METHODS

Tissue culture methods

Cultures of primary rat Schwann cells, purified DRG neurons and dissociated myelinating DRG–cultures were done as previously described[1]. Viral infection was carried out for two hours with 8 μg ml^{-1} polybrene on the first, second and third day after plating with undiluted viral stock; myelination was induced after 10 days in culture and proceeded for additional 10–12 days before analysis. Cell–lines (Cos7, HEK–293T and Phoenix–Eco) were grown in DMEM and 10% FCS. Fc–fusion proteins were produced in HEK 293T cells as described previously[1].

Constructs and antibodies

All the plasmids used in this study are detailed below. Expression plasmid for mouse Necl2[2] was obtained from Y. Takai. Polyclonal antibodies to Necl1 and Necl4 were generated by immunizing rabbits with GST–fusion proteins containing the cytoplasmic domains of rat Necl1 and rat Necl4, respectively. Affinity–purified antibodies were prepared by first absorbing each serum on a column containing the non–immunizing GST–fusion (e.g. GST–Necl4ct for anti–Necl1ct serum and GST–Necl1ct for anti–Necl4c serum) and then purifying the unbound antibodies on column containing the GST–fusion proteins used for immunization. Antibodies to gliomedin[1], Caspr[3] were previously described. The following antibodies were obtained from commercial sources: rat anti–MBP (Chemicon), mouse anti–MAG (Roche), rabbit and mouse anti–S100, mouse anti–Na^+ channels, mouse anti–β–dystroglycan, mouse anti–GFAP and mouse anti–neurofilaments (Sigma), mouse anti–βIII tubulin and mouse anti–L1 (Covance).

siRNA and transfection of Schwann cells and DRG–neurons

RNA–oligonucleotides were purchased from Dharmacon. The following target–sequences were used for rat Necl4 (derived from siGENOME SMARTpool Upgrade #MU–081963–00): Oligo #4 (CTATAGTCGTCATTCAGAATT) reduced the expression of Necl4, whereas Oligo #3 (GCCCAAGGCCGATGAAATCTT) was ineffective and used as a negative control. For rat Necl1, the following target–sequence was used (derived from siGENOME SMARTpool Upgrade #MU–083108–00): Oligo #1 (CCAGCAGACTCTATACTTT); for these experiments, we used the si*CONTROL* Non–Targeting siRNA Pool (#D–001206–13) as a negative control. Transfection was done using oligofectamine (Gibco) for Schwann cells and Amaxa's Rat Neuron Nucleofector Kit according to the respective manufacturer's instructions.

Immunoprecipitation and immunoblot analysis

Brain membrane preparation, immunoprecipitation, and immunoblot analysis were done as described previously[3]. Immunoprecipitation from Schwann cells was done using one confluent 10cm–plate per sample. For immunoblot–analysis of rat sciatic nerves, two sciatic nerves of 12–week–old rats were dissected, desheathed on ice–cold PBS, cut into 2–3–mm pieces, and frozen in liquid nitrogen. The nerves were then crushed in 400 µl of 25 mM Tris, pH 8.0, and 1 mM EDTA 2% SDS, boiled for 15 min, and spun for 15 min at 15°C. Protein concentration was measured using the BCA protein assay reagent (Pierce Chemical Co.) and 100 µg of protein was used per sample.

Immunofluorescence

Rat or mouse sciatic nerves were fixed for 25 min in 4% PFA or for 10 min in Zamboni's fixative and then teased, postfixed and permeabilzed in cold methanol for 5 min at –20^{O}C. Slides were blocked for 1 hour with PBS containing 5% normal goat serum and 0.1% Triton X–100 (blocking solution). The samples were incubated overnight at 4^{O}C with different primary antibodies diluted in blocking solution, washed with PBS and then incubated for 45 min at room–temperature with secondary anti–mouse–Cy3 (Jackson Laboratories), anti–rabbit–488 (Molecular Probes), and anti–rat–Cy5 (Jackson laboratory). Slides were mounted with elvanol and analyzed using a Nikon Axioplan microscope or a BioRad confocal microscope. Myelinated cultures were fixed for 15 min in 4% PFA, permeabilized for 10 min in ice–cold methanol at –20^{O}C, blocked for 1 hour at room–temperature and then processed as above. For immunolabeling using two rabbit antibodies, the first antibody was detected with Cy3–coupled anti–Rabbit Fab–fragment. The sample was then refixed in 4% PFA for 15 minutes, blocked as described above and stained with the second antibody, followed by an Alexa488–coupled anti–Rabbit Fab–fragment.

Adhesion–assay of rat Schwann cells to purified Fc–fusion proteins

The adhesion–assay was done as previously described[4]. Substrates were prepared by placing 2 μl containing 50 μg ml^{-1} of proteins for 1 hour on a polystyrene dish followed by three washes and blocking with 2% of heat–inactivated BSA for 1 hour. In competition–experiments, the second Fc–fusion protein was applied before the blocking step. 2x10^{4} Schwann cells were allowed to adhere for 2 hours at room–temperature; non–adhering cells were then washed away with PBS and the

remaining cells were fixed with 4% PFA and stained with Crystal Blue; low–magnification images were acquired with a CCD camera, and the cells were counted using the ImageJ software. Experiments were repeated 3 times with duplicates for each Fc–fusion in each experiment. The amount of cells bound in each set of duplicates and repetition was normalized with the amount of cells bound to Necl1–Fc representing 100%; statistical analysis (ANOVA) was made using the Instat3 software (GraphPad).

References

1. Eshed, Y. et al. Gliomedin mediates Schwann cell-axon interaction and the molecular assembly of the nodes of Ranvier. Neuron 47, 215-29 (2005).
2. Shingai, T. et al. Implications of nectin-like molecule-2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1 in cell-cell adhesion and transmembrane protein localization in epithelial cells. J Biol Chem 278, 35421-7 (2003).
3. Poliak, S. et al. Caspr2, a new member of the neurexin superfamily, is localized at the juxtaparanodes of myelinated axons and associates with K+ channels. Neuron 24, 1037-47 (1999).
4. Adamsky, K., Schilling, J., Garwood, J., Faissner, A. & Peles, E. Glial tumor cell adhesion is mediated by binding of the FNIII domain of receptor protein tyrosine phosphatase beta (RPTPbeta) to tenascin C. Oncogene 20, 609-18 (2001).

RT-PCR Necls: Primers

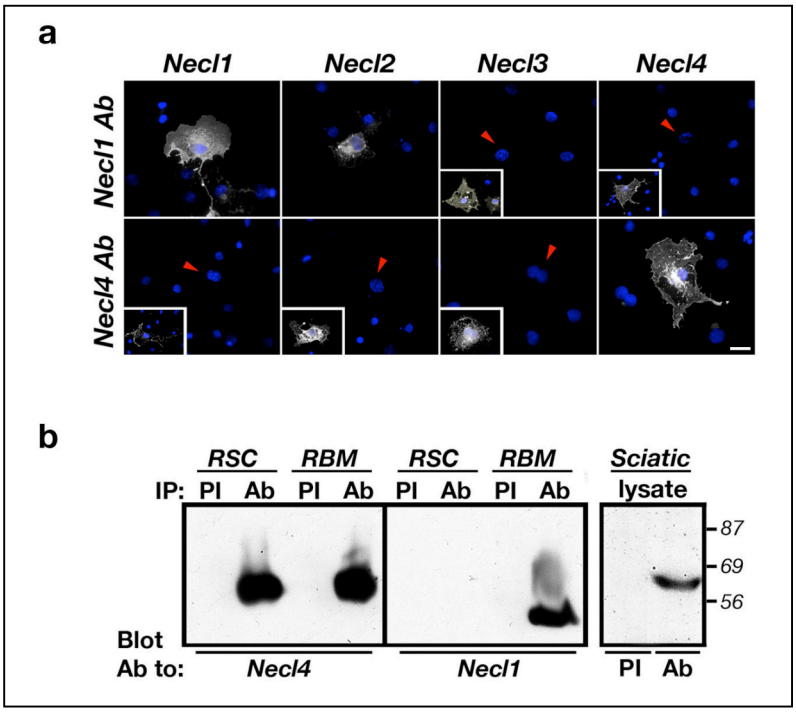
Gene	Accesion	5' seq	3'seq
Necl1	XM_341157	CTCGGAATCCCACAGAAACC	GCTGAGAGGTGGATCTGTC
Necl2	NM_001012201	CGATATCCAGAAAGACACGG	CTGCACTTCTAGATACCGCTG
Necl3	XM_340958	CTTTTCCACAAGAAGGACAGG	CTGCGCTGCTTTGACCAATG
Necl4	XM_344870	CGTGGAAATGGGAATTCTG	GGTCAAGATGGAGGGAAGGG
Gliomedin	AY266116	AGAGAGTCTGCTAACAGGAG	GGTATGTGGTATTGATGTGC
Actin		GAGCACCCTGTGCTGCTCACCGAGG	GTGGTGGTGAAGCTGTAGCCACGCT

Construction of Necl-plasmids and Controls

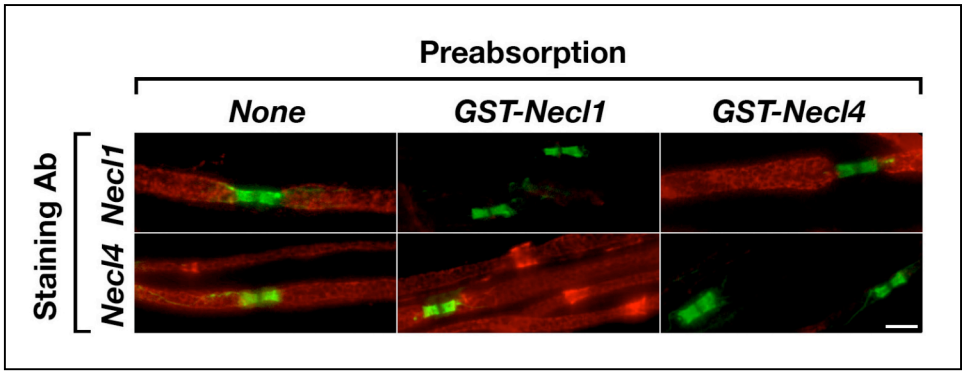
Plasmid-name	Gene	Species	Accession	Purpose	*bp* (aa)	Vector	Template	5' seq	3' seq
pGEM-Necl1-ISH	Necl1	rat	XM_341157	Riboprobe	*404-1443*	pGEM-T Easy	REX-SST-3rSN	GGGCCAATCTTTCCCAGG	CCCTCCAATGATGGCGTGG
pGEM-Necl2-ISH	Necl2	rat	NM_001012201	Riboprobe	*293-1383*	pGEM-T Easy	pC3.1-rNecl2	GCCGCGGCCCAGCCGGCCACAGGTGATGGGCAGAATC	CACCGGTACCTCCACTGCCCCAATGGCG
pGEM-Necl3-ISH	Necl3	mouse	NM_178721	Riboprobe	*594-1657*	pGEM-T Easy	pCX-Necl3	GCACGCTAGCGTTCGCTCAACCAGCATCTC	AGCAGGATCCGGGCCATTCTGGCCAGCC
pGEM-Necl4-ISH	Necl4	rat	XM_344870	Riboprobe	*610-2217*	pGEM-T Easy	REX-SST-iSC	GAGGGTGGGGTGGCTGAG	GGTCAAGATGGAGGGAAGGG
pC3-Necl1	Necl1	rat	XM_341157	Expression	1-430	pCDNA3	rBrain	CGCCGCTAGCAAGCTTGGGAGGTGGCCAGGAAGC	CGCACTCGAGGTGTGGGTGCCCCTAGATG
pC3.1-Necl3-myc/his	Necl3	mouse	NM_178721	Expression, myc-fusion	1-404	pCDNA3.1-myc/his	mBrain	GCACGCTAGCGTTCGCTCAACCAGCATCTC	CTGAGGATCCAATGAAATACTCTTTTTTCTCTTCG
pC3.1-Necl4	Necl4	Mouse rat	NM_153112	Expression	1-436	pCDNA3.1-myc/his	mBrain, rBrain	GTATCTGCAGATAACGGCACC	CAATCTCGAGGCTGGCTCCTTGCAGCTGG
pSX-Necl1	Necl1	rat	XM_341157	Fc-Fusion	23-360	pSX-Fc	rBrain	GGGCGGGCCCAGCCGGCCAATCTTTCCCAGGAC	GGCGGGATCCGTACTGGAGGATGAGGGCAC
pSX-Necl2	Necl2	mouse	NM_207675	Fc-Fusion	43-383	pSX-Fc	pCAGIPuro-Necl2-flag	CGGCGGCCCAGCCGGCCCCCACAGGTGATGGACAG	GTCCGGATCCCCAATGGTCCCCTCTTC
pCX-Necl3	Necl3	mouse	NM_178721	Fc-Fusion	1-334	pCX-Fc	mBrain	GCACGCTAGCGTTCGCTCAACCAGCATCTC	AGCAGGATCCGGGCCATTCTGGCCAGCC
pCX-Necl4	Necl4	mouse	NM_153112	Fc-Fusion	1-321	pCX-Fc	mBrain	TGGCGCTAGCAAGCTTGCCACCATGGGCCGGGCCC	AATGAGATCTGGAACCGATGTCTGAGCCTC
pCX-Zlg1	Zlg1/ HepaCM	mouse	NM_175189	Fc-Fusion		pCX-Fc	mBrain	TGCTGCTAGCAAGCTTCAGAGCTGGAGAACGCCAC	CACTGTGTATAGAAGAAGCTCGGATCCTATC
pGX-Necl1ct	Necl1	rat	XM_341157	GST-Fusion	384-430	pGEX-6P1	rSN	ATTGAATTCGGACACTATTTGATCCGGCAC	GTGGCTCGAGCTAGATGAAATATTCCTTCTTGTC
pGX-Necl4ct	Necl4	rat	XM_344870	GST-Fusion	395-436	pGEX-6P1	pGEM-Necl4-ISH	ATGGAATTCTGCTCTGTCCGACAGAAGG	GGGACTCGAGTCAAATGAAGAACTCTTCTTTCCG
pMX-GFP-Nf155ct	Neurofascin155	rat	AY061639	Infection	1066-1174	pMX-GFP-C	pCMV-Nf155-HA	GAATTCAAGAGGAGTCGTGGCGG	CTCGAGGCTCCATCAGGCCAGGGA
pMX-GFP-Necl4ct	Necl4	rat	XM_344870	Infection	395-436	pMX-GFP-C	pGEM-Necl4-ISH	ATGGAATTCTGCTCTGTCCGACAGAAGG	GGGACTCGAGTCAAATGAAGAACTCTTCTTTCCG
pMX-EGFP	EGFP			Infection-control		pMX	pEGFP-N1	GAATTCGTCGCCACCATGGTGAGC	TGATGTCGACCTCGAGTCGCGGCCGCTTAAC

New Name	**CADM1**	**CADM2**	**CADM3**	**CADM4**
Older Names	Necl2 SynCAM1 Igsf4a TSLC1 IGSF4 RA175 SgIGSF	Necl3 SynCAM2 Igsf4d	Necl1 SynCAM3 Igsf4b TSLL1	Necl4 SynCAM4 Igsf4c TSLL2

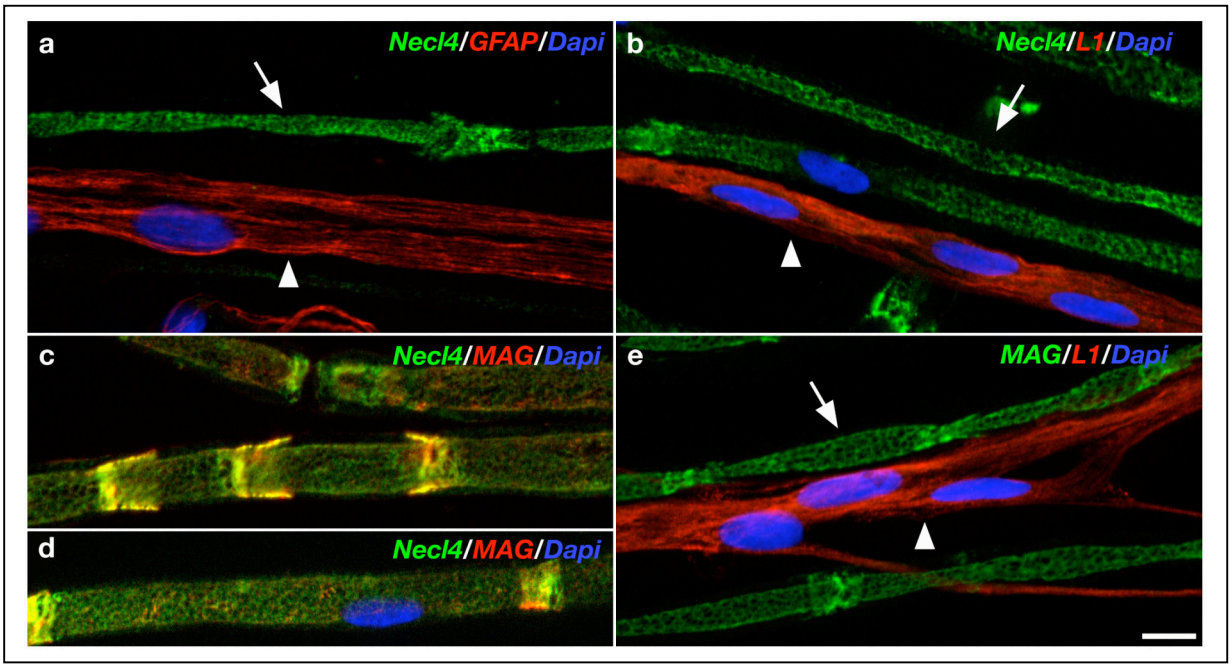
Supplementary Table 1. Summary of the different names used for the CADM gene family. New names refer to gene name, whereas older names refer to the proteins. New human genes are named according to HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature); new mouse names are according to the Mouse Genome Informatics provided by the Jackson Laboratory (http://www.informatics.jax.org).



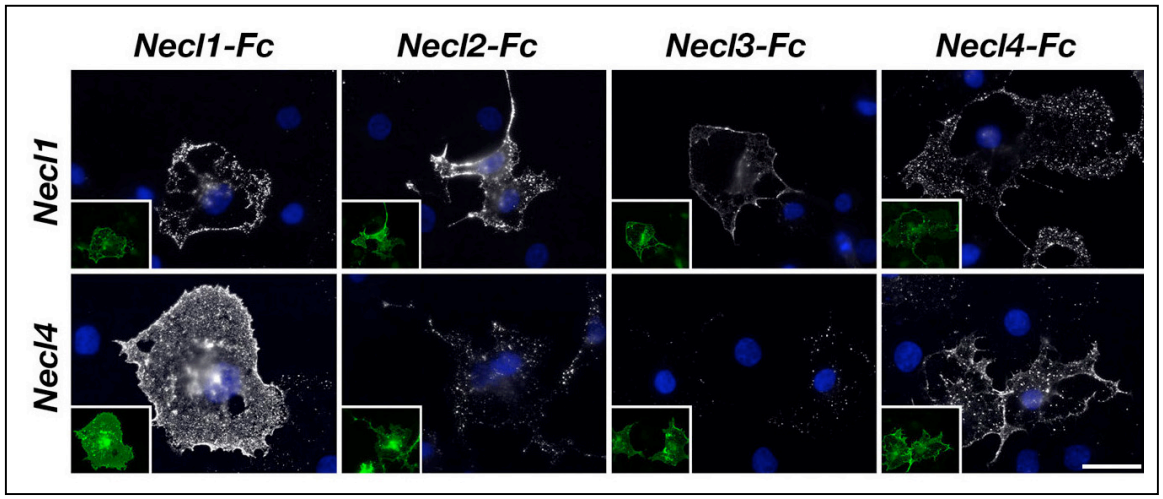
Supplementary Figure 1. Specificity of the antibodies to Necl4.
(a) Immunocytochemistry. COS-7 cells transfected with Necl1-Necl4 cDNAs as indicated, and immunolabeled with an affinity purified antibody against Necl1 (upper row) or Necl4 (lower row). The insets show that the different Necls were expressed, as determined using an antibody to flag-tag (Necl2), myc-tag (Necl3), or binding of Necl4-Fc (Necl1) or Necl1-Fc (Necl4). Nuclei were labeled with DAPI (blue) and the location of the cells shown in the inset is marked with a red arrowhead. The Necl4 antibody recognized Necl4 but not Necl1-3, while the Necl1 antibody reacted with Necl1, more weakly with Necl2, but not with Necl3 or Necl4. **(b)** Western blot analysis. Rat brain membranes (RBM) or rat Schwann cells (RSC) were immunoprecipitated (IP) with the antibodies against Necl1 or Necl4 (Ab), or preimmune serum (PI) as indicated on the top of the figure, followed by immunoblotting with the same antibody indicated on the bottom of each panel. The panel on the right shows an immunoblot of sciatic nerve lysate using the Necl4 antibody or the preimmune serum. The location of molecular weight markers is shown on the right in kDa. Scale bar: 30 μm.



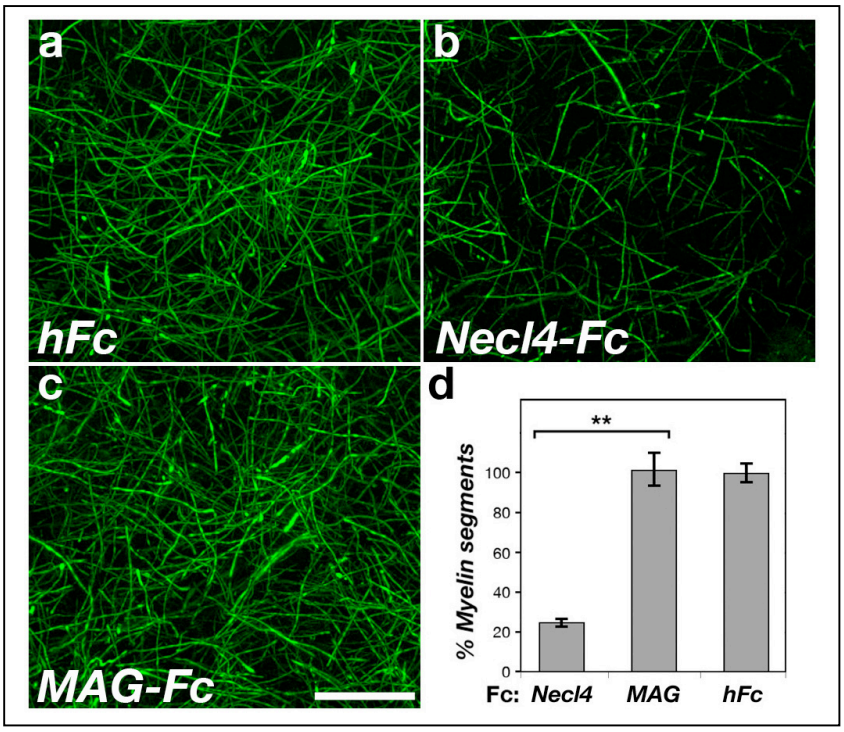
Supplementary Figure 2. Specific immunolabeling for Necl1 and Necl4 in sciatic nerve.
Teased rat sciatic nerves were immunolabeled with antibodies to Caspr (green) and Necl1 (red) or Necl4 (red) as indicated. To examine antibody specificity, the antibodies were used directly (none) or pre-incubated with the GST-fusion protein containing the intracellular domain of Necl1 (GST-Necl1) or Necl4 (GST-Necl4) that was used as antigen for immunization. Scale bar: 10 μm.



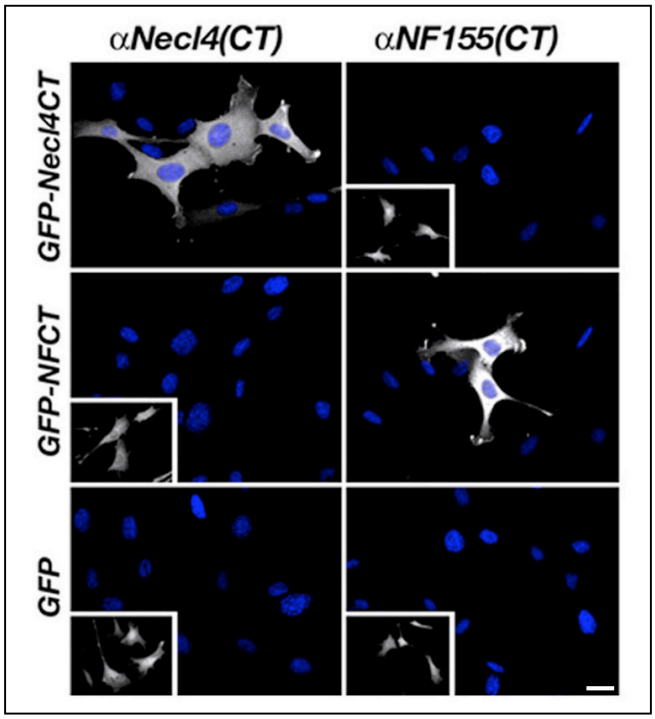
Supplementary Figure 3. Necl4 is expressed by myelinating, but not by non-myelinating Schwann cells in sciatic nerve.
(**a-d**) Immunofluorescence staining of teased adult rat sciatic nerves for Necl4 and (**a**) GFAP, (**b**) L1, or (**c-d**) myelin-associated glycoprotein (MAG) as indicated. (**e**) Double immunolabeling of teased rat sciatic nerves with antibodies to MAG and L1. Schwann cell nuclei were labeled with Dapi (blue). Note that Necl4 is expressed by myelinating Schwann cells (arrows) but is absent from non-myelinating Schwann cells (arrowheads), which are labeled with GFAP and L1. Scale bar: 10 µm.



Supplementary Figure 4. Homophilic and heterophilic binding of Necls.
Fc-fusion proteins of Necl1-Necl4 were added to COS7 cells transfected with Necl1 or Necl4 cDNA, and visualized with an antiserum to human IgG. Transfected cells were identified by immunostaining with rabbit antibodies to Necl1 or to Necl4 (insets) as indicated. Note that Necl1-Fc bound to cells expressing Necl1-Necl4, while Necl4-Fc bound better to cells expressing Necl1 and Necl4 than to those expressing Necl2 or Necl3. Scale bar: 40 µm.



Supplementary Figure 5. Addition of Necl4-Fc, but not MAG-Fc to myelinating cultures inhibits myelination.
Dissociated DRG-cultures were grown in the presence of Fc-fusion proteins (50 µg/ml) containing the extracellular domain of Necl4 (Necl4-Fc; **b**), MAG (MAG-Fc; **c**) or only human Fc (h-Fc; **a**) as control. Fresh Fc-fusion proteins were added every second day for an additional 10 days, then the cells were fixed, immunostained for MBP (green), and MBP-positive myelin sheaths were counted. The number of MBP-positive segments present in each condition is shown as a percentage of that in the h-Fc-treated cultures (**e**). Cultures grown in the presence of Necl4-Fc contained significantly (**p<0.0001) fewer myelin segments compared to the control h-Fc and MAG-Fc-treated cultures. Note that in contrast to Ncl4-Fc, MAG-Fc had no effect on myelination. Scale bar: 100 µm.



Supplementary Figure 6. Expresson of GFP-Necl4CT and GFP-NF155CT. 3T3 cells were infected with retroviruses that direct the expression of green fluorescence protein (GFP) or GFP-fusion proteins containing the cytoplasmic domain of either Necl4 (GFP-Necl4CT) or neurofascin 155 (GFP-NF155CT). The expression of GFP-Necl4CT and GFP-NF155CT was examined three days later by immunostaining the C-terminus of Necl4 or neurofascin 155; both are localized in the cytoplasm of infected cells. Inset: lower magnification of each field showing GFP-fluorescence. Scale bar: 30 μm.