Nr-CAM and neurofascin interactions regulate ankyrin G and sodium channel clustering at the node of Ranvier

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Voltage-dependent sodium (Na$^+$) channels are highly concentrated at nodes of Ranvier in myelinated axons and play a key role in promoting rapid and efficient conduction of action potentials by saltatory conduction. The molecular mechanisms that direct their localization to the node are not well understood but are believed to involve contact-dependent signals from myelinating Schwann cells [1] and interactions of Na$^+$ channels with the cytoskeletal protein, ankyrin G [2]. Two cell adhesion molecules (CAMs) expressed at the axon surface, Nr-CAM and neurofascin, are also linked to ankyrin G and accumulate at early stages of node formation, suggesting that they mediate contact-dependent Schwann cell signals to initiate node development [3]. To examine the potential role of Nr-CAM in this process, we treated myelinating cocultures of DRG (dorsal root ganglion) neurons and Schwann cells with an Nr-CAM-Fc (Nr-Fc) fusion protein. Nr-Fc had no effect on initial axon-Schwann cell interactions, including Schwann cell proliferation, or on the extent of myelination, but it strikingly and specifically inhibited Na$^+$ channel and ankyrin G accumulation at the node. Nr-Fc bound directly to neurons and clustered and coprecipitated neurofascin expressed on axons. These results provide the first evidence that neurofascin plays a major role in the formation of nodes, possibly via interactions with Nr-CAM.

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Results and discussion

We have utilized an in vitro myelination system comprised of DRG (dorsal root ganglion neurons) and Schwann cells [4, 5] to investigate the role of Nr-CAM and neurofascin in axon-Schwann cell interactions, including node formation. Nr-CAM and neurofascin appropriately localize at nodes of Ranvier in this culture model (Figure 1), as we have previously shown for Na$^+$ channels and ankyrin G [4], indicating that this culture system recapitulates the key events of node formation. Like ankyrin G and Na$^+$ channels [4], Nr-CAM and neurofascin are also located in heminodes that form on either side of individual myelinated internodes in these cocultures. In addition, neurofascin extends partially into the paranodes by day 6 (Figure 1), consistent with reports that a glial isoform is a component of the paranodal junctions [6]. Interestingly, Caspr, a key neuronal component of the paranodal junctions [7], is minimally concentrated in the paranodes at this stage (see Figure S1 in the Supplementary material), although it is highly concentrated in the paranodes by day 9, in agreement with our earlier findings [5]. These results indicate that nodal clustering occurs prior to, and independent of, paranodal junction formation.

To examine the role of Nr-CAM in axon-Schwann cell interactions, including formation of the node of Ranvier, we added exogenous Nr-CAM to the cocultures. For these studies, we used a recombinant protein corresponding to most of the extracellular region of Nr-CAM fused to an Fc domain; this soluble construct (Nr-Fc) exhibits robust binding activity and perturbs normal Nr-CAM interactions in an axon guidance assay in vivo [8]. We hypothesized that this Nr-CAM construct might competitively block interactions of Nr-CAM in *trans* with ligands on the Schwann cell and/or in *cis* with ligands on the axon. We incubated neuron-Schwann cell cocultures, with or without Nr-Fc, for 6 days in myelin-promoting media; we have previously shown that myelination is initiated and

Figure 1
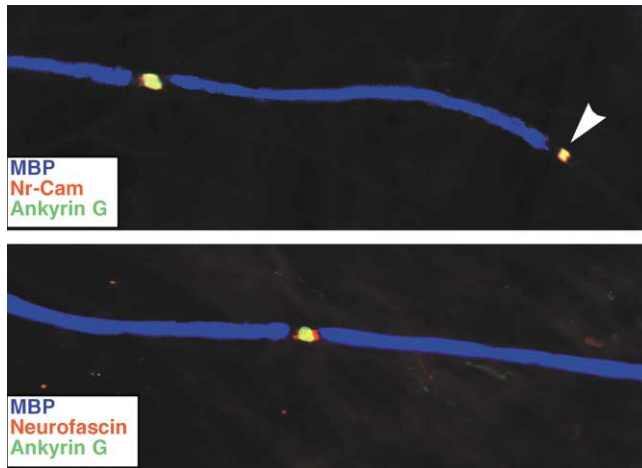
Expression of nodal markers in vitro. DRG neuron-Schwann cell cocultures, after 6 days in myelin-promoting media, were fixed and stained for MBP (blue channel) and antibodies to Nr-CAM and ankyrin G (top panel) or neurofascin and ankyrin G (bottom panel). Nr-CAM (red) and ankyrin G (green) colocalize at nodes of Ranvier and heminodes (indicated with arrowhead) and appear yellow in the merged image; neurofascin (red) is present at nodes of Ranvier and also extends partially into the paranodes.

nodes first form over this time period [4]. In parallel, we treated other cultures with L1-Fc and human IgG as CAM and Fc controls, respectively.

Cultures treated with 50 μg/ml Nr-Fc myelinated comparably to controls, as evidenced by both phase microscopy and by staining for myelin basic protein (MBP), a marker of compact myelin. Strikingly, treated cultures had significantly fewer nodes of Ranvier, or heminodes, enriched in Na^+ channels or ankyrin G (Figure 2b,e). To quantitate this effect, every MBP^+ myelin sheath in the cultures was identified and scored for the presence or absence of associated ankyrin G and Na^+ channel clusters (Figure 2g,h). In control cultures, most myelin sheaths were bordered by clusters of Na^+ channels or ankyrin G, in contrast to Nr-Fc-treated cultures. This is a specific effect, as an L1-Fc fusion protein that binds avidly to neurons did not inhibit the accumulation of these proteins at the node (Figure 2c,f). Nr-Fc did not downregulate the expression or increase the turnover of Na^+ channels or ankyrin G, as staining for these proteins in neuron cultures was not altered even after 6 days of treatment (data not shown). These findings strongly suggest that Nr-Fc specifically inhibited the formation of the nodes without affecting overall Na^+ channel or ankyrin G expression.

To investigate further the specificity of Nr-Fc in this system, we examined whether it had any effect on Schwann cell proliferation or myelination. Neurons induce Schwann cell proliferation via a mechanism that requires direct cell-cell contact [9]; therefore, a nonspecific effect of Nr-Fc on the association between neurons and Schwann cells might reduce Schwann cell proliferation. To examine this possibility, Schwann cells were seeded onto DRG neurons; Nr-Fc (50 μg/ml) was added to the media for 72 hr, and BrdU (bromodeoxyuridine) was added during the last 4 hr. There was no difference in Schwann cell proliferation between Nr-Fc-treated and control cultures (Figure 2i), underscoring the specificity of the effects of Nr-Fc. Similarly, we quantitated the number of myelin sheaths that formed in control and Nr-Fc-treated cultures to determine whether Nr-Fc affected myelination. The number of myelin sheaths, and the intensity of MBP staining, in Nr-Fc-treated and control cultures was comparable (Figure 2j), indicating that there was not a general effect on compact myelin formation. We also investigated whether the failure of nodes to form in the treated cultures affected the length of myelin sheaths. We detected no differences in the lengths of myelin sheaths between Nr-Fc-treated and control cultures (data not shown). These results suggest that the absence of nodes does not affect internode length, as might be expected if the position of the nodes and internode length are intrinsically determined by the axon [10]. Taken together, these data indicate that Nr-Fc specifically perturbs clustering of ankyrin G and Na^+ channels at the node without affecting myelination or other axon-Schwann cell interactions.

To determine whether Nr-Fc inhibited node formation by a primary effect on neurons or Schwann cells, we characterized its binding specificity. Nr-Fc bound robustly to DRG neurons, but not to Schwann cells, either grown alone (Figure 3) or in combination with neurons (data not shown). The binding of Nr-Fc to neurons is heterophilic, as it bound equally well to neurons isolated from wild-type mice and neurons isolated from the recently generated [11] Nr-CAM-deficient mouse (data not shown). To identify axonal proteins to which Nr-Fc binds, we examined whether several candidate CAMs coclustered with the bound Nr-Fc. We incubated live neuron cultures with Nr-Fc, induced clustering of the bound Nr-Fc by incubating with polyclonal antibodies to the Fc domain, and then fixed and immunostained cultures for CAMs to which Nr-CAM is known to bind. We found that neurofascin specifically colocalized with the bound Nr-Fc, whereas L1 did not (Figure 4a). These results indicate that Nr-CAM associates with, and possibly binds directly to, neurofascin. Nr-Fc did not cocluster with TAG-1, another candidate ligand [12], and still bound robustly to DRG neurons after phosphatidylinositol-specific phospholipase C treatment to remove TAG-1 and other GPI-anchored proteins (data not shown).

The potential interaction of Nr-CAM with neurofascin was investigated further in coprecipitation studies. We

Figure 2

Nr-Fc inhibits node formation in neuron-
Schwann cell cocultures. Incubation of
cocultures in myelinating media with 50 μg/ml
chick Nr-Fc, L1-Fc, and human Ig for 6 days
showed specific inhibition by Nr-Fc of **(a–c)**
Na$^+$ channel and **(d–f)** ankyrin G clustering
at heminodes defined by MBP-positive myelin
sheaths. Quantitation of **(g)** ankyrin G and
(h) Na$^+$ channel clustering in Nr-Fc-treated
(n = 8) and control cultures (n = 5) showed
statistically significant inhibition of clustering
(Student's t test; p < .05). **(i)** Schwann cell
proliferation was measured following a 4-hr
pulse of 20 μM BrdU and staining with FITC-
linked anti-BrdU. There was no effect on
Schwann cell proliferation of Nr-Fc (n = 3)
compared to control Ig (n = 3). **(j)** The total
numbers of MBP-positive myelin sheaths
were counted in each of the five cultures on
coverslips treated with Nr-Fc or control Ig;
no effect of Nr-Fc treatment was observed.
The scale bar represents 40 μm.
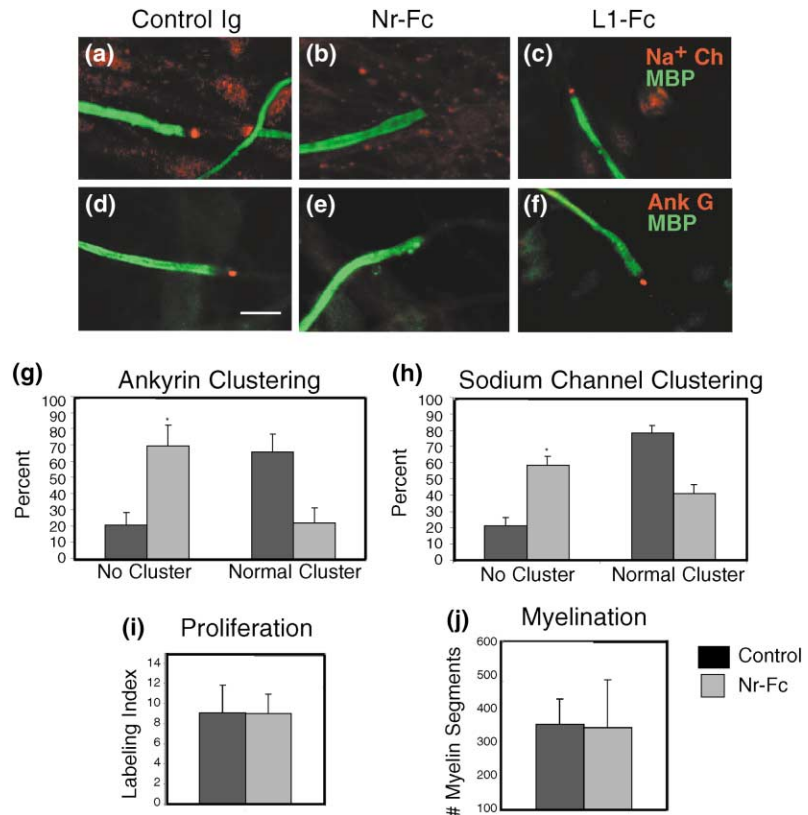
incubated Nr-Fc with detergent extracts of DRG neurons,
followed by precipitation with protein A beads and immu-
noblotting with antibodies to identify associated proteins.
Anti-neurofascin antibodies recognized an ∼190-kDa
band in the Nr-Fc-treated DRG neuron extracts (Figure
4b). The migration in SDS gels of this neurofascin band
resembles that of the 186-kDa isoform containing the
mucin-like domain that is found selectively on the axo-
lemma at nodes of Ranvier [13]. Previous studies in the
chick also indicated that Nr-CAM interacts with neurofas-
cin via its Ig domains [14], which are present in all of
the characterized isoforms of neurofascin. The combined
results indicate that neurofascin interacts with Nr-CAM;
although, these results do not distinguish between a direct
interaction or one mediated through intermediary mole-
cules.

To address this issue and to determine whether neurofas-
cin can act as a cell surface receptor for Nr-CAM, we
expressed neurofascin in CHO cells and tested for binding
of Nr-Fc. Nearly all the neurofascin-positive cells exhib-
ited Nr-Fc binding, while neurofascin-negative cells did
not bind (Figure 4c). The observation that the binding
of Nr-Fc to CHO cells was dependent on neurofascin
expression supports a direct interaction between these
two CAMs and extends previous studies in the chick

that demonstrated that Nr-CAM can act as a cell surface
receptor for neurofascin [14]. The failure of Nr-Fc to bind
Schwann cells, which express neurofascin isoforms in the
paranodal region [6], appears to reflect the much lower
levels of neurofascin expressed by Schwann cells com-
pared to DRG neurons (Figure S2).

The inhibition of ankyrin G and Na$^+$ channel clustering
in Schwann cell-neuron cocultures by Nr-Fc provides the
first functional evidence for a role of CAMs in the develop-
ment of the node of Ranvier. Such a role is consistent
with the localization of neurofascin and Nr-CAM in na-
scent nodes prior to clustering of Na$^+$ channels and an-
kyrin G [3]. Neurofascin and Nr-CAM can interact with
ankyrin G via their cytoplasmic domains [2] and may
thereby recruit voltage-gated Na$^+$ channels to the nodes,
as these channels also bind to ankyrin, possibly via their
β subunits [15]. Further evidence for the role of Nr-CAM
in node formation has emerged from initial analysis of
Nr-CAM-null mice that display abnormalities at the node
during the first postnatal week (data not shown). The
latter findings appear to reflect a principal role of Nr-CAM
on the axon, as we did not detect Nr-CAM expression by
Schwann cells (data not shown).

It is likely that neurofascin on the axon is a major target

Figure 3
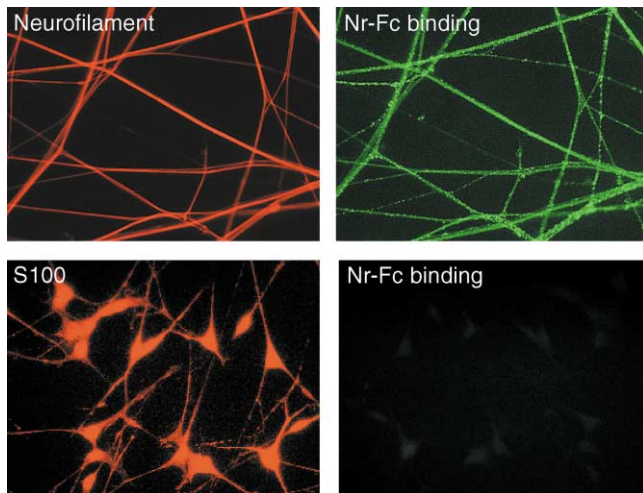
Nr-Fc binds to neurons, but not to Schwann cells. Cultures of purified rat Schwann cells and neurons were incubated for 1 hr at 4°C with Nr-Fc and then with goat anti-human Fc (FITC conjugated) for 1 hr. Cultures were then fixed for 15 min with 4% paraformaldehyde and were permeabilized at −20°C with methanol for 15 min. Neurons were stained with anti-neurofilament monoclonal antibodies and goat anti-mouse IgG (TRITC) each for 1 hr at room temperature; Schwann cells were stained with anti-S100 and goat anti-rabbit IgG (Alexa 568).

of the inhibitory effects of Nr-Fc on node formation. This is consistent with the coclustering and coprecipitation of neurofascin with Nr-CAM and its ability to serve as a cell surface receptor for Nr-CAM. Binding of Nr-Fc to neurofascin may inhibit node formation by two distinct mechanisms. Binding of Nr-Fc to neurofascin on the axon could inhibit *cis* interactions of neurofascin with other axonal components, such as Nr-CAM or the β subunits of the Na^+ channel [16], and thereby block complex formation required for node formation. Potentially, intracellular interactions of neurofascin or Nr-CAM with PDZ domain proteins, such as syntenin-1 [17], might similarly be affected. Alternatively, binding of Nr-Fc may block putative *trans* interactions of neurofascin with ligands at the tips of the extending Schwann cell that are required to organize the forming node. In agreement, recent studies indicate that nodes of Ranvier form in direct contact with overlying Schwann cell processes [18]. In addition, we have observed that neurofascin 186 binds robustly to Schwann cells, but not to axons (M. Grumet, unpublished data), which is in contrast to Nr-CAM, which binds to axons, but not to Schwann cells, as shown here.

We suggest that binding of Nr-Fc to neurofascin on the axon blocks interactions of neurofascin with its cognate receptor(s) on the Schwann cell that normally promote node formation. In the future, identification of the neurofascin ligand(s) expressed by Schwann cells and genetic strategies to disrupt neurofascin expression on the axon

should provide important additional insights into the role of neurofascin-Schwann cell interactions in node formation.

Materials and methods

Antibodies and recombinant proteins
Mouse monoclonal antibodies used in these studies included the anti-myelin basic protein (MBP) SMI 94, anti-neurofilaments SMI 31 and SMI 32 (Sternberger Monoclonals), and BrdU 1202693 (Roche Molecular Biochemicals). Rabbit polyclonal antibodies included anti-S100 (DAKO), anti-neurofascin NFC1 (gift of Dr. Peter Brophy), anti-neurofascin (anti-ABGP) [13], anti-pan Na^+ channel [4], anti-ankyrin G [4], anti-Caspr [5], anti-L1 [19], and antibodies against mouse Nr-CAM generated against the mouse Nr-Fc fusion protein [19]. Secondary antibodies included affinity-purified, rhodamine-conjugated donkey anti-rabbit IgG and fluorescein-conjugated donkey anti-mouse IgG (Chemicon International), affinity-purified, fluorescein-conjugated goat anti-human IgG Fc, goat anti-rabbit IgG Alexa 568, goat anti-mouse IgG Alexa 568, affinity-purified, rhodamine-conjugated goat anti-rabbit, fluorescein-conjugated goat anti-rabbit, fluorescein-conjugated goat anti-mouse IgG, and coumarin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories).

Nr-Fc was produced as described previously [19]. L1-Fc was generously provided by Dr. Jeffrey Haspel; its production is described elsewhere [20]. Normal human Ig was purchased from Jackson ImmunoResearch Laboratories.

Cell culture and Fc inhibition studies
Schwann cells were isolated from rat postnatal day 2 sciatic nerves and were expanded with 5 ng/ml glial growth factor (gift of Cambridge Neuroscience) and 4 μM forskolin (Sigma); DRG neurons were isolated from embryonic day 16–17 rats and were cultured as described [5]. Myelinated Schwann cell-DRG neuron cocultures were generated by adding 200,000 Schwann cells to each coverslip of DRG neurons, switching the cocultures to serum-free media for 3 days to allow the Schwann cells to repopulate the neurites. Cultures were then switched to MEM plus 10% FCS and 50 μg/ml ascorbic acid (Sigma) to promote basal lamina formation and myelination.

To determine the effect of Fc constructs on node formation, media was supplemented with 50 μg/ml Nr-Fc, L1-Fc, or normal human IgG when the cultures were switched to myelin-promoting media and were maintained for 6 days. Cultures were then fixed and stained for ankyrin G or Na^+ channels and were double stained for MBP. All fields were then counted and scored for the presence or absence of ankyrin G and Na^+ channel clusters adjacent to MBP segments.

Immunofluorescence microscopy
Cultures were fixed and stained as described previously [5] and were examined with an Axiophot microscope (Carl Zeiss). Photographs were taken either with slide film (Elite II; Eastman Kodak) and were scanned using the Nikon SF-20 slide scanner (Nikon) or they were taken with a Hamamatsu C4742-95 digital camera (Hamamatsu) and were processed using the Openlab 2.2 software package (Improvision).

Binding assays
Cultures of purified rat Schwann cells and neurons were incubated for 1 hr at 4°C with Nr-Fc (diluted in L-15 to a final concentration of 10 μg/ml), and then with goat anti-human Fc fluorescein (1:100) for 1 hr. Cultures were then fixed for 15 min with 4% paraformaldehyde and were permeabilized at −20°C with methanol for 15 min. Neurons were stained with anti-neurofilament antibodies, each used at 1:1000, and goat anti-mouse IgG rhodamine was used at 1:100 for 1 hr at room temperature. Schwann cells were stained with anti-S100 at 1:100 and goat anti-rabbit IgG Alexa 568 at 1:100.

For the cocapping experiments, cultures of purified rat DRG neurons

Figure 4

(a)

(b)

(c)
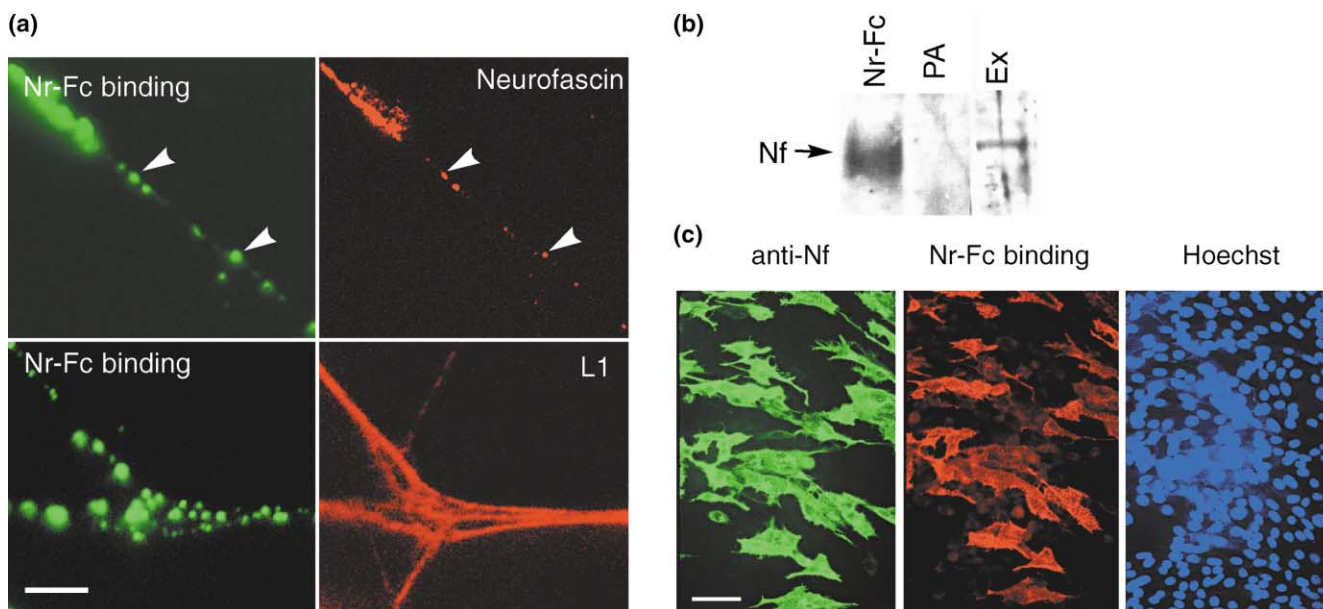
Nr-Fc binds to and interacts with neurofascin. **(a)** Neurofascin coclusters with bound Nr-Fc. Cultures of purified rat DRG neurons were incubated with Nr-Fc or human Ig at room temperature for 20 min, followed by fluorescein-linked anti-human Fc for 20 min at room temperature, and were then stained for neurofascin or L1. Note that clustered Nr-Fc colocalizes with neurofascin (two clusters are indicated with arrowheads), but not with L1, which remains diffuse. **(b)** Neurofascin coprecipitates with Nr-Fc. Detergent extracts of DRG neurons were incubated with Nr-Fc, collected by protein A, fractionated on SDS gels, and immunoblotted with anti-neurofascin

antibodies (Nr-Fc); controls incubated with protein A alone (PA) are also shown. The anti-neurofascin antibody recognized a band of ~190 kDa in the extract (Ex) as well as in the precipitate with Nr-Fc. **(c)** Nr-Fc binds directly to neurofascin. Monolayers of CHO cells expressing neurofascin were incubated with Nr-Fc for 1 hr, followed by goat anti-human Fc (rhodamine); cultures were then fixed and stained for neurofascin visualized with goat anti-rabbit antibodies conjugated to FITC. Note that nearly all of the cells that bound anti-neurofascin (anti-Nf, green) also exhibited binding of Nr-Fc (red).

were incubated in L-15 medium containing 20 µg/ml Nr-Fc or human Ig at room temperature for 20 min. The medium was removed and replaced with L-15 containing 1 µg/ml fluorescein-linked anti-human Fc for 20 min at room temperature. Coverslips were then washed 3 times in L-15 and fixed for 15 min in fresh 4% paraformaldehyde in PBS. Cells were permeabilized and stained with rabbit anti-neurofascin (anti-ABGP) at 1:700 and with goat anti-rabbit IgG Alexa 568 or with anti-L1 (1:300).

For the Sindbis virus infections, confluent monolayers of CHO cells on coverslips were infected with 4.4 µl Sindbis virus containing the 155-kDa isoform of neurofascin in 130 µl alphaMEM (Life Technologies) plus 1% FBS (HyClone Laboratories) plus 2 mM glutamine (Life Technologies). After 1 hr, an additional 300 µl medium was added. After 13 hr, the cells were washed in L-15 medium at 4°C and were incubated for 1 hr with 10 µg/ml Nr-Fc in L-15 and then for 1 hr with goat anti-human Fc fluorescein diluted 1:100 in L-15. Then the cultures were fixed with 4% paraformaldehyde for 15 min and were permeabilized at −20°C with methanol for 15 min. After blocking in L-15 plus 10% heat-inactivated FBS, incubations were done in the same buffer with a 1:1000 dilution of anti-neurofascin NFC1 for 75 min, followed by 3 washes and treatment with a 1:100 dilution of goat anti-rabbit IgG fluorescein for 1 hr. The coverslips were washed with buffer, PBS, and water and were then mounted with Citifluor containing Hoechst.

Coimmunoprecipitations
Cultures of DRG neurons were extracted in 500 µl buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, 20 mM PMSF, 10 µg/ml leupeptin, 0.1 M NaF, 10 mM Na^+ tetraphosphates, and 0.6 mM Na^+ vanadate and

were cleared for 30 min in a microfuge. A total of 0.25 ml of the extract was incubated overnight with 20 µl Protein A (PA) beads with or without preincubation with 15 µg Nr-Fc. After washing the beads with HNTG buffer, which contains 50 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol, the beads were boiled in SDS sample buffer, resolved on SDS gels, and immunoblotted with anti-neurofascin antibodies.

Proliferation assays
The proliferation of Schwann cells in myelinating cocultures was determined using a BrdU nuclear labeling assay according to the manufacturer's instructions (Boehringer Mannheim).

Supplementary material
Supplementary material available at http://images.cellpress.com/supmat/supmatin.htm demonstrates that nodes of Ranvier form before Caspr clusters in the paranodal region and the pattern of neurofascin expression in cultured neurons and Schwann cells.

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