Perlecan is recruited by dystroglycan to nodes of Ranvier and binds the clustering molecule gliomedin

Cristina Colombelli,1* Marilena Palmisano,1,2,3* Yael Eshed-Eisenbach,4 Desirée Zambroni,1 Ernesto Pavoni,1 Cinzia Ferri,1 Stefania Saccucci,1 Sophie Nicole.5,6,7,8 Raija Soininen,9 Karen K. McKee,10 Peter D. Yurchenco,10 Elior Peles,4 Lawrence Wrabetz,1,2,3 and M. Laura Feltri1,2,3

1Division of Genetics and Cell Biology, San Raffaele Hospital, 20132 Milan, Italy
2Department of Biochemistry and 3Department of Neurology, Hunter James Kelly Research Institute, School of Medicine and Biomedical Sciences, University at Buffalo, Buffalo, NY 14203
3Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel
4Institut du Cerveau et de la Moelle Épinière, 75013 Paris, France
5Institut National de la Santé et de la Recherche Médicale, U1127, 75019 Paris, France
6Sorbonne Universités, Université Pierre et Marie Curie, UMR5127, 75252 Paris, France
7Centre National de la Recherche Scientifique, UMR 7225, 75013 Paris, France
8Oulu Center for Cell-Extracellular Matrix Research, University of Oulu, 90014 Oulu, Finland
9Oulu Center for Cell–Extracellular Matrix Research, University of Oulu, 90014 Oulu, Finland
10Rutgers University, Piscataway, NJ 08854

Fast neural conduction requires accumulation of Na+ channels at nodes of Ranvier. Dedicated adhesion molecules on myelinating cells and axons govern node organization. Among those, specific laminins and dystroglycan complexes contribute to Na+ channel clustering at peripheral nodes by unknown mechanisms. We show that in addition to facing the basal lamina, dystroglycan is found near the nodal matrix around axons, binds matrix components, and participates in initial events of nodogenesis. We identify the dystroglycan-ligand perlecan as a novel nodal component and show that dystroglycan is required for the selective accumulation of perlecan at nodes. Perlecan binds the clustering molecule gliomedin and enhances clustering of node of Ranvier components. These data show that proteoglycans have specific roles in peripheral nodes and indicate that peripheral and central axons use similar strategies but different molecules to form nodes of Ranvier. Further, our data indicate that dystroglycan binds free matrix that is not organized in a basal lamina.

Introduction

Nodes of Ranvier are located at gaps in myelin, where the axolemna is endowed with high densities of voltage-gated Na+ channels that ensure the regeneration of action potentials during saltatory conduction (Ranvier, 1871; Hodgkin and Huxley, 1952). The way Na+ channels accumulate at these focal sites is the matter of intense studies. Neurofascin 186 (NF186) is a pioneer molecule that traps Na+ channels at nodes by linking to glial molecules and to the axonal cytoskeleton (Sherman et al., 2005; Zonta et al., 2008; Thaxton et al., 2011). Additional mechanisms such as barriers formed by adjacent paranodal junctions are required to safeguard node integrity (Feinberg et al., 2010). Peripheral and central nodes have common and distinct features, the most notable being the difference in the overlying glial cell: oligodendrocytes and astrocytic processes in the central nervous system (CNS) versus Schwann cell (SC) microvilli in the peripheral nervous system (PNS; Elfvin, 1961; Peters, 1966; Hildebrand, 1971; Hildebrand and Waxman, 1984; Raine, 1984; Waxman and Black, 1984; Ichimura and Ellisman, 1991).

Both PNS and CNS nodes are embedded in a matrix enriched in nonsulfated mucopolysaccharides, hyaluronic acid, and proteoglycans. Recent work showed that proteoglycans in the CNS constitute a third redundant protection for nodes, such that disruption of more than one mechanism is required to impair Na+ channel localization and maintenance (Susuki et al., 2013).
mice lacking SC laminins and in a merosin-deficient muscular dystrophy patient (Occhi et al., 2005). SC DG comprises an α subunit that binds laminins, agrin, and perlecan in basal laminae and a transmembrane β subunit linked to the cytoskeleton through different dystrophin isoforms. DG and the 116-kD dystrophin (Dp116) are in microvilli, whereas laminins 211 and 511 are enriched in the basal lamina over nodes (Occhi et al., 2005). It is unknown whether DG is required for the formation or maintenance of Na+ channel clusters and by which mechanism. Here we show that DG is recruited to nascent nodes and is required for the formation of normal heminodes and Na+ channel clusters. By immunoelectron microscopy (IEM), α- and β-DG are localized at SC microvilli facing both the basal lamina and the axon, suggesting that in addition to its known role as a basal lamina receptor, DG could interact with components of the perinodal matrix. Indeed, we find that the DG ligand perlecan is a novel proteoglycan found in PNS nodes, and that perlecan localization at nodes requires DG. Perlecan binds gliomedin and enhances clustering of nodal components by gliomedin. This work identifies perlecan as a HSPG that binds gliomedin and highlights similarities and differences with the assembly of central nodes.

Results

α- and β-DG are early markers of SC microvilli

α-DG, β-DG, and Dp116 are found at nodes of Ranvier, and laminins 211 and 511 are enriched in the basal lamina over microvilli (Occhi et al., 2005). Ablation of DG in SCs results in small and abnormally shaped Na+ channel clusters at nodes (Saito et al., 2003; Occhi et al., 2005). To explore if this is
Absence of DG does not interfere with the timing of Na⁺ channel cluster formation
We counted the number of clusters flanked by Caspr-positive paranodes at different ages in sciatic nerves of mice deficient in SC-DG and found that at each time point the number of clusters per field of view was similar to wild-type animals (Fig. 2 A). Therefore, the absence of DG does not affect the timing of cluster formation.

Newly formed clusters are already abnormal in the absence of DG
To determine whether DG is required to form or stabilize clusters, we evaluated the morphology of newly formed clusters in mutant nerves at P4 and P7 by immunostaining with anti–pan-Na⁺

caused by defective cluster formation during development or by degeneration of normal clusters over time, we first asked if DG is found in developing nodes by immunostaining sciatic nerve teased fibers. Both α- and β-DG colocalize with ezrin, an early nodal marker, soon after birth (Fig. 1). Western blot analysis confirmed that both glycosylated α-DG, recognized by the glyco-specific antibody IIH6, and the cleaved (β-DG31) and uncleaved form of β-DG (β-DG43) are present at postnatal day 2 (P2; Fig. 1; Yamada et al., 2001; Martin, 2003; Hnia et al., 2006; Zhong et al., 2006; Cai et al., 2007; Court et al., 2011; Walko et al., 2013). In contrast, laminin α5 at P2–P5 is found predominantly in perinuclear regions, and it acquires its nodal localization starting at P10 (Fig. S1). Thus, laminin 511 localizes at nodes after they are formed.

Figure 2. DG is required for proper Na⁺ channel clustering at nascent nodes and heminodes. (A) Na⁺ channel clustering is not delayed in DG-deficient nerves. Number of paranodes (Caspr staining) containing (shaded bars) or devoid (open bars) of Na⁺ channels in nerves. Total sites analyzed for wild type were P2, 89; P5, 362; and P10, 1,107; and for dgko, P2, 74; P5, 336; and P10, 786. Results are reported as mean ± SEM of three mice/genotype. (B and C) Na⁺ channel clusters form abnormally without DG. Sciatic nerve fibers from P4 and P7 mice stained for pan-Na⁺ channels. (B) Mutant clusters are smaller and irregular. (C) More clusters are abnormal in DG mutants [P4, n = 83, 79%; P7, n = 41, 69.5% in mutant; P4, n = 27, 23%; P7, n = 15, 23.8% in wild type]. P < 0.001 mutant versus wild type by χ² test; n = 224 (P4) and n = 122 (P7). (D) Abnormal or absent Na⁺ channel clusters at heminodes are more frequent in dgko (n = 186; 40%) than control (n = 49; 14%). P < 0.001 by χ² test; n = 823 nodes. (E) Teased nerve fibers from P6 nerves labeled for Na⁺ channels (red), Caspr (green), and neurofilament (NF; blue). Arrowheads point to the position of developing heminodes/nodes. (F) Staining with S100 (green) shows that SC cytoplasm covers the space between heminodes (arrows). Bars: (B) 4.5 µm; (E and F) 17.5 µm.
the nodal matrix that binds NF186 and NrCAM, is required for formation of heminodes (Feinberg et al., 2010). To ask if DG is also involved, we stained developing nerves from wild-type and DG-deficient mice at P6, when both nodes and heminodes can be detected (Schafer et al., 2006; Fig. 2 E). Two independent investigators counted heminodal clusters blindly (identified by the presence of only one flanking Caspr-positive paranode) and classified them as normal or abnormal/absent. The frequency of abnormal or absent clusters was higher in mutants than controls (Fig. 2 D). Thus, DG contributes to the clustering of Na+ channels at heminodes.

Proper clustering of Na+ channels at heminodes requires DG

In development, Na+ channels are first redistributed from local axolemma pools to heminodes, through interaction with extracellular molecules (Feinberg et al., 2010; Zhang et al., 2012). Longitudinal growth of the glial processes, which entirely covers the space between heminodes (Fig. 2 F), displaces these heminodal clusters, probably bringing them closer until they fuse and form a single node (Boiko et al., 2001; Pedraza et al., 2001; Eshed-Eisenbach and Peles, 2013). Gliomedin, a component of the nodal matrix that binds NF186 and NrCAM, is required for formation of heminodes (Feinberg et al., 2010). To ask if DG is also involved, we stained developing nerves from wild-type and DG-deficient mice at P6, when both nodes and heminodes can be detected (Schafer et al., 2006; Fig. 2 E). Two independent investigators counted heminodal clusters blindly (identified by the presence of only one flanking Caspr-positive paranode) and classified them as normal or abnormal/absent. The frequency of abnormal or absent clusters was higher in mutants than controls (Fig. 2 D). Thus, DG contributes to the clustering of Na+ channels at heminodes.

The absence of paranodal junctions slightly worsens the nodal abnormalities of DG-deficient mice

Several overlapping mechanisms ensure Na+ channel accumulation at nodes. The pioneer molecule NF186 is first redistributed from local pools to heminodes by interacting with NrCAM and gliomedin (Sherman et al., 2005; Feinberg et al., 2010; Thaxton et al., 2011; Zhang et al., 2012). Here NF186 recruits Na+ channels,
which are then stabilized through ankyrin G and βIV spectrin in the axon (Komada and Soriano, 2002; Lacas-Gervais et al., 2004; Yang et al., 2004; Koticha et al., 2006; Dzhashiashvili et al., 2007) and by formation of paranodes (Feinberg et al., 2010). Disruption of at least two of these mechanisms is necessary to impair Na⁺ channel clustering (Feinberg et al., 2010).

To ask if disruption of paranodes further impaired nodal clustering in DG mutants, we crossed them with mice lacking the paranodal protein Caspr. Caspr null mice exhibit a progressive neurological defect, but have a normal life span (Bhat et al., 2001; Gollan et al., 2003). Homozygous dgko/Caspr−/− mice were similar to Caspr−/− mice without worsening of the neurological phenotype. By EM, double mutants showed more severe and frequent nodal defects than single DG mutants. Microvilli were more disorganized, did not attach to the nodal axolemma, and penetrated the space between paranodal loops and axons (Fig. 3 F–I). Axonal protrusions that contained mitochondria and vesicles invaded the nodal gap in place of atrophic microvilli (Fig. 3 I) and were detected with higher frequency (Fig. 3 K). Despite these abnormalities, nodal length was not increased (Fig. 3 J) and nodal clustering of Na⁺ channels was not worsened (Fig. 3 A–E).

α- and β-DG localize in the nodal gap

By confocal microscopy, DG colocalizes with ezrin/radixin/moesin (ERM) proteins at SC microvilli (Occhi et al., 2005). To address whether DG is inserted solely in the SC membrane facing the basal lamina, or also in the nodal gap facing the axon, we performed IEM on sciatic nerves. Both α- and β-DG are found in SC microvilli not only adjacent to the basal lamina but also in the nodal gap abutting onto the axon (Fig. 4, A and B). Only occasional gold grains were found in DG-deficient mice (Fig. 4).

In light of the dual localization of α- and β-DG, we hypothesize two different, but not mutually exclusive, mechanisms through which DG aids Na⁺ channel clustering. First, by bridging the basal lamina to the cytoskeleton, DG induces cytoskeletal rearrangements required for microvilli to grow radially and internodes to grow longitudinally adjacent to nodes, thus mechanically restricting Na⁺ channel. Indeed, DG deficiency in SCs causes microvillar hypotrophy (Saito et al., 2003) and short internodes (Court et al., 2009), and in epithelia DG interacts with ezrin to form cellular protrusions (Spence et al., 2004). The second possibility is that DG also organizes the free ECM in the nodal gap and favors the local presentation of adhesion molecules important for Na⁺ channel clustering. The combination of these multiple mechanisms would explain why Na⁺ channel cluster abnormalities are more frequent at nodes than at heminodes in the absence of DG (Fig. 2).

Microvillar ERM proteins are reduced in DG-deficient nerves

The first hypothesis is supported by the fact that even mutants for the DG ligand laminin 211 show microvillar and Na⁺ channel clustering defects similar to those found in DG-deficient mice (Occhi et al., 2005). Microvillar hypotrophy could be caused by a deficient linkage with ezrin, which is located in microvilli and interacts with β-DG (Spence et al., 2004). In DG-deficient nerves, Western blot revealed a slight decrease in the levels of total and phosphorylated ERMs (Fig. 5 A) after normalization to neurofascin to account for the higher numbers of nodes in mutants caused by short internodes (Court et al., 2009). By
immunofluorescence, although ezrin is normally restricted to nodes, we observed a higher number of cytoplasmic puncta along the fiber, suggesting an impairment of ERM transport or increased endocytosis (Fig. 5 C, arrows). Indeed, when the image was overexposed, we detected some intermodal ERM puncta along microtubules also in normal nerves (Fig. 5 F), suggesting that they may be transported along microtubules in the cytoplasmic channels named Cajal bands (Court et al., 2004). Cajal bands and microtubular tracks are disrupted in SCs lacking laminin 211 and DG (Court et al., 2009), providing a possible common mechanism resulting in impaired ERM transport. Indeed ERM-P nodal staining was frequently reduced in DG-deficient nodes (Fig. 5, E and G), the absence of ERM-P at nodes correlated with Na⁺ channel abnormalities at P6, but not at P90 (Fig. 5, H and I). Thus, the absence of DG decreases ERM accumulation in a subset of nodes, and this may contribute to abnormal Na⁺ channel clustering during development.

α-DG can be shed after proteolysis cleavage and binds SC and the ECM, but not axons

The second possibility is that DG favors the concentration of molecules that promote Na⁺ channel clustering in the nodal gap. This could be mediated by DG directly or by DG ligands HSPGs, which have been proposed to mediate the incorporation of gliomedin into the nodal ECM (Eshed et al., 2007). To address this, we first asked if fragments of the ligand binding α-DG could be released in the nodal gap and if they bound molecules on axons, SCs, or the ECM. Metalloproteinase 2 and 9 cleave α-DG releasing the N-terminal domain of α-DG. This cleavage is active and regulated in SCs (Court et al., 2011) and could shed the whole α-DG molecule in the nodal gap. In addition, α-DG is cleaved by a furin protease in cell lines, releasing the N-terminal domain α-DG-N (Singh et al., 2004; Saito et al., 2008). Because gliomedin is also secreted upon furin proprotein convertase cleavage (Eshed et al., 2007; Maertens et al., 2007; Feinberg et al., 2010), we asked if...
α-DG-N was released by SCs to potentially be deposited in the nodal gap. Rat SCs cultured in the presence of the furin inhibitor I (CMK) were analyzed by Western blot. In the absence of CMK, α-DG in cell lysates had a molecular weight of ~120 kDa using the IIH6 antibody, which recognizes the mucin-like domain of α-DG, whereas conditioned medium contained an ~35–40-kDa fragment detected by an anti-α-DG-N-specific antibody (Saito et al., 2008; Fig. 6 A). After treatment with CMK the IIH6 antibody detected α-DG with a larger molecular mass of ~160 kDa in cell lysates, whereas the 35–40-kDa band became undetectable in culture medium (Fig. 6 A). Finally, treatment of dorsal root ganglia (DRG)/SC co-cultures with CMK inhibited myelination and reduced Na⁺ channel clustering at nodes (Fig. S2). Thus α-DG-N is excised by furin and shed, and furin cleavage could control the composition of the nodal gap. To ask if α-DG can bind SC, matrix, or axonal molecules around nodes, we used α-DG fusion proteins and performed binding assays. DGFc5 represents the whole α-DG-N, DGFc6 has a deletion in the N-terminal domain, whereas DGFc2 encompasses the N-terminal domain (Fig. 6 B). In cultured SCs, both DGFc5 and DGFc6, but not DGFc2, bound to the cell membrane and were deposited in the ECM, similar...
Perlecan is at nodes of Ranvier and is selectively lost in the absence of DG. Sciatic nerve fibers from wild-type and dgko mice. Staining for agrin (agr), perlecan (pcan), or syndecan-3 (syn-3; green; A–J) and Caspr (merged confocal images in A’–J’). Perlecan is enriched at wild type developing (A and C) and adult (E) nodes (arrows). In the absence of DG, perlecan is lost at nodes, but not in the basal lamina (B, D, and F; asterisks). 68% and 87% Caspr-positive paranodes flank perlecan-positive nodes at P6 and P10 in wild-type nerves. Only 4% and 6% of nodes are perlecan positive in DG-deficient age-matched animals. (G–J’) Agrin (G and H) and syndecan-3 (I and J) are at nodes, but they are retained in the absence of DG (G’–J’). Bars, 17.5 μm.

Perlecan is reduced in DG-deficient nodes, but two different perlecan mutants have normal Na⁺ channel clusters

We next asked if proteoglycans that are known DG ligands are found at nodes and discovered that the HPSGs agrin and perlecan (Gesemann et al., 1998; Talts et al., 1999) are enriched at nodes of Ranvier (Fig. 7, E–G). Notably, perlecan is absent in the majority of nodes deficient in DG (Fig. 7 F), indicating that DG is required to localize perlecan at nodes. Perlecan is at nodes early in postnatal development (Fig. 7, A–C) and absent in DG-deficient nerves as early as P6 (Fig. 7, B–D), supporting a potential role in the formation of Na⁺ channel clusters. To test this, we analyzed peripheral nerves from two different perlecan mutant mice: Hspg2<sup>3/3</sup>, lacking the attachment sites of heparan sulfate (HS) side chains (Rossi et al., 2003), and Hspg2<sup>KI/KO</sup>, compound heterozygote carrying a null allele and a point mutation found in Schwartz-Jampel syndrome (Stum et al., 2008; Bangratz et al., 2012), which results in a severe hypomorph. Hspg2<sup>KI/KO</sup> mutants display congenital nerve hyperexcitability and muscle stiffness caused by dysfunction of muscles and neuromuscular junctions (Stum et al., 2008; Bangratz et al., 2012). Clusters of gliomedin and Na⁺ channels were not altered in adult Hspg2<sup>3/3</sup> mice (Fig. 8, A–D) or in developing and adult Hspg2<sup>KI/KO</sup> nodes and heminodes, despite the marked reduction of perlecan in P6 mutant nerves (Fig. 8, E–F’). Overall, these results indicate that DG is required to concentrate perlecan in the nodal gap, but absence of perlecan alone is not sufficient to impair Na⁺ channel clustering. Indeed, in addition to perlecan and agrin, syndecan-3 and -4 and versican-1 are present at nodes (Goutebroze et al., 2003; Melendez-Vasquez et al., 2005), and their localization at nodes is maintained in the absence of DG and perlecan (Fig. 7, H and J; and Fig. S3). Thus, it is probable that significant redundancy among proteoglycans exists in PNS nodes, similar to a recent study in the CNS (Susuki et al., 2013).
Perlecan links the matrix at nodes of Ranvier

Perlecan binds gliomedin and favors Na⁺ channel cluster formation

It was recently reported that the proteoglycans versican2, brevican, and Bral1 stabilize Na⁺ channels at CNS nodes by binding to NF186 and Na⁺ channels (Hedstrom et al., 2007; Susuki et al., 2013). We thus assessed whether perlecan also binds NF186 or other nodal components. Recombinant perlecan was purified (Fig. 9 A) and spotted at increasing concentrations for Far
binding was revealed. As predicted, the positive control α-DG bound to perlecan, whereas empty Fc did not bind. In addition, gliomedin ECD-Fc bound strongly to perlecan (Fig. 9 C).

Western assay. The membrane was overlaid with recombinant Fc fusion proteins for the extracellular domains of neurofascin, NrCAM, gliomedin (ECD-Fc), or α-DG (DG-Fc5) and binding was revealed. As predicted, the positive control α-DG bound to perlecan, whereas empty Fc did not bind. In addition, gliomedin ECD-Fc bound strongly to perlecan (Fig. 9 C).
In contrast, and differently from CNS proteoglycans, we could not detect binding of NF186 or NrCAM to perlecan. It was shown that gliomedin ECD-Fc induces Na⁺ channel clustering. It was shown that gliomedin ECD-Fc induces Na⁺ channel clustering on isolated DRG neurons, provided that the Fc domain is preaggregated with anti-Fc antibodies (Eshed et al., 2005). Gliomedin ECD contains two domains that are both required to cluster nodal proteins: the olfactomedin domain (OLF), which binds NF186 and NrCAM; and the collagen-like domain that mediates multimerization and incorporation into the ECM by binding to HSPGs (Eshed et al., 2007; Labasgue et al., 2011). We postulated that perlecan could be one of the HSPGs that incorporates gliomedin into the ECM. To test this, we first asked if deletion of either the olfactomedin or the collagen-like domain prevented binding to perlecan, and found that indeed the collagen domain of gliomedin was necessary and sufficient (Fig. 9, B and C). Finally the HS chains of perlecan contributed binding, because binding was reduced in the presence of heparin (Fig. 9 C). Treatment of perlecan with heparinase decreased, but not abolished binding (Fig. S4), indicating that gliomedin binds to both the perlecan protein core and HS chains. We next repeated the Far Western by spotting constant amounts of perlecan and overlaying increasing amounts of purified Fc fusion proteins. As shown in Fig. 9 D, perlecan bound gliomedin in a dose-dependent fashion and with higher affinity than DG. Solid-phase assay experiments predict apparent dissociation constants of 8 nM for gliomedin and 41 nM for DG (Fig. S4). Next, we confirmed that perlecan and gliomedin colocalize at nodes of Ranvier in teased fibers in vivo (Fig. 9 E). Finally, we asked if the addition of recombinant perlecan to DRG treated with gliomedin Fc influenced gliomedin binding to neurons and the clustering of nodal components. Strikingly, perlecan increased the formation of nodal-like gliomedin clusters that contained βIV spectrin (Fig. 9, H–J). Binding of gliomedin Fc and cluster formation was specific, because Fc alone did not bind, irrespective of the presence of perlecan (unpublished data). Na⁺ channels and βIV spectrin, but not Caspr coclustered with gliomedin (Fig. 9, F and G) and βIV spectrin was diffusely localized along axons before clustering (Fig. 9 L). Perlecan localized to clusters and colocalized with Gldn-ECD, but not with Gldn-OLF (Fig. 9, F and G). Addition of perlecan alone did not induce Na⁺ clustering (unpublished data). As expected, the collagen-like domain of gliomedin alone was not able to induce Na⁺ channel clustering, with or without perlecan (unpublished data). These data show that perlecan is one HSPG that favors binding of gliomedin to axons and formation of nodal-like clusters, possibly by optimizing gliomedin multimerization and presentation to NrCAM and NF186.

**Discussion**

Multiple mechanisms underlie the formation of nodes of Ranvier and adjacent paranodes and juxtaparanodes. One is incorporation of gliomedin in the perinodal gap, where it is postulated to interact with proteoglycans and to form a net that favors clustering of Na⁺ channels. Here we show that perlecan is the proteoglycan that interacts with gliomedin at nodes and favors gliomedin binding to axons and clustering of Na⁺ channels. We also show that DG favors the initial events of node formation by recruiting perlecan and organizing the SC cytoskeleton in microvilli. Multiple proteoglycans are redundant in PNS nodes (Melendez-Vasquez et al., 2005; this paper). This is reminiscent to what was recently described in CNS nodes (Susuki et al., 2013), although a different set of molecules are used.

**DG participates in the initial events of nodes of Ranvier formation**

DG and laminins are required for proper clustering of Na⁺ channels at PNS nodes. Specific laminins (211 and 511) and dystrophin complexes (Dp116) localize at nodes (Occhi et al., 2005), suggesting that a unique basal lamina–cytoskeletal linkage promotes nodal architecture. Our data exclude that laminin 511 participates in node formation because it localizes over nodes after they are formed. In contrast, Na⁺ channels cluster abnormally at nodes and heminodes in the absence of DG, suggesting that DG is involved in the formation of Na⁺ channel clusters.

**DG helps Na⁺ channel clustering via two distinct mechanisms**

Our data suggest two nonmutually exclusive mechanisms by which DG favors nodal Na⁺ channel clustering (Fig. 10): a canonical interaction with the ECM organized in the basal lamina above nodes (i.e., laminin 211); and a non-canonical interaction with amorphous ECM in the nodal gap (i.e., perlecan). In the first case, DG may influence the architecture of the node indirectly by regulating transport in Cajal bands and organization of ERM proteins in the microvilli cytoskeleton. In the second instance, DG is required locally to retain perlecan at nodes.

**Microvilli, Cajal band transport, and nodes of Ranvier**

DG and its dystrophin partners’ utrophin and DRP2/Periaxin are crucial for the organization of internodes by regulating Cajal band formation and internodal length (Court et al., 2004, 2009). Cajal bands probably endow the extremely long myelinating cell with specialized cytoplasmic channels in which microtubule-mediated transport of molecules and organelles is optimized (Court et al., 2004, 2009). In support of this, DG-null internodes contain numerous ERM puncta along microtubule tracks, suggesting that ERM transport to microvilli is impaired. We postulate that this may cause microvilli to be hypotrophic in DG nodes. Similarly, because DG-deficient internodes are short, it is conceivable that paranodes exert less mechanical force to fuse heminodal clusters into nodes.

**Perlecan, gliomedin, and clustering of Na⁺ channels**

Growing evidence shows a direct effect of the ECM, such as perineuronal nets and the nodal substance, on neuronal function (Kwok et al., 2011). The nodal gap ECM, earlier termed “cementing disc of Ranvier,” contains proteoglycans and nonsulfated mucopolysaccharides (Hess and Young, 1952; Landon and Langley, 1971) and is emerging as a region of communication between glia and the axolemma critical for Na⁺ channel clustering. In the PNS, gliomedin binds NF186 when shed into...
Mutually exclusive, mechanisms. At the side of basal lamina, DG intertwining at nodes. DG aids axonal Na⁺ channel clustering via different, not Na⁺ channels were still localized at nodes, suggesting that perlecan to NF186. In nerves mutant for perlecan, gliomedin and This in turn is needed to favor binding of the olfactomedin do-
modulate the interactions of gliomedin with the nodal ECM. impaired by heparin, suggesting that the HS chains of perlecan polymerization. This interaction is through the core protein but collagen domain of gliomedin, possibly contributing to its po-
yerization. It is possible that two sequential cleavages, by MMPs and furin, release two forms of α-DG with distinct functions in Na⁺ channel clustering.

Figure 10. Model for DG and perlecan participation in Na⁺ channel clustering at nodes. DG aids axonal Na⁺ channel clustering via different, not mutually exclusive, mechanisms. At the side of basal lamina, DG interaction with laminin 211 regulates the formation of Cajals bands, which may favor ERM transport and remodeling of the microvilli. In the nodal gap, α-DG retains perlecan, which binds gliomedin and favors its binding to axons. It is not known if α-DG at nodes is anchored to the membrane of microvilli or released after cleavage by furin or metalloproteinases. It is possible that two sequential cleavages, by MMPs and furin, release two forms of α-DG with distinct functions in Na⁺ channel clustering.

the nodal manner and incorporated in a multimolecular complex in an HS-dependent manner (Eshed et al., 2007). Here we show that perlecan is retained by DG in the nodal gap and binds the collagen domain of gliomedin, possibly contributing to its polymerization. This interaction is through the core protein but impaired by heparin, suggesting that the HS chains of perlecan modulate the interactions of gliomedin with the nodal ECM. This in turn is needed to favor binding of the olfactomedin domain to NF186. In nerves mutant for perlecan, gliomedin and Na⁺ channels were still localized at nodes, suggesting that perlecan is not the only HSPG interacting with gliomedin. Other HSPGs may be redundant with perlecan in modulating the binding of gliomedin to the ECM or to axons. The function of perlecan at nodes resembles that of HSPGs in other locations, which trap growth factors to prevent proteolysis, favor high-affinity receptor binding, or modulate release and bioavailability (Bishop et al., 2007; Bix and Iozzo, 2008). However, not all roles of perlecan are mediated by growth factors. Perlecan binds other molecules, including ECM components like collagens, via either the HS or the core protein (Whitelock et al., 2008; Farach-Carson et al., 2014). In PNS nodes, our data suggest that the interaction between perlecan and collagens (gliomedin) favors Na⁺ channel clustering.

Similarities and differences between CNS and PNS nodes

Our data reveal that SCs and central glia use similar strategies at nodes, in that both adopt a set of proteoglycans to form a functionally redundant network that ensures formation and maintenance of high Na⁺ channel density. However, the specific set of proteoglycans used is different, probably reflecting the differing cellular origin of the matrix and the lack of basal lamina in CNS myelin.

In contrast to the HSPG perlecan, CNS nodes rely on chondroitin-sulfate proteoglycans and hyaluronan-binding and linker proteins: versican V2, brevican, neurocan, phosphacan, and Bral1 (Melendez-Vasquez et al., 2005; Hedstrom et al., 2007; Dours-Zimmermann et al., 2009; Bekku et al., 2010). Similar to perlecan, genetic loss of single CNS proteoglycans does not affect clustering of Na⁺ channels, although ionic diffusion, buffering, and conduction velocity may be affected (Weber et al., 1999; Brakebusch et al., 2002; Dours-Zimmermann et al., 2009; Bekku et al., 2010). A core of proteoglycans (brevican, versican V2, and Bral1) maintains localization of the others and interacts with NF186 (Dours-Zimmermann et al., 2009; Bekku et al., 2010; Susuki et al., 2013). Deletion of these core proteins plus another redundant mechanism (i.e., paranodal or nodal adhesion molecules) prevents Na⁺ channel clustering (Susuki et al., 2013). The function of perlecan in the PNS and of these core CNS proteoglycans appears different. CNS proteoglycans bind NF186 and can initiate Na⁺ channel clustering, but are accumulated at nodes after Na⁺ channels are clustered, similar to laminin 511 in PNS nodes, and are therefore considered stabilizers of nodes (Susuki et al., 2013). In contrast, PNS perlecan does not bind NF186, but is localized early at nodes and may participate in the initial phases of clustering by favoring incorporation of gliomedin into the matrix.

Despite these differences there are also similarities. For example, perlecan, gliomedin, agrin, and other HSPGs are secreted by oligodendrocytes or astrocytes and some accumulate at nodes (Kaplan et al., 2001; Winkler et al., 2002; Eshed et al., 2007). When astrocytes or oligodendrocytes are analyzed separately in culture, they show similarities and differences from SCs at nodes. For example, oligodendrocytes secrete a Na⁺ channel clustering activity, which remains elusive (Kaplan et al., 1997). However gliomedin binds the ECM produced by SCs, but not that of astrocytes (Eshed et al., 2007). Assuming that SCs perform the role of both oligodendrocytes and astrocytes at paranodes and nodes, it is possible that similar experiments conducted with oligodendrocytes and astrocytes together may reveal that a combination of molecules secreted by oligodendrocytes (e.g., an HSPG fragment) and astrocytes (e.g., gliomedin) accounts for the elusive oligodendrocyte-secreted clustering activity (Kaplan et al., 1997).
Materials and methods

Mice

The generation of transgenic and knockout mice was previously described (Moore et al., 2002). In brief, the Dag1 floxed allele was created using a targeting construct with a floxed PGKNeo vector that resulted in the insertion of a floxed neomycin (neo) cassette at the Sall site in intron 5 of exon 2 and a loxP site at an EcoRV site 3’ of exon 2 of mouse Dag1 (a gift from K. Campbell, University of Iowa, Iowa City, IA; Moore et al., 2002). PCOcre mice were generated using the mP, an encoding recombinase under the control of a complete mouse Mpf gene with 6 kb of promoter, all exons and introns, and a natural polyadenylation site (Feltli et al., 1999). Caspr null mice (casp-/-) were generated using a replacement vector in which a neo resistance gene replaces an Sphi-BssHII fragment containing Caspr exon 1, including the initiator methionine and the sequence signal (Gall寡 et al., 2003). Hspg2fl/+/mice were generated using a targeting construct that replaces a 130-bp KpnI-BamHI fragment containing Caspr exon 1 with a PGK neo cassette (Rossi et al., 2003). The Hspg2Zfl allele was generated by introducing the c.4595G>A (p.C1532Y) point mutation into exon 36 and a floxed PGK neo cassette containing a polyadenylation signal into intron 36 in the same orientation as Hspg2, 86 bps downstream from exon 36 (Stum et al., 2008; Bangratz et al., 2012). The Hspg2Zfl allele was produced using a gene-trapping strategy (Skarnes et al., 1995), which results in a fusion protein between truncated perlecan and LacZ, retained within the ER. These mice do not have detectable perlecan mRNA and they are phenotypically similar to perlecan-deficient mice described by Arikawa-Hirasawa et al., 2005) and were congenic in C57BL6/N. The P0Cre gene with 6 kb of promoter, all exons and introns, and a natural polyadenylation site (Feltri et al., 1999). antibodies included the following: rabbit anti–mouse Caspr (Ab5270; 1:200) and mouse anti-gliomedin (Mab94; 1:50; Eshed et al., 2008; Moore et al., 2002). In brief, nerves from mutant and control mice were dissected and either fixed by immersion in ice-cold 4% paraformaldehyde for 30 min and stored in PBS at 4°C until teasing or washed in PBS and teased immediately. After perineurium removal, nerve fibers were gently separated with tungsten pins (Fine Science Tools) and transfected onto 3-aminoethylisothiouronium-sulfate–treated slides. For immunostaining, fibers were permeabilized in cold acetone for 5–10 min at −20°C. After blocking with 5% fish skin gelatin and 0.5% Triton X-100 in PBS, fibers were labeled with primary antibodies, followed by appropriate secondary antibodies, washed, dried, and mounted with Vectashield (Vector Laboratories). Western blot analysis

Sciatic nerves were dissected from rats or mice, frozen and homogenized in a metal pestle, and then lysed with lysis buffer containing 25 mM Tris, pH 7.4, 95 mM NaCl, 10 mM EDTA, pH 8, 2% SDS, 1 mM NaF, 1 mM Na3VO4, and 1% protease inhibitor cocktail (Sigma-Aldrich). After rocking at 4°C for 30 min, samples were spun at 13,200 rpm in a microcentrifuge for 10 min to eliminate insoluble material. The supernatant was recovered and stored at −80°C until use. Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Equal amounts of homogenates (containing 5–10 µg of protein) were added to reducing or non-reducing 10% SDS–polyacrylamide gels, and electrophoresed onto a polyvinylidene fluoride microporous membrane (PerkinElmer). Membranes were stained with Ponceau red to verify equal loading of proteins. Blots were blocked with 0.1% Tween and 5% dry milk in PBS and incubated with the appropriate antibody in 0.1% Tween and 1% dry milk in PBS. HRP-conjugated secondary antibodies were visualized using the ECL method with autoradiography films (GE Healthcare).

Immunohistochemistry on teased nerve fibers

Teased nerve fibers from sciatic nerves were prepared and immunostained as previously described (Occhi et al., 2003). In brief, nerves from mutant and control mice were dissected and either fixed by immersion in ice-cold 4% paraformaldehyde for 30 min and stored in PBS at 4°C until teasing or washed in PBS and teased immediately. After perineurium removal, nerve fibers were gently separated with tungsten pins (Fine Science Tools) and transfected onto 3-aminoethylisothiouronium-sulfate–treated slides. For immunostaining, fibers were permeabilized in cold acetone for 5–10 min at −20°C. After blocking with 5% fish skin gelatin and 0.5% Triton X-100 in PBS, fibers were labeled with primary antibodies, followed by appropriate secondary antibodies, washed, dried, and mounted with Vectashield (Vector Laboratories). Western blot analysis

Sciatic nerves were dissected from rats or mice, frozen and homogenized in a metal pestle, and then lysed with lysis buffer containing 25 mM Tris, pH 7.4, 95 mM NaCl, 10 mM EDTA, pH 8, 2% SDS, 1 mM NaF, 1 mM Na3VO4, and 1% protease inhibitor cocktail (Sigma-Aldrich). After rocking at 4°C for 30 min, samples were spun at 13,200 rpm in a microcentrifuge for 10 min to eliminate insoluble material. The supernatant was recovered and stored at −80°C until use. Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Equal amounts of homogenates (containing 5–10 µg of protein) were added to reducing or non-reducing 10% SDS–polyacrylamide gels, and electrophoresed onto a polyvinylidene fluoride microporous membrane (PerkinElmer). Membranes were stained with Ponceau red to verify equal loading of proteins. Blots were blocked with 0.1% Tween and 5% dry milk in PBS and incubated with the appropriate antibody in 0.1% Tween and 1% dry milk in PBS. HRP-conjugated secondary antibodies were visualized using the ECL method with autoradiography films (GE Healthcare).

EM and IEM

EM was performed as previously described (Occhi et al., 2003). For EM, adult wild-type and DG-deficient mice (used as negative controls) were anesthetized and perfused with 4% paraformaldehyde/0.05% glutaraldehyde in 0.01 M Na+ periodate, 0.1 M Myseine, and 3% sucrose in 0.1 M phosphate buffer (P/S), pH 7.4. Sciatic nerves were further fixed for 2 h at room temperature, left overnight in 3.5% sucrose in 0.1 M phosphate buffer P/S at 4°C, and then dissected. Tissues were stained with 0.25% tannic acid in P/S for 1 h, washed in P/S, quenched in 50 mM HCl in P/S, washed in 4% sucrose in 0.1 M maleate buffer, pH 6.2, and incubated for 1 h with 2% uranyl acetate in 0.1 M maleate buffer/4% sucrose (all steps at 4°C). Nerves were dehydrated in 50% (30 min at 4°C), 70%, and 90% ethanol (45 min at 20°C). Tissues were infiltrated at −20°C with LRGold resin (Polysciences)/ethanol (1:1 ratio; 7:3 ratio; 100% LRGold resin; 1 h each), and left in 100% LRGold resin overnight at −20°C. Next, tissues were infiltrated in fresh LRGold resin with 0.5% benzoin methyl ether, first for 1 h and then overnight, embedded in gelatin capsules, and polymerized by UV irradiation (365 nm) for 48 h at −20°C. Sections were collected on nickel formward/carbon grids and treated differently for staining for α- or β-DG. Immunostaining for β-DG was performed using mouse anti–β-DG (Novocastra). Grids were washed twice in PBS, blocked with 0.25% fish skin gelatin and 0.1% Triton X-100 in PBS at 1 h at room temperature, incubated overnight at 4°C with antibodies, rinsed with PBS, and incubated for 1 h with goat anti–mouse antibody conjugated with 10-nm gold particles (British BioCell). Sections were then washed with PBS and distilled water. Staining α-DG was performed using mouse anti–glycosylated α-DG (IHH; according to Eshed et al., 2010). In brief, grids
were first incubated with TBS buffer for 10 min at room temperature, and then treated with 0.1% trypsin in TBS, pH 7.0, for 4 min. Next, grids were rinsed with TBS, blocked with 1% BSA in TBS, pH 7.4, for 10 min at room temperature, and incubated overnight with primary mouse anti-DG antibody at room temperature. The next day, grids were washed with TBS and incubated for 1 h with secondary antibodies (goat anti-mouse IgG coupled to 10 nm gold particles). Finally, grids were counterstained with saturated uranyl acetate and lead citrate. Images were acquired using a transmission electron microscope (912AB; Carl Zeiss).

SC cultures
SCs were isolated from the sciatic nerves of 3-d-old Sprague-Dawley rats and cultured as previously described (Feltli et al., 1992, 1994). In brief, sciatic nerves were dissected and dissociated in 1% collagenase and 2.5% trypsin. Pelleted cells were plated on 100-mm2 tissue culture plates coated with poly-l-lysine, in DMEM, supplemented with 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, 2 mM furosemide, and 2 ng/ml β-nerve growth factor. Fibroblast growth was inhibited using 10-µM Ara-C, and by complement with human Fc and were produced as described previously (M. Grumet, Rutgers University, Piscataway NJ; Lustig et al., 2001; Koticha et al., 2002). Two DRGs were seeded in coverslips coated with collagen in a drop of C-medium (MEM, 4 g/l glucose (Sigma-Aldrich), 50 ng/ml NGF (Harlan Laboratories, Inc.), and penicillin/streptomycin (Gibco). Cultures were then treated with 0.1% trypsin in TBS, pH 7.0, for 4 min. Next, grids were washed with 3% BSA in 50 mM Tris, pH 7.4, 90 mM NaCl, 0.15 M sodium bicarbonate overnight at 4°C. An aliquot (7.5 µg in 35 µl) of perlecan was treated with heparinase (Seikigaku-Kogyo/Amsbio) which contains the signal sequence of the α2 chain of human IgG, and then neutralized immediately with 1 M Tris, pH 9.0. The Fc-tagged proteins were eluted with 4 bed-volumes of 0.1 M glycine, pH 2.6, and then dialyzed in TBS buffer (50 mM tris, pH 7.4, with 90 mM NaCl and 0.15 M sodium bicarbonate). The resulting protein was dialyzed against 300 mM NaCl (in 50 mM tris, pH 7.4, and 1 mM EDTA) and eluted with 500 mM NaCl (in 50 mM tris, pH 7.4, and 1 mM EDTA). The eluate was diluted to 150 mM NaCl and purified using a colbalt HisPur column (Sigma-Aldrich). This column was washed with 50 mM sodium phosphate buffer, 300 mM NaCl, and 10 mM imidizol. Elution was obtained with 400 mM imidizol in the same buffer. The resulting protein was dialyzed in TBS buffer (50 mM tris, pH 7.4, with 90 mM NaCl and 0.125 mM EDTA) and then concentrated using an Amicon Ultra 100K MWCO filter (EMD Millipore).

For Western analysis
Fc-tagged proteins were purified from the medium by adding 0.150 ml of protein A Sepharose beads (IAP-400HC; Repligen) per 50-ml tube. The tubes were placed on a rotating wheel at 4°C overnight, and then spun at 1,000 rpm (1,000 g) for 5 min. Next, the supernatant was decanted and the beads were resuspended in the remaining liquid and transferred to a chromatography mini-column (Bio-Rad Laboratories). After washing in PBS, the proteins were eluted with 4 bed-volumes of 0.1 M glycine, pH 2.6, and neutralized immediately with 1 M Tris, pH 9.0. The Fc-tagged proteins were dialyzed in TBS buffer (50 mM tris, pH 7.4, with 90 mM NaCl and 0.125 mM EDTA) and quantified using a BCA kit (Invitrogen). Recombinant perlecain or BSA were spotted on nitrocellulose; blocked with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20, and 10% milk; washed; and incubated with purified Fc fusion prey proteins. After washing, the bound Fc proteins were detected using protein G–HRP.

ELISA analysis
Wells were coated with 0.5 µg/ml of recombinant perlecain, and purified Fc fusion proteins were added for 2 h, washed, treated with anti–human Fc–HRP for 1 h, and revealed for 5 min with TMB ELISA Substrate (Thermo Fisher Scientific).

Heparinase treatment and solid-phase perlecain binding assay
Perlecain was coated onto the wells of high-binding Costar dishes at 5 µg/ml in 0.15 M sodium bicarbonate overnight at 4°C. An aliquot (7.5 µg in 35 µl) of perlecain was treated with heparinase (Seikigaku-Kogyo/Amsbio) at 37°C for 20 h with 25 µl enzyme before coating. Dishes were blocked for 1 h at room temperature with 3% BSA in 50 mM Tris, pH 7.4, 90 mM NaCl, 0.1% Ultra-Low IgG FBS (Invitrogen), and 2 mM l-glutamine.

and 1 mM CaCl₂. Gliomedin-Fc protein was added to the perlecan-coated dishes at the indicated concentrations in blocking buffer and incubated for 1 h at room temperature. After washing off unbound protein, the bound species was detected with 1:5,000 anti–Fc-HRP (Thermo Fisher Scientific) and Ultra TMB substrate (Thermo Fisher Scientific). Absorbance was determined at 450 nM with a Spectra Fluor multichannel plate reader.

**Image acquisition, processing, and quantification**

Immunofluorescence stainings of teased fibers and cultures were imaged at room temperature using one of the following four microscopes. UltraView ERS spinning disk confocal microscope (PerkinElmer) with the Velocity acquisition software, adapted with a Plan Apochromat 63x (NA 1.4) Oil objective and a camera [C9100-02, Hamamatsu Photonics]. TCS-SP2 or SP5 confocal fluorescence microscope (Leica) equipped with a Plan Apochromat 63 Å/1.4 or HCX PLAPO 100x/1.44 oil CORR CS oil-immersion objectives and using the LCS confocal acquisition software (LAS AF Version 2.6.3.8173; Leica). Observer (Carl Zeiss) equipped with Apotome.2 and AviXion software v. 4.8.2.0, a 40x/1.30 M27EC Plan-Neofluar objective and a high resolution microscopy camera (AxioCam MRK Rev. 3 FireWire). FITC, TRITC, and cy5 fluorophores were excited with an Ar laser (488 nm), a He/Ne laser (568 nm), and a red diode laser (640 nm), respectively.

Figures were assembled using Photoshop software [Adobe]. None or minimal modification to the images was performed with Photoshop; all the images in each panel were treated identically at every step. To quantify the frequency of abnormal Nav clusters, images were photographed using a Plan Apochromat 63 Å/1.4 oil-immersion objective and using the LCS confocal acquisition software (Leica). The gain of Nav fluorescence detection was maintained below the threshold of fluorochrome saturation. z-axis confocal acquisition software (Leica). The gain of Nav fluorescence detection was maintained below the threshold of fluorochrome saturation. z-axis confocal acquisition software (Leica). The gain of Nav fluorescence detection was maintained below the threshold of fluorochrome saturation. z-axis confocal acquisition software (Leica). The gain of Nav fluorescence detection was maintained below the threshold of fluorochrome saturation. z-axis confocal acquisition software (Leica).

**Online supplemental material**

Fig. S1 shows the expression of laminin α5 during nerve and node development. Fig. S2 shows that pharmacological inhibition of furin impairs myelination and nodogenesis in DRG co-cultures. Fig. S3 shows the expression of perlecan, β-DG, versican 1, syndecan 3, and agrin in perlecan mutant nerves. Fig. S4 shows the binding affinity of glicomedin to perlecan with or without heparinase treatment and in comparison to α-DG. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201403111/DC1.


Hildebrand, C. 1971. Ultrastructural and light-microscopic studies of the de-


ing and its clustering at the developing nodes of Ranvier. J. Biol. Chem. 286:42246–422434. http://dx.doi.org/10.1074/jbc.M111.26353


Lambert, S., J.Q. Davis, and V. Bennett. 1997. Morphogenesis of the node of Ranvier: co-clusters of ankyrin and ankyrin-binding integral proteins de-


Liendo, P., and P.T. Martin. 2005. Differential expression of proteoglycans at central and per-


Mandel, S. and J.Q. Davis, and V. Bennett. 1997. Morphogenesis of the node of Ranvier: co-clusters of ankyrin and ankyrin-binding integral proteins de-


Nico, B., R. Tamma, T. Annese, D. Mangieri, A. De Luca, P. Corsi, V. Belluardo, V. Longo, D. Rinaldo, and D. Ribatti. 2010. Glial dystrophin-associated proteins, laminin and agrin, are downregu-


Pan, Z., T. Kao, Z. Horvath, J. Lemos, J.Y. Sui, S.D. Cramton, V. Bennett, S.S. Scherer, and E.C. Cooper. 2000. A common ankyrin-G-based mechanism retains KCNQ and Na V channels at electrically active do-


