI4 and *Lgi1a/b*. Visual inspection of LGI protein alignments identified three amino acids within the second half of the EPTP domain that were identical in LGI4 proteins from mammals and *Lgi1* proteins from zebrafish, but were different in the other LGI proteins (highlighted in yellow in Fig. 5A and supplementary material Fig. S4). A structure for the LGI4 EPTP domain was predicted on the basis of its high primary amino acid sequence homology with the WDR5 protein using the online HHpred tool (Soding et al., 2005). Mapping the position of these three amino acids onto this structure shows that their side chains are solvent exposed and form a potential interaction interface at the side of the slightly conical, donut-shaped EPTP domain (Fig. 5B). The three amino acids (RR-M) contribute two positive charges (the two

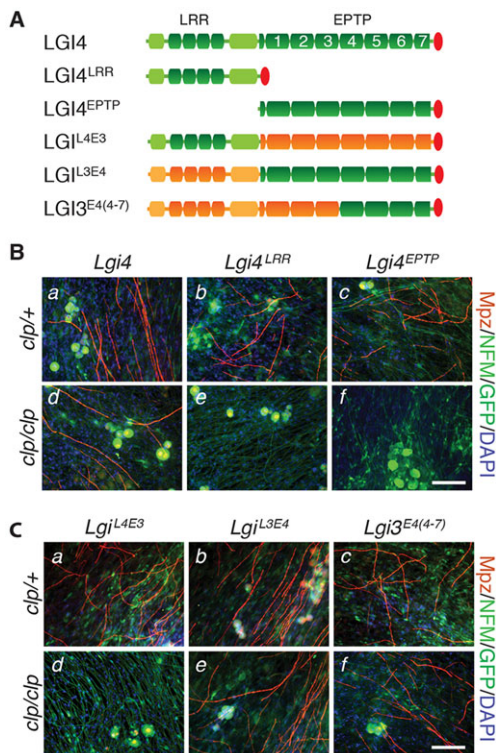


Fig. 3. The myelin-promoting function of *Lgi4* depends on the carboxyl terminal half of the EPTP domain. (A) Schematic of the constructs used for infection in B and C. *Lgi4* domains are indicated in green, *Lgi3* domains are indicated in orange. Red oval represents a V5 and 6xHis tag. (B) *Lgi4* function cannot be reduced to its LRR or EPTP domain. *Clp/+* and *clp/clp* co-cultures were infected with retrovirus containing *Lgi4^{LRR}* or *Lgi4^{EPTP}* and maintained in myelinating conditions for 19 days and then stained for MPZ (myelin) and NFM (neurons). Infected Schwann cells express GFP as the retroviral construct carries an IRES-GFP cassette. *Clp/+* cultures (a-c) and *Lgi4*-infected *clp/clp* cultures (d) express normal levels of MPZ, whereas *Lgi4^{LRR}* (e) and *Lgi4^{EPTP}* (f) infected *clp/clp* cultures show no MPZ expression. (C) *Lgi4* function is associated with its EPTP domain. *Clp/+* and *clp/clp* cultures were infected with retrovirus containing *Lgi^{L4E3}*, *Lgi^{L3E4}* or *Lgi^{3E4(4-7)}*. *Clp/+* co-cultures (a-c) express normal levels of MPZ, whereas *Lgi^{L4E3}*-infected *clp/clp* cultures show no MPZ expression (d). Numerous myelin segments are evident in *Lgi^{L3E4}* (e) and *Lgi^{3E4(4-7)}* (f) infected *clp/clp* cultures. More than six cultures of each genotype were infected per construct. Scale bars: 100 μm.

arginine R-groups) and a hydrogen acceptor (the sulfur atom of methionine) to this putative interaction surface.

To assess the relevance of this putative three amino acid (RR-M motif) interaction surface for the myelin-promoting activity of LGI4, we generated a mouse *Lgi1* expression cassette (*Lgi1^{RR-M}*, Fig. 5C) in

which the identity of the three amino acids was changed into those found in LGI4, i.e. N422R, Q423R and S464M (Fig. 5A,C). LGI1^{RR-M} was found to be secreted and to bind ADAM22 (Fig. 5C; supplementary material Fig. S3), indicating that the amino acid replacements do not affect the proper processing and folding of the LGI1^{RR-M} protein. Transduction of *clp/clp* cultures with *Lgi1^{RR-M}* restored myelination (Fig. 5D), demonstrating that these amino acid side chains determine LGI4 specificity and contribute to an interaction interface that is crucial for LGI4 function in PNS myelination.

As mammalian LGI1 is normally glycosylated at position N422 (Sirerol-Piquer et al., 2006), it is possible that these sugar side chains obstruct a potential myelin-promoting interface. The simple destruction of this glycosylation may unmask it, and, thus, we generated a LGI1^{N422Q} mutant protein. A previous study showed that mutating this N-linked glycosylation site does not affect the proper processing or secretion of LGI1 (Sirerol-Piquer et al., 2006). Indeed, we found that LGI1^{N422Q} is normally secreted from transfected cells. *clp/clp* and *clp/+* DRG co-cultures were transfected with LGI1^{N422Q}. Myelination was observed in *clp/+*, but not in *clp/clp*, cultures, showing that removal of the sugar modifications on this side of the LGI1 protein does not unmask a latent myelin-promoting activity. Therefore, the three amino acids we identified here are an essential part of an interaction interface that is functionally significant for PNS myelination.

DISCUSSION

LGI proteins play important roles in key developmental and functional aspects of the vertebrate nervous system, but their mechanism of action is still poorly understood. Here, we have investigated whether a common or distinct mechanism(s) underlies LGI protein function during nervous system development. We demonstrate here that LGI4 functions in the peripheral nervous system through a distinct mechanism that involves a unique interaction surface on the outer circumference of the EPTP domain.

In contrast to the proposed models for LGI1 function in the central nervous system, LGI4 does not require the ADAM23 receptor to drive myelination in the PNS. A model in which LGI4 links the apposing axonal membrane and Schwann cell membrane through interaction with ADAM22 and ADAM23, respectively, seems therefore unlikely. However, we found that ADAM23 accumulates at the juxtaparanode (JXP) of myelinated axons, which is characterized by a high density of shaker type potassium channels and the cell adhesion molecules CASPR2, TAG-1 and ADAM22. The accumulation of Kv1 channels at the JXP depends on CASPR2 and TAG-1 interactions in the apposing membranes and interactions between the CASPR2 cytoplasmic domain and Protein 4.1B (EPB4.1L3 – Mouse Genome Informatics) that link the complex to the axonal cytoskeleton. The

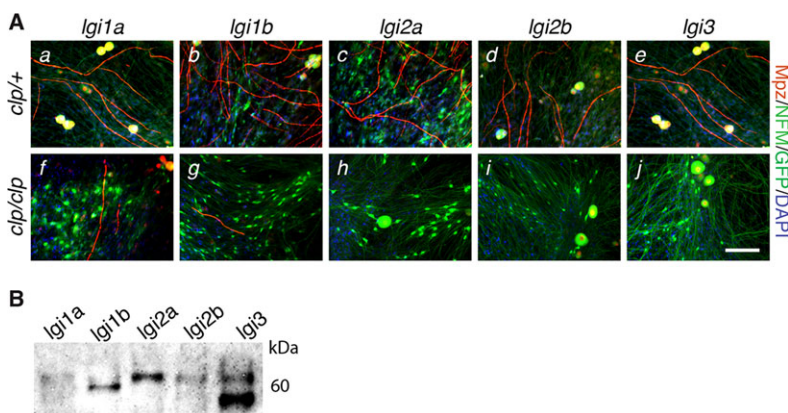


Fig. 4. *lgi1a* and *lgi1b* are functional homologs of mammalian LGI4.

(A) *Clp/+* and *clp/clp* cultures were infected with retroviruses containing zebrafish *Lgi* proteins and maintained in myelinating conditions for 19 days and then stained for MPZ (myelin) and NFM (neurons). Infected Schwann cells express GFP. *Clp/+* cultures show normal amounts of myelin figures (a-e). *lgi1a* and *lgi1b* restore myelination in *clp/clp* cultures (f,g). *lgi2a*, *lgi2b* and *lgi3* infected *clp/clp* cultures do not show any myelin (h-j). More than six cultures of each genotype were infected per construct. Scale bar: 100 μm. (B) The zebrafish *Lgi* proteins are recovered from the conditioned medium of the cultures shown in A three days after infection and detected by western blot using a V5 antibody.

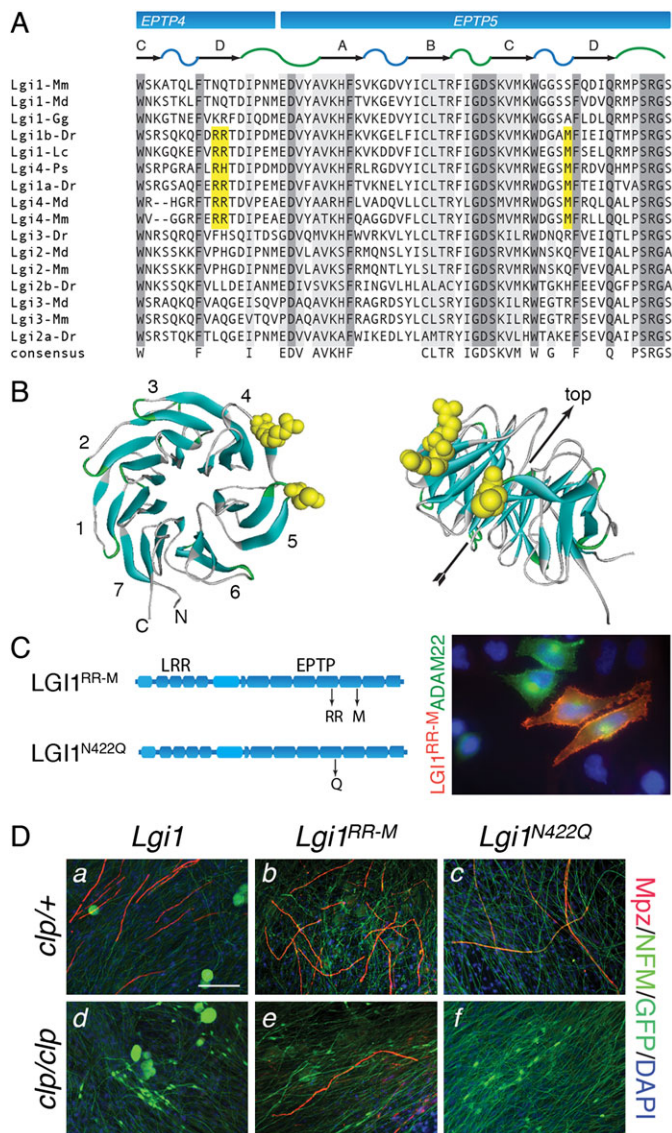


Fig. 5. LGI4-specific function depends on a conserved three amino acid motif. (A) LGI protein alignments identified three amino acids within the second half of the EPTP domain that were identical between LGI4 proteins from mammals (mouse: Mm, *Mus musculus*; opossum: Md, *Monodelphis domestica*) and Lgi1 proteins from zebrafish (Dr, *Danio rerio*) and coelacanth (Lc, *Latimeria chalumnae*), but were different in other LGI proteins. Chicken: Gg, *Gallus gallus*; Chinese softshell turtle: Ps, *Pelodiscus sinensis*. (B) A structure for the LGI4 EPTP domain was predicted on the basis of its high primary amino acid sequence homology with the WDR5 protein using the online HHpred tool (Soding et al., 2005). Mapping the position of the three identical amino acids onto this structure shows that their side chains are solvent-exposed and form a potential interaction interface at the outer surface of the EPTP domain. (C,D) LGI1 mutant construct containing the identical amino acids (LGI1 RR-M) binds ADAM22 overexpressing HeLa cells (C) and stimulates myelination in *clp/clp* cultures (D). More than six cultures of each genotype were infected per construct. Scale bar: 100 μ m.

presence of the ADAM22 and ADAM23 receptors in apposing membranes of the JXP is compatible with the suggestion that they contribute to the formation or stability of the JXP complexes, possibly in an LGI4-dependent fashion, a hypothesis that will be the subject of future experiments.

Despite the fact that both LGI4 and LGI1 bind with relative high affinity to the ADAM22 receptor, only LGI4 is capable of restoring

myelination in our *in vitro* myelination system derived from LGI4 mutant mice. Thus, ADAM22-ligand binding per se is not sufficient to elicit the biologically relevant response in this system. Differential outcome of different ligands binding to the same receptor is not an uncommon phenomenon. For example, binding of NGF or NT3 (also known as NTF3) to the TrkA receptor (NTRK1) has common but also different biological effects that correlate with different ligand-induced receptor membrane mobility and internalization (Marchetti et al., 2013). One other example is provided by the Type I interferons (16 different forms), which elicit different physiological responses through the same heterodimeric receptors IFNAR1 and IFNAR2 (Thomas et al., 2011). Interferons bind to this receptor through common 'anchor points' that are interspersed among ligand-specific interactions that tune ligand affinities, a mechanism referred to as 'ligand proofreading'. This mechanism involves conformational changes in the receptor. The crystal structure of the ADAM22 ectodomain has been resolved and it was suggested that considerable movement between the metalloproteinase-like (M) domain and the disintegrin/cysteine-rich/EGF-like (DCE) domain might provide a basis for ligand discrimination and/or ligand-induced conformational changes (Liu et al., 2009). It is thus conceivable that part of the specificity of the LGI4-ADAM22 interaction results from such ligand-induced conformational adaptation. As crystal structures for LGI proteins or the LGI-ADAM ectodomain complex are not available, such a mechanism remains highly speculative.

Computational modeling of LGI1 structure led Leonardi and colleagues to suggest that LGI proteins bind to the ADAM receptor through the top surface of its EPTP domain (Leonardi et al., 2011). The interaction surface we have identified here in LGI4 maps to the circumference of the EPTP domain and is thus distinct from the prospective ADAM22/23 interaction interfaces common to all LGI proteins. The seven-bladed propeller structure of the EPTP domain provides a stable scaffold to present multiple interaction surfaces and our results underscore its versatility. The seven-bladed propeller structure is found in a wide range of proteins involved in different cellular processes and, in general, it functions as a multivalent interaction hub (Xu and Min, 2011). For example, the WDR5 protein, to which the EPTP domain of LGI proteins is most closely related, is part of the mammalian Trithorax (TrxG) complex. It interacts with the histone H3 tail and the RBBP5 core complex subunit through the top and bottom part of its seven-bladed propeller, effectively presenting the histone H3 tail for K4 methylation by the MLL1 histone methyltransferase (also known as KMT2A) (Avdic et al., 2011). Likewise, LGI4 function in the PNS appears to depend on interactions with the ADAM22 receptor through the top surface of the EPTP domain and possibly with accessory proteins through the surface on the side, created by the amino acid side chains of propeller blades 4 and 5. Alternatively, these side chains in blades 4 and 5 provide specific additional ADAM22 contacts to fine tune the interaction and confer specificity (Fig. 5C). As ADAM22 is also an integrin receptor, the binding of LGI ligand may potentiate interactions with integrins on the axonal Schwann cell membrane, thereby stimulating migration of the Schwann cell lamellipodia over the axonal surface to initiate myelination.

In addition to the interaction platform provided by the EPTP domain, LGI proteins contain an LRR domain that is engaged in protein-protein interactions. Indeed, the LRR domain of LGI4 is essential for full LGI4 activity, as the LGI4 EPTP domain by itself is secreted but does not restore myelination in LGI4-deficient *clp/clp* cultures (Fig. 3). Our experiments did not reveal a specific role for the LGI4 LRR domain, as that of LGI1 or LGI3 could replace it

without loss of myelin-promoting activity. What generic role the LRR domain fulfills in LGI4 function, or in any of the other LGI proteins, is unclear. The demonstration that LGI proteins can dimerize through their LRR domains might be of functional significance (Fukata et al., 2006).

Our functional phylogenetic analysis allows us to reconstruct the evolutionary history of this interaction surface in ancestral LGI proteins. *Lgi* genes are only found in the genomes of chordates (with the exception of urochordates), suggesting that they evolved in the early vertebrate lineage 525 million years ago. The primitive extant chordates *Amphioxus* (*Brachyostoma floridae*) and Lamprey (*Petromyzon marinus*) have one *Lgi* gene that encodes proteins that miss the RR-M motif identified here (supplementary material Fig. S4). By contrast, all extant jawed-vertebrates (gnathostomes) have LGI proteins that carry this motif. The evolution of this motif then coincides with the proposed first appearance of compact myelin in the peripheral nervous system of jawed vertebrates ~425 million years ago (Yoshida and Colman, 1996). A duplication event of an ancestral *Lgi1* gene in the early tetrapod lineage then gave rise to the *Lgi1* and *Lgi4* genes present in the genomes of all modern day tetrapods, dating the birth of the *Lgi4* gene to ~365 million years before present day. On the basis of this reconstruction and our complementation assays, we predict that the *Lgi1 a/b* proteins in zebrafish are expressed in Schwann cells and promote sorting and myelination of peripheral nerve fibers. Our analysis also emphasizes the importance of combining functional data as generated here with computational methods to reconstruct reliable evolutionary relationships.

MATERIALS AND METHODS

Mice

C57BL/6J *clp*^{+/+} (*Lgi4*^{clp^{+/+}) mice were originally obtained from the Jackson Laboratory and the *clp* allele has subsequently been maintained on a FvB/N and C57BL/6 background. Genotypes were determined by PCR using the Expand Long Template PCR system (Roche) and the following primers: 5'-AGAGAGCCAAGACCTTGGCTAC-3' and 5'-GTCATCTGAAGTTCG-AGGATGGC-3' (*Lgi4*^{clp} allele 550 bp and wt *Lgi4* allele 750 bp). An *Adam23* conditional knock-out allele (*Adam23*^{LoxP}) was generated through homologous recombination in embryonic stem cells (ESCs) using standard techniques (Jaegle et al., 2003). A mouse line was generated from a correctly recombined ESC line and *Adam23*^{LoxP/+} mice were crossed with a transgenic mouse expressing Cre recombinase in the germline to generate offspring with an *Adam23* null allele (*Adam23*^{Δ1/+}) in which the first exon plus promoter of *Adam23* were deleted. *Adam23*^{LoxP/+} mice were intercrossed to generate *Adam23*^{Δ1/Δ1} mice. Mice were genotyped by PCR using the following primers: A23-S 5'-GATCCCAGTGGCTCAGTGCC-3' and A23-aS1 5'-GATCTGGCAACAACCTTGCCAAG-3'. The wild-type allele will yield a product of 256 bp and the *LoxP* allele (*ADAM23*^{Lox}) will yield a 300-bp product. The *ADAM23*-null allele (*ADAM23*^{Δ1}) will yield a 421-bp PCR product using the A23-S primer as above in combination with the following anti-sense primer: A23-aS2 5'-TGATTGCTCATTATGACAAGC-3'. Animal experiments were performed according to protocols approved by the independent Animal Experimentation Committee (DEC) and in compliance with institutional guidelines at Erasmus University Medical Center.}

DNA constructs

All mouse *Lgi* cDNAs were amplified from cDNA produced from E13.5 DRG total RNA, cloned in Zero-Blunt TOPO vector (Invitrogen) and fully sequenced. Zebrafish *Lgi* cDNAs were amplified from zebrafish embryos at three days post fertilization. RNA was isolated using RNA-Bee reagent (Tel-Test) and converted into cDNA using Superscript III (Invitrogen) and cDNA was amplified using Phusion polymerase (Finnzymes).

The different domain swap LGI expression constructs were created with overlap PCR. Point mutations were introduced using segments of DNA synthesized by Genscript. All *Lgi* expression constructs were cloned in the retroviral vector pBMN-IRES-GFP (G. Nolan, Stanford University, CA, USA,

obtained through Addgene), which was modified to extend the LGI open reading frame with a V5 and 6xHis tag. Furthermore, all LGI open reading frames start with a signal peptide derived from the mouse kappa light chain immunoglobulin gene replacing the LGI protein's own signal peptide. Adam22-Fc constructs have been previously described (Ozkaynak et al., 2010).

Virus production

HEK293T cells were co-transfected with pcl-ECO packaging vector (IMGENEX Corporation) and pBMN-LGI-IRES-GFP constructs, using the polyethylenimine (PEI) method (Boussif et al., 1995). Virus was collected in DMEM/F10, 10% heat-inactivated fetal calf serum (FCS) and Penicillin + Streptomycin (Invitrogen) (PS) and was ready for use 2 days after transfection. When virus was not used directly, it was snap frozen in liquid nitrogen and stored at -80°C. Viral supernatants were titred using primary rat Schwann cells.

Primary cell culture and virus infection

DRGs were isolated from individually genotyped E13.5 mouse embryos (Kleitman et al., 2002) and dissociated in trypsin. Cells were plated onto Matrigel (BD Biosciences)/poly-D-lysine (Sigma)-coated 18 mm coverslips (Thermo Scientific) and maintained in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), Glutamax (Invitrogen), 4 g/l D-glucose, 100 ng/ml NGF (Harlan), 10% horse serum (Invitrogen) and PS. The next day, medium was replaced with BN medium [BME medium (Invitrogen) containing insulin-transferrin-selenium-A supplement (Invitrogen), Glutamax, 0.2% bovine serum albumin (BSA), 4 g/l D-glucose, 100 ng/ml NGF, PS] for one day. The following day, cultures were infected with retrovirus in BN medium supplemented with 3.75 µg/ml Polybrene (Sigma) and incubated overnight. The virus-containing medium was replaced with fresh BN medium and cultures were incubated for another day before medium was switched to M1 medium [MEM (Invitrogen), 3% FCS, 100 ng/ml NGF, PS]. One week after DRG isolation, myelination was induced by refreshing co-cultures every other day with MEM (Invitrogen), 10% FCS, 100 ng/ml NGF, PS, 50 µg/ml ascorbic acid (Sigma) for 19 days, after which the cultures were fixed in 4% paraformaldehyde (PFA)/4% sucrose in PBS for 15 min at 4°C. During the culture period, conditioned medium from the cultures was collected to assess the expression of the LGI proteins by western blotting.

Immunohistochemistry, western blotting and antibodies

Transfected HEK293T or HeLa cells were fixed with 4% PFA/4% sucrose in PBS for 15 min at 4°C, then washed with PBS and blocked for at least 20 min at room temperature. Blocking solution contained 1% FBS, 0.2% fish skin gelatin (Sigma), 0.5% BSA in PBS. Primary antibody incubation was carried out at room temperature for 2 h or at 4°C overnight. Next day, cells were extensively washed with PBS and incubated for 45 min at room temperature with secondary antibody. Cells were mounted in Mowiol containing DAPI for DNA staining. Samples were examined under a Zeiss AxioImager Z1 microscope (63× magnification) and images were captured with an AxioCam MRm digital camera, and analyzed with AxioVision software. Brightness and contrast levels were adjusted in Adobe Photoshop CS4.

DRG cultures were essentially processed as described above. Additionally, cultures were fixed with ice-cold methanol for 20 min in -20°C to facilitate P0 staining. After fixation, cultures were washed with PBS/0.1% Triton X-100 and blocked in 1% FBS, 0.2% fish skin gelatin, 0.5% BSA in PBS, for 1 h at room temperature. First and secondary antibody incubation and analysis was the same as described for HEK293T and HeLa cells, except after incubation with antibodies cultures were washed five times with PBS/0.1% Triton X-100. Cultures were microscopically analyzed as described above using 20× magnification. Western blot experiments were performed as previously described (Jaegle et al., 2003).

Primary antibodies were from the following sources: chicken anti-MPZ [PZO, Aves Labs; immunofluorescence (IF), 1:1000; western blot (WB), 1:10,000], mouse anti-neurofilament M (2H3 monoclonal antibody developed by Jessell and colleagues obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department

of biology, University of Iowa, Iowa City, IA, USA; IF, 1:100), rabbit anti-c-Myc (SC-789, Santa Cruz Biotechnology; WB, 1:1000), mouse anti-V5 [05025C5, Absea Biotechnology; WB, 1:100; and 05025D5, Absea Biotechnology; IF, 1:10], mouse-anti-MBP (SAB5300427, Sigma; WB, 1:1000), mouse anti-acetylated alpha tubulin (clone C3B9, European Collection of Cell Cultures; WB 1:10,000). The mouse anti-CASPR antibody was a kind gift of Dr E Peles (Weizmann Institute of Science, Israel) and was used at 1:200 dilution. The ADAM23 antibody was raised in rabbits against a peptide corresponding to the 15 amino acid cytoplasmic tail of the protein. The antibody was affinity purified and was used at a 1:200 dilution for western blots and at 1:50 dilution in immunohistochemistry. The following secondary antibodies were used: Alexa594-conjugated donkey anti-chicken and DyLight488-conjugated donkey anti-mouse (Jackson ImmunoResearch; IF, 1:300), Alexa488-conjugated donkey anti-mouse, Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch; IF, 1:600), HRP-conjugated goat anti-rabbit (Dako; WB, 1:5000), goat anti-human (GE Healthcare; WB, 1:5000), alkaline phosphatase-conjugated goat anti-chicken (Aves Laboratory; WB, 1:1000), alkaline phosphatase-conjugated goat anti-mouse (DAKO WB 1:1000), alkaline phosphatase-conjugated goat anti-rabbit (DAKO; WB, 1:1000).

Immunoprecipitation

For LGI-ADAM22 binding assays, media conditioned for 4 days with either ADAM22-Fc or LGI-V5-His were collected and filtered. A portion of the LGI-V5-His conditioned medium was adjusted to 20 mM imidazole and the His-tagged LGI proteins were bound to Nickel beads (Qiagen) overnight at 4°C (input). Conditioned medium from ADAM22-Fc transfected cells was adjusted with Tris pH 8 to a final concentration of 0.1 M and mixed with LGI-V5-His protein conditioned medium to allow binding overnight at 4°C. ADAM22-Fc-LGI-V5-His protein complexes were bound to Protein A beads (RepliGen) and precipitated. Beads were washed in PBS/0.1% Triton X-100 and bound proteins were separated on a 4-12% gradient SDS-PAGE gel (Invitrogen) and analyzed by western blotting.

LGI-V5-His proteins from conditioned media of retrovirally transduced *clp/clp* cultures were precipitated using a V5 antibody (050210D11, Absea Biotechnology) covalently coupled to Protein A beads (RepliGen). Bound proteins were separated on a 4-12% gradient SDS-PAGE gel and analyzed by western blotting using a V5 monoclonal antibody (clone 5C5).

Light microscopy

Mice were deeply anesthetized with sodium pentobarbital and transcardially perfused with PBS followed by 4% PFA/1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Sciatic nerves were dissected and placed in the same fixative at 4°C for at least 16 h. Tissues were washed with 0.1 M phosphate buffer and osmicated overnight at 4°C in 1% osmium tetroxide/ferricyanide in phosphate buffer. Tissues were embedded in plastic resin and 1 µm sections were cut, mounted and stained with paraphenylenediamine (PPD), as described previously (Estable-Puig et al., 1965; Ozkaynak et al., 2010). Sections were examined using an Olympus BX40 microscope and pictures were collected using a ColorviewIIIu camera. Images were processed using Adobe photoshop CS4.

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Competing interests

The authors declare no competing financial interests.

Author contributions

L.K. performed most of the experiments and S.D., M.J., E.A., K.L. and D.M. assisted with the experiments. Y.F., M.W. and M.F. provided valuable unpublished reagents. L.K., M.J. and D.M. designed the study and wrote the manuscript.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.107995/-/DC1>

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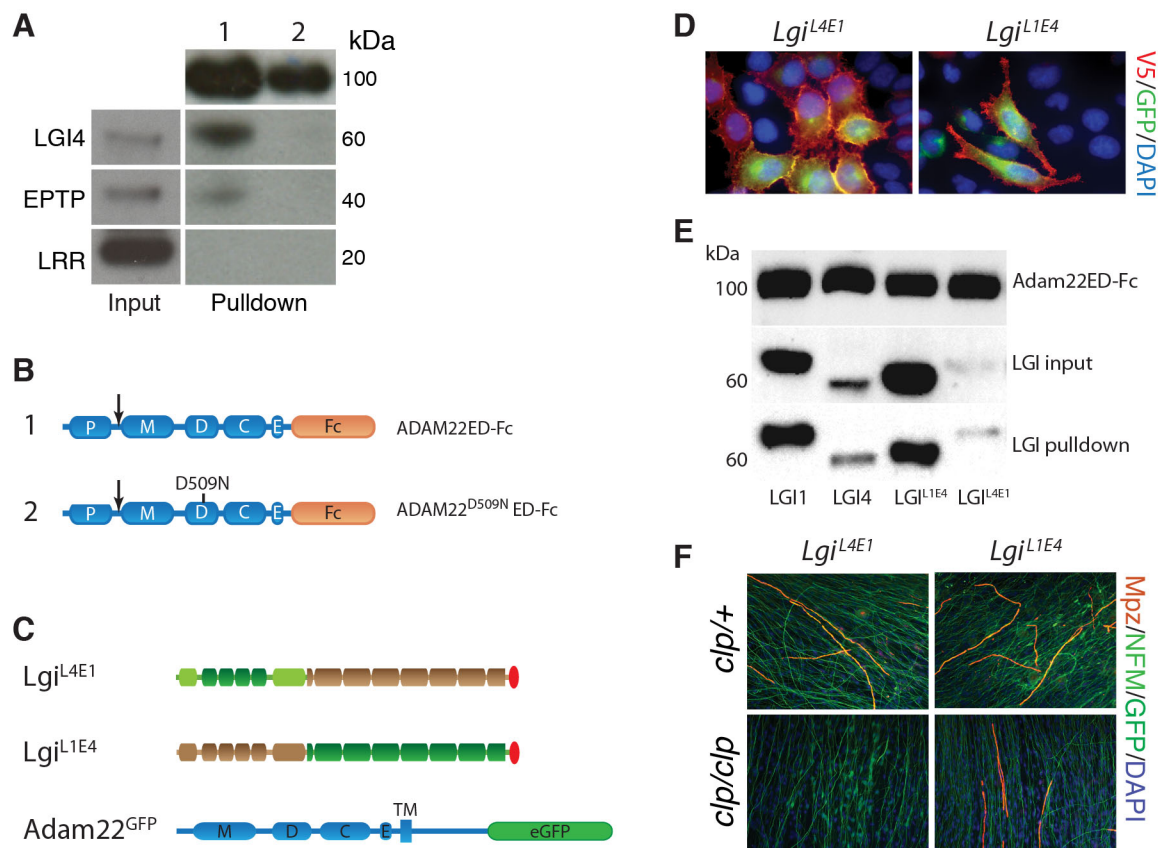
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Supplementary Figure 1.

The EPTP domain of LGI4 is required for binding to the extracellular domain of ADAM22 and myelination. (A) LGI4 and the LGI4 EPTP (EPTP) domain are efficiently precipitated from conditioned medium with a soluble form of the ADAM22 ectodomain (ADAM22ED-Fc; construct number 1 in B), but not by its mutant form ADAM22^{D509N}ED-Fc (construct 2 in B). The LGI4 LRR domain (LRR) has no affinity for the ADAM22 extracellular domain. All proteins are overexpressed in HEK293T cells. The LGI4, LRR and EPTP domain carry a Myc-His tag at their carboxyl terminal for detection and purification. The structure of Fc fusion proteins 1 and 2 is schematically depicted in B. P=Prodomain, M=metalloproteinase domain, D=Disintegrin domain, C= Cystein-rich domain and E is the EGF domain. The transmembrane and cytoplasmic domains of ADAM22 are replaced by the human IgG heavy chain domain (Fc). The D509N mutation in the disintegrin domain abrogates LGI ligand interactions as previously described (Fukata et al., 2006). The arrow indicates the position where convertases cleave the prodomain.

(C) Schematic representation of the chimeric LGI4/LGI1 expression cassettes. LGI^{L4E1}; the LGI4 LRR domain (green) fused to the LGI1 EPTP domain (brown). LGI^{L1E4}; The LGI1 LRR domain (brown) fused to the LGI4 EPTP domain (green). The red oval represents the carboxyl terminal V5/His tag. The ADAM22 protein is fused at its cytoplasmic tail with enhanced GFP to yield ADAM22^{GFP}. (D) Both LGI^{L4E1} and LGI^{L1E4} bind to ADAM22^{GFP} expressed at the cell surface of transfected HeLa cells. LGI proteins are detected by virtue of their V5 tag (red).

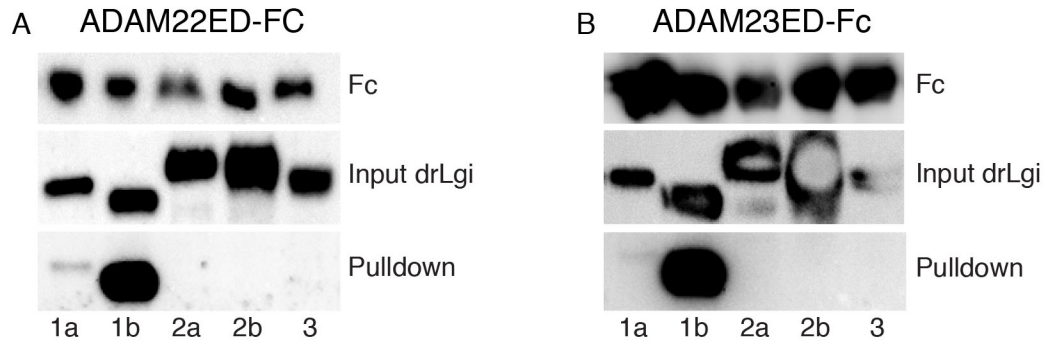
(E) The secreted LGI proteins from transfected HEK cells (LGI input) were precipitated (LGI pull down) with the soluble ADAM22ED-Fc fusion protein. (F) Retroviral transduction of *Lgi^{L1E4}* in *clp/clp* cultures restores myelination. *Clp/+* and *clp/clp* DRG cultures are infected with retrovirus containing *Lgi^{L4E1}* or *Lgi^{L1E4}* and maintained in myelinating conditions for 19 days and then stained for MPZ to reveal compact myelin segments and neurofilament (NFM) to reveal neurites. Infected Schwann cells express GFP as the retroviral construct carries an IRES-GFP cassette. Bar = 100 μ m.



Supplementary Figure 2.

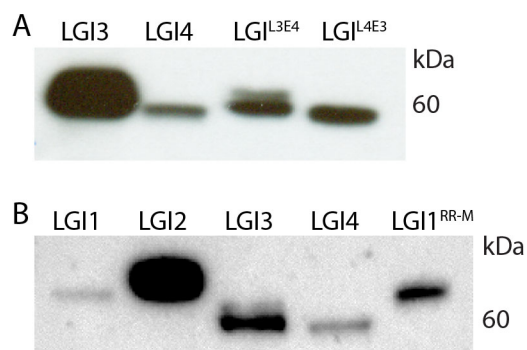
Zebrafish *Lgi1a* and *Lgi1b* bind directly to the extracellular domain of mouse ADAM22 and ADAM23. Conditioned medium containing mouse ADAM22ED-Fc (A) or mouse ADAM23ED-

Fc (B) was mixed with conditioned medium containing zebrafish Lgi protein to allow the formation of complexes, which were subsequently precipitated using proteinA agarose beads. The ADAM ectodomain fusion proteins are detected with an anti human Fc antibody. The level of zebrafish Lgi protein expression (input) was determined by purification of Lgi protein from conditioned medium using Ni-NTA beads. The zebrafish Lgi proteins are detected by virtue of their V5 tag. The Lgi proteins are labeled 1a=Lgi1a, 1b=Lgi1b, 2a=Lgi2a, 2b=Lgi2b and 3=Lgi3.



Supplementary Figure 3.

Mutant LGI proteins are expressed and secreted from transfected HEK cells. The mutant proteins used in Figure 3 (LGI^{L3E4} and LGI^{L4E3}) and 5 (LGI^{RR-M}) can be recovered from the medium of transfected HEK cells. Proteins are detected by Western blot using a V5 antibody.



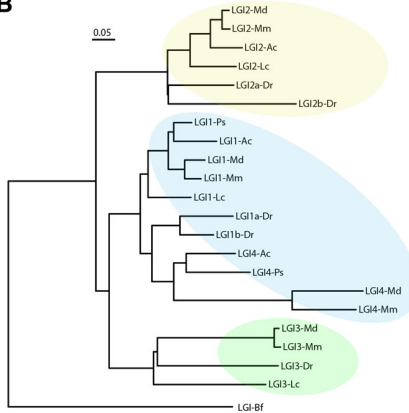
Supplementary Figure 4.

Phylogenetic analysis reveals a close evolutionary relationship between LGI1 and LGI4 proteins. Protein sequence alignments of LGI proteins from a wide range of organisms were performed using the DNAMAN software package (Lynnon corporation). LGI protein sequences encompassing the EPTP4 to EPTP7 repeat from a placental mammal (mouse *Mus musculus* Mm), a marsupial (opossum *Monodelphis domestica* Md), reptiles (green Anole lizard *Anolis carolinensis* Ac and Chinese softshell turtle *Pelodiscus sinensis* Ps), a lobe-finned fish (Coelacanth *Latimeria chalumnae* Lc), a teleostfish (Zebrafish *Danio rerio* Dr) are compared with the one LGI protein encoded in the genome of the primitive chordate *Branchiostoma floridae*, a lancelet. The three amino acids that are identical in zebrafish Lgi1a and Lgi1b and LGI4 are highlighted in yellow. Note that the Lgi1 protein encoded in the Coelacanth genome has the same three amino acids as LGI4 and Lgi1a/1b. On the basis of this sequence alignment a phylogenetic tree was constructed using the maximum likelihood method of Jones, Taylor and Thornton (Jones et al., 1992). The *Branchiostoma floridae* Lgi protein was used as an outgroup to root the tree. The tree suggests that a last common ancestor to the modern day vertebrates had three *Lgi* genes (*Lgi1*, *Lgi2* and *Lgi3*) and that *Lgi4* arose in the tetrapod lineage through duplication of an ancestral *Lgi1* gene.

A

	EPTP4	EPTP5	EPTP6	EPTP7
LG12-Md	RDTDAEFVDIDG----	KSHLLLSRSQVPIILQNNKSSKRFVPHGDI	PNMEDVLAVKSRMQNTLYLSLTRFIGDSRVHKN	NS--KQFVEIQALPSRGAMTLQRF
LG12-Mm	RDTDAEFVDIDG----	KSHLLLSRSQVPIILQNNKSSKRFVPHGDI	PNMEDVLAVKSRMQNTLYLSLTRFIGDSRVHKN	NS--KQFVEIQALPSRGAMTLQRF
LG12-Ac	RDTDAEFVDIDG----	KSHLLLSRSQVPIILQNNKSSKRFVPHGDI	PNMEDVLAVKSRMQNTLYLSLTRFIGDSRVHKN	NS--KQFVEIQALPSRGAMTLQRF
LG12-Lc	RDTDAEFVDIDG----	KSHLLLSRSQVPIILQNNKSSKRFVPHGDI	PNMEDVLAVKSRMQNTLYLSLTRFIGDSRVHKN	NS--KQFVEIQALPSRGAMTLQRF
LG12a-Dr	RDTDAEFVNLGD----	KAHLLLASRSQVPIYQNSRSTQKFTLQGEI	PNMEDVAVKAKETIKEDLYLAMTRYIGDSKVLH	MTA--KEFEVQAI
LG12b-Dr	RDTDAEFVNLGD----	KAHLLLASRSQVPIYQNSRSTQKFTLQGEI	PNMEDVAVKAKETIKEDLYLAMTRYIGDSKVLH	MTA--KEFEVQAI
LG11-Lc	RDTDAEFVLDIG----	KPHLLLSRSQVPIYQNSKGTSNFIDPVDI	PDMDVYAVKHKVKKEDIYVCLTRFIGDSKVMK	GG--SFKLDLQRP
LG11-Ac	RDTDAEFVLDIG----	KPHLLLSRSQVPIYQNSKGTSNFIDPVDI	PDMDVYAVKHKVKKEDIYVCLTRFIGDSKVMK	GG--SFKLDLQRP
LG11a-Dr	RDTDAEFVLDIG----	KPHLLLSRSQVPIYQNSKGTSNFIDPVDI	PDMDVYAVKHKVKKEDIYVCLTRFIGDSKVMK	GG--SFKLDLQRP
LG11b-Dr	RDTDAEFVLDIG----	KPHLLLSRSQVPIYQNSKGTSNFIDPVDI	PDMDVYAVKHKVKKEDIYVCLTRFIGDSKVMK	GG--SFKLDLQRP
LG14-Ac	RDTDAEFVLDIG----	KPHLLLSRSQVPIYQNSKGTSNFIDPVDI	PDMDVYAVKHKVKKEDIYVCLTRFIGDSKVMK	GG--SFKLDLQRP
LG14-Ps	RDTDAEFVLDIG----	KPHLLLSRSQVPIYQNSKGTSNFIDPVDI	PDMDVYAVKHKVKKEDIYVCLTRFIGDSKVMK	GG--SFKLDLQRP
LG14-Md	RDTDAEFVLDIG----	KPHLLLSRSQVPIYQNSKGTSNFIDPVDI	PDMDVYAVKHKVKKEDIYVCLTRFIGDSKVMK	GG--SFKLDLQRP
LG14-Mm	RDTDAEFVLDIG----	KPHLLLSRSQVPIYQNSKGTSNFIDPVDI	PDMDVYAVKHKVKKEDIYVCLTRFIGDSKVMK	GG--SFKLDLQRP
LG13-Md	RDTDAEFVLDIG----	KPHLLLSRSQVPIYQNSKGTSNFIDPVDI	PDMDVYAVKHKVKKEDIYVCLTRFIGDSKVMK	GG--SFKLDLQRP
LG13-Mm	RDTDAEFVLDIG----	KPHLLLSRSQVPIYQNSKGTSNFIDPVDI	PDMDVYAVKHKVKKEDIYVCLTRFIGDSKVMK	GG--SFKLDLQRP
LG13-Lc	RDTDAEFVLDIG----	KPHLLLSRSQVPIYQNSKGTSNFIDPVDI	PDMDVYAVKHKVKKEDIYVCLTRFIGDSKVMK	GG--SFKLDLQRP
LG13-Dr	RDTDAEFVLDIG----	KPHLLLSRSQVPIYQNSKGTSNFIDPVDI	PDMDVYAVKHKVKKEDIYVCLTRFIGDSKVMK	GG--SFKLDLQRP
LG1-Bf1	HEADFEFFVDIT----	QPVLTINLSGRHVALYKSRGQRQIF	FEADIPFEEFVAHS--EVLDDVVYCLTQF	IGESKLLRNMTSTSGQVYKFSRGMVLCQLQV

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