The cellular form of the prion protein (PrP\textsuperscript{C}) is a highly conserved glycoprotein mostly expressed in the central and peripheral nervous systems by different cell types in mammals. A misfolded, pathogenic isoform, denoted as prion, is related to a class of neurodegenerative diseases known as transmissible spongiform encephalopathy. PrP\textsuperscript{C} function has not been unequivocally clarified, and it is rather defined as a pleiotropic protein likely acting as a dynamic cell surface scaffolding protein for the assembly of different signaling modules. Among the variety of PrP\textsuperscript{C} protein interactors, the neuronal cell adhesion molecule (NCAM) has been studied in vivo, but the structural basis of this functional interaction is still a matter of debate. Here we focused on the structural determinants responsible for human PrP\textsuperscript{C} (HuPrP) and NCAM interaction using stimulated emission depletion (STED) nanoscopy, SPR, and NMR spectroscopy approaches. PrP\textsuperscript{C} co-localizes with NCAM in mouse hippocampal neurons, and this interaction is mainly mediated by the intrinsically disordered PrP\textsuperscript{C} N-terminal tail, which binds with high affinity to the NCAM fibronectin type-3 domain. NMR structural investigations revealed surface-interacting epitopes governing the interaction between HuPrP N terminus and the second module of the NCAM fibronectin type-3 domain. Our data provided molecular details about the interaction between HuPrP and the NCAM fibronectin domain, and revealed a new role of PrP\textsuperscript{C} N terminus as a dynamic and functional element responsible for protein-protein interaction.

A misfolded form of the host-encoded cellular prion protein (PrP\textsuperscript{Sc}) is the causative agent for a class of human and animal neurodegenerative diseases denoted as transmissible spongiform encephalopathies. PrP\textsuperscript{C} is a sialoglycoprotein, tethered to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. Soluble, natively \(\alpha\)-helix-folded monomers of PrP\textsuperscript{C} may adopt an aggregated protease-resistant conformation known as PrP\textsuperscript{Sc} (1). The mature human PrP\textsuperscript{C} (HuPrP) is composed of 209 residues including a largely unstructured N-terminal part and a globular \(\alpha\)-helix-rich C-terminal domain (2). Conversely, PrP\textsuperscript{Sc} is \(\beta\)-sheet-enriched, partially protease-resistant, insoluble, and multimeric (3). The insoluble nature of PrP\textsuperscript{Sc} and its propensity to aggregate have hampered the use of high-resolution techniques, and therefore different PrP\textsuperscript{Sc} models currently exist (4).

Despite the fact that PrP\textsuperscript{C} is highly conserved among different species, its physiological function has not been fully clarified. Defining PrP\textsuperscript{C} function remains one of the main challenges in prion biology, and it is also an absolute requirement for understanding prion diseases. It is now being accepted that PrP\textsuperscript{C} is a pivotal molecule with diverse roles in brain development and in neural plasticity in the adult (5–8). Proposed PrP\textsuperscript{C} functions range from neuronal growth and differentiation (9), synaptic plasticity (10, 11), cell signaling (12,
PrP<sup>C</sup> N Terminus-mediated Cross-talk with NCAM

13), NMDA receptor regulation (14, 15), and brain metal homeostasis (16). Putative functions are based on PrP<sup>C</sup>-interacting molecules present on the cell surface, such as the 37-kDa/67-kDa laminin receptor, the stress-inducible protein 1, and vitronectin (17–19). Among the different PrP<sup>C</sup> protein interactors, the neuronal cell adhesion molecule (NCAM) has been extensively characterized in vitro, in cell-based assays, and in vivo (13, 20–23).

NCAM belongs to Ig superfamily cell adhesion molecules (CAMs), and it is present on the cell surface of neurons, astrocytes, and oligodendrocytes, where it mediates homophilic and heterophilic cell adhesion (24). NCAM is involved in neuronal migration, axon growth, and guidance, as well as in synaptic plasticity associated with learning and memory (25). Alternative splicing of the NCAM1 gene results in isoforms of three size classes that differ in their membrane attachment and cytosolic regions: NCAM-180, NCAM-140 (with intracellular domains consisting of 386 and 120 amino acids, respectively), and NCAM-120 (lacking the intracellular domain and linked to the membrane by a GPI anchor). NCAM isoforms share an extracellular domain consisting of five Igs and two fibronectin type-3 (FNIII1,2) domains. Variable use of alternative exons in the extracellular domain results in small insertions into Ig4 or between the FNIII1,2 domains. NCAM function is further regulated by an unusual posttranslational modification consisting of the addition of polysialic acid to Ig5 (26, 27). Although a complete picture has yet to emerge, it appears that NCAM physiological function is mediated by multiple modes of homophilic interaction through the NCAM Ig domains (28). Additionally, NCAM is also engaged in heterophilic interactions, i.e. NCAM can bind other protein partners modulating different functions. Different groups have investigated the structural and molecular basis governing the interaction between the NCAM FNIII1,2 domain and the fibroblast growth factor receptor 1 (FGFR1) (29, 30).

Besides the interaction with FGFR1, NCAM can bind PrP<sup>C</sup> and engages different cellular responses. PrP<sup>C</sup>-NCAM binding induces NCAM redistribution in lipid rafts, leading to FYN tyrosine kinase activation and neurite outgrowth (13). ELISA experiments using an NCAM-derived peptide library against the recombinant full-length mouse prion protein (MoPrP) have shown that the FNIII1,2 domain shares highest affinity for MoPrP (21). As revealed by in situ cross-linking of MoPrP deletion mutants expressed in cell models, the PrP<sup>C</sup>-binding site seems to be formed by the N terminus and a segment comprising α-helix 1 (21). However, PrP<sup>C</sup>-NCAM interacting surfaces have not been described at the structural and biophysical levels yet. Recently, it has been observed that PrP<sup>C</sup> controls polysialylation of NCAM during cellular morphogenesis (23). Current evidence strengthens the hypothesis that the FNIII1,2 domain plays a role for NCAM-mediated heterophilic interaction; therefore, this domain may represent a useful model for structural studies aiming at understanding the molecular determinants of PrP<sup>C</sup>-NCAM interaction.

Here we have performed cell biology, biophysical, and structural investigations on the interaction between the FNIII1,2 domain and HuPrP. Despite the inherent structural disorder of the PrP<sup>C</sup> N-terminal domain, which can mediate its interaction with both metals and cellular polyanions (e.g. sulfate proteoglycans) (31), binding with physiological protein partners has not been proved yet. HuPrP segments that are able to interact with the FNIII1,2 domain were identified by SPR experiments. NMR chemical shift perturbation experiments allowed us to identify binding sites on the FNIII1,2 domain involved in the interaction with the HuPrP. The module 2 (FNIII2) of the fibronectin type-3 domain and peptides covering different parts of the HuPrP were employed as a model system. Additionally, we examined the effect of a pathological amino acid substitution, P102L, the prototypical mutation linked to a genetic form of human prion disease denoted as Gerstmann-Straussler-Scheinker disease (GSS), on binding to FNIII2. Interestingly, this mutation also unveiled a new hidden binding site corresponding to the non-octarepeat copper-binding site (32). These results may provide a structural understanding of the interaction of NCAM with PrP<sup>C</sup> and where the binding site is located. Besides, the novel insight of the effect of HuPrP bearing the pathogenic point mutation P102L on its interaction with the FNIII2 domain is introduced here.

Results

NCAM Co-localizes with PrP<sup>C</sup>—Functional interactions of PrP<sup>C</sup> with its binding partner(s) have been suggested previously (13): cis and trans interactions between NCAM and PrP<sup>C</sup> promote neurite outgrowth, and the disruption of these interactions indicates that PrP<sup>C</sup> is involved in nervous system development cooperating with NCAM as a signaling receptor.

In this study, we used stimulated emission depletion (STED) nanoscopy to confirm the association between PrP<sup>C</sup> and NCAM in mouse hippocampal culture. We determined simultaneously the cellular distribution of PrP<sup>C</sup>, NCAM, and actin (Fig. 1A). PrP<sup>C</sup> and NCAM share very similar distributions along the neurite and in hippocampal growth cones (GC). The staining for PrP<sup>C</sup> and NCAM was preferentially localized in the central domain and transition zone of the GC membrane (Fig. 1A, arrows). By using STED nanoscopy, we were able to detect very low co-localization between PrP<sup>C</sup> and NCAM, whereas treatment with 1 µM nerve growth factor (NGF) results in increased co-localization (Fig. 1B). The observed higher association between PrP<sup>C</sup> and NCAM in treated cultures suggested that these proteins might functionally cooperate to transduce signals into the cell interior, which in turn trigger the neurite growth.

FNIII1,2 Domain Binds to HuPrP N-terminal Domain with High Affinity—SPR experiments were used to analyze the binding of the FNIII1,2 domain to different HuPrP and MoPrP constructs, including full-length HuPrP and MoPrP (i.e. from residues 23 to 231 and 23 to 230, respectively), the HuPrP N-terminal domain (residues 23–144), and the truncated C-terminal HuPrP and MoPrP (residues 90–231 and 89–230, respectively). We observed strong interaction between the FNIII1,2 domain and the HuPrP N-terminal domain with K<sub>d</sub> of 5.4 nM. Also the full-length HuPrP binds to the FNIII1,2 domain (K<sub>d</sub> of 337 nM), whereas for the truncated HuPrP, we were not able to report any interaction. The full-length MoPrP displayed a weak affinity for the FNIII1,2 domain (K<sub>d</sub> 3.8 µM), whereas the truncated MoPrP behaved almost iden-
tically to the truncated HuPrP, displaying no interaction with the FNIII1,2 domain (Table 1 and Fig. 2). Additionally, we confirmed that a short NCAM peptide, named BCL, corresponding to the sequence of residues 620–635 in the FNIII1,2 domain, is able to interact with HuPrP using ELISA (Fig. 3). Peptide BCL also binds to MoPrP (21), and it is considered an NCAM mimetic peptide employed as NCAM surrogate in pharmacological experiments (34). These data add more insights about the HuPrP regions involved in the

**TABLE 1**
Dissociation constants for the interaction between FNIII (immobilized) and different HuPrP and MoPrP constructs

| Protein          | $K_{d}$  | $K_{on}$    | $K_{off}$  |
|------------------|---------|-------------|------------|
| HuPrP(23–231)    | 377     | 2.00E+05    | 0.0674     |
| HuPrP(90–231)    | NA      | NA          | NA         |
| HuPrP(23–144)    | 5.4     | 1.42E+06    | 7.66E-03   |
| MoPrP(23–230)    | 3800    | 2.58E+04    | 0.0983     |
| MoPrP(89–230)    | NA      | NA          | NA         |

NA, not applicable.
interaction with the FNIII1,2 domain, showing that this binding is largely mediated by the unstructured N-terminal HuPrP domain.

**FNIII2 Domain Was Used for NMR Characterization of Binding between HuPrP and NCAM**—NMR spectroscopy is an excellent tool to evaluate and characterize the binding properties of HuPrP and NCAM in solution at atomic level. Unambiguous assignment of HuPrP(23–144) residues was not possible because of high signal overlap in the $^{15}$N HSQC spectrum, which was due to the high percentage of glycine residues, octarepeats, and intrinsically disordered properties of the unstructured N terminus (supplemental Fig. S1A). Thus, we focused of the HuPrP interaction partner instead. $^{15}$N,$^{13}$C-labeled FNIII1 and FNIII2 domains of NCAM were produced to find a suitable candidate for the evaluation of HuPrP-NCAM binding properties. The $^{15}$N HSQC spectra of the FNIII1,2 (supplemental Fig. S1, B–D), FNIII1, and FNIII2 (Fig. 4) domains were recorded. The FNIII1,2 and FNIII1 domains did not exhibit suitable NMR properties for further analysis, whereas for the FNIII2 segment (with a molecular mass of 11.8 kDa), a complete NMR analysis has been carried out.

The $^{15}$N HSQC spectrum of the FNIII2 domain demonstrated good dispersion of cross-peaks, indicating the potential for detailed structure characterization (Fig. 4). The sequence-specific assignment was performed with the use of standard triple resonance NMR experiments to obtain the structure at atomic resolution. The structure of the FNIII2 domain was calculated using 778 intra-residual and sequential restraints, 108 medium-range and 772 long-range distance restraints, and 136 backbone torsion angle restraints (Table 2). The calculated

![Graphs A, B, C](image_url)
three-dimensional model showed that the FNIII2 domain consists of six antiparallel β-strands, marked A–F on Fig. 5. They are arranged into two right-handed β-sheets that form a β-sandwich. The first β-sheet is composed of strands A and D (residues Ser616–Asn620 and His657–Lys661, respectively), whereas the second β-sheet is composed of strands B, C, E, and F (residues Ile631–Ala640, Ile649–Pro652, Tyr669–Asn677, and Ala685–Phe687, respectively).

NMR approaches of biomolecular complexes are usually challenging because of the increase of molecular weight due to the interaction between partners. Clearly, a large number of similar cross-peaks will be observed and will result in the overlapped spectra and increased linewidths associated with the slower tumbling of a high molecular mass complex. Note that increased line widths of cross-peaks are also expected in the spectrum. Moreover, sometimes, macromolecular interactions lead to several line broadenings through the enhanced relaxation that resulted in decrease and even loss of cross-peaks of interest (35, 36).

Multidimensional heteronuclear NMR experiments were used to evaluate binding properties between the FNIII2 domain and N-terminal HuPrP(23–144). Interestingly, no amide chemical shift changes were observed in the 15N HSQC spectrum of the FNIII2 domain.

## Table 2

| NMR restraints and structural statistics for the ensemble of 20 lowest-energy structures of the FNIII2 domain |
|---------------------------------------------------------------|
| **NOE upper distance limits**                                  |
| Total                                                         | 1658 |
| Intra-residue ($|i-j| = 0$)                                     | 337  |
| Sequential ($|i-j| = 1$)                                      | 441  |
| Medium-range ($|i-j| < 5$)                                   | 108  |
| Long-range ($|i-j| > 5$)                                    | 772  |
| **Torsion angle restraints**                                  |
| Backbone ($\phi/\psi$)                                    | 136  |
| **RMSD to the mean coordinates (Å)**                          |
| Ordered backbone atoms (595–691)                             | 0.28 ± 0.09 |
| Ordered heavy atoms (595–691)                                | 0.78 ± 0.08 |
| **Ramachandran plot (595–691)**                              |
| Residues in most favored regions (%)                         | 88.8  |
| Residues in additional allowed regions (%)                   | 11.2  |
| **Structure Z-scores**                                       |
| 1st generation packing quality                               | −0.419 ± 0.568 |
| 2nd generation packing quality                               | 7.386 ± 2.149 |
| Ramachandran plot appearance                                 | −3.376 ± 0.339 |
| y-1/γ-2 rotamer normality                                    | −6.283 ± 0.259 |
| Backbone conformation                                        | −0.441 ± 0.137 |
| **RMS Z-scores**                                              |
| Bond lengths                                                  | 1.201 ± 0.004 |
| Bond angles                                                   | 0.581 ± 0.008 |
| ω angle restraints                                            | 0.758 ± 0.030 |
| Side chain planarity                                          | 0.437 ± 0.029 |
| Improper dihedral distribution                                | 0.779 ± 0.016 |
| Inside/Outside distribution                                   | 1.029 ± 0.011 |

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#### Notes:

- None of the 20 structures exhibit distance violations over 0.2 Å and torsion angle violation over 5°.
- Ensemble of structures was analyzed by the PROCHECK-NMR and WhatIF programs incorporated in the CING structure evaluation package (54) and PSVS (55).
domain during a titration experiment with HuPrP(23–144) at pH 7.0 (Fig. 6A). However, the FNIII2 sample became blurred upon rising concentration of HuPrP(23–144), presumably because of interactions between partners that also led to insoluble aggregates. Analysis of cross-peak linewidths and absence of chemical shift changes in the $^{15}$N HSQC spectra of FNIII2 resulted in the conclusion that cross-peaks correspond to the unbound state of the labeled FNIII2 domain. Additionally, we tried to thermally disrupt multimeric complex at 45 °C to gain the monomeric form of the complex. Although the sample cleared up, indicating that the multimers fell apart, the $^{15}$N HSQC spectrum did not improve after thermal treatment. Certainly, even smaller multimeric complexes were still too big to be observed by NMR. Titration was replicated at pH 6. The pH was decreased to achieve favorable conditions where the complex would not precipitate and would be stable often enough that we could observe chemical shift changes. The $^{15}$N HSQC spectra of the FNIII2 domain (Figs. 6, 7, and 8) led us to conclude that residues Tyr$^{669}$, Val$^{673}$, His$^{686}$, Phe$^{687}$, and Val$^{688}$ of the FNIII2 domain exhibit negligible chemical shift changes. Other cross-peaks of the FNIII2 domain exhibit negligible chemical shift changes.
Phe687, and Val688 of the FNIII2 domain are involved in the interaction with HuPrP.

**Interactions between FNIII2 Domain and HuPrP(93–114, P102L)—**
Because peptide HuPrP(93–114, P102L) exhibited the strongest interactions with the FNIII2 domain, we examined the effects of the interaction on the peptide as well. The 15N HSQC, NOESY, TOCSY, and 13C HSQC spectra of aliphatic and aromatic regions were used for chemical shift assignment of unlabeled peptide. 13C-15N ω₁-filtered 2D NOESY/TOCSY and 13C-15N ω₁-filtered 3D 15N HSQC-NOESY were used to determine inter- and intramolecular contacts in complex. Upon binding, no intermolecular contacts were observed in 13C-15N ω₁-filtered 2D NOESY. The largest intramolecular chemical shift changes were observed for cross-peaks in 13C-15N ω₁-filtered 2D TOCSY of amino acids Trp99, His111, Met112, and Ala113 of HuPrP(93–114, P102L).

Experimental data therefore indicate that amino acid residues Tyr669, Val673, His686, Phe687, and Val688 of the FNIII2 domain and Trp99, His111, Met112, and Ala113 of HuPrP(93–114, P102L) are most probably involved in the interaction of the NCAM domain and WT HuPrP. These data were used as docking restraints for HADDOCK calculation (37, 38). 131 lowest-energy structures were grouped into 13 clusters according to RMSD values. The numbers of structures in the 10 best clusters varied from 4 to 34 with their HADDOCK scores ranging from 71.8 to 53.5. The clusters could be split into two groups of similar size. The first model describes the interactions between Trp99 on HuPrP(93–114, P102L) and the FNIII2 domain, whereas the second one describes the interactions of His111–Ala113 with HuPrP(93–114, P102L) and the FNIII2 domain (Fig. 9).

**Discussion**
Among the variety of PrPC protein interactors (37), PrPC associates with NCAM in vivo. Both molecules have been independently implicated in nervous system development and may play a role in neural stem cells (9, 38, 40). PrPC recruits to and stabilizes the transmembrane NCAM isoforms (NCAM-180 and NCAM-140) in lipid-rich microdomains. This activates FYN kinase and promotes neurite outgrowth by cis and trans interactions (13, 41). The interaction of these two molecules and their relation to specific signaling pathways during neurodevelopment merits further investigation at the structural and molecular level, not the least because the physiological role of PrPC may provide novel insights into the neuropathology of prion diseases.

Along this line, the structural basis of the cross-talk between PrPC and NCAM has not been previously investigated. In this study, we have performed a structural investigation on the interaction between the recombinant FNIII2 domain of NCAM and different peptides originating from N-terminal human PrPC using different experimental approaches.

The in vitro experiments designed to confirm the co-localization of PrPC and NCAM in hippocampal neuron cultures led us toward a better understanding of this interaction. We have identified the HuPrP and MoPrP segments able to interact with the FNIII2 domain by means of SPR experiments. These experiments have unveiled that the N-terminal HuPrP domain mediates the FNIII2 domain binding with high affinity.

X-ray approaches have been previously applied to better understand the structural properties of the FNIII2 domain (42). Interestingly, the comparison between the FNIII2 NMR structure and the corresponding x-ray structure revealed local structural differences in the length of β-strands. In particular, although the NMR structure features two short β-strands (A and D) on one protein side and four β-strands (B, C, E, and F) on the other, the x-ray structure is characterized by seven
Structural discrepancies between diffraction and NMR data are to be expected considering the differences of the two methods in terms of spatial distribution of the molecules in the sample and time scales accessible to each method. Notably, solution NMR data represent an average over semi-randomly oriented mol-

\[ \Delta \delta(H,N) \] values were calculated using Equation 1 (see "Experimental Procedures").

\[ \frac{\Delta \delta(H,N)}{\text{ppm}} \]

FIGURE 8. Chemical shift changes $\Delta \delta(H,N)$ for FNIII2 domain upon interaction with peptides originating from HuPrP at pH 6.0. A, HuPrP(23–144) peptide at ratio 1:0.5. B, HuPrP(93–114, P102L) at ratio 1:2. C, HuPrP(23–89) at ratio 1:2. The $\Delta \delta(H,N)$ values were calculated using Equation 1 (see "Experimental Procedures").

\[ \Delta \delta(H,N) \] values were calculated using Equation 1.

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Cells in solution detected in a nanosecond-to-second time regime, whereas diffraction data represent an average over molecules arranged in a periodic crystal lattice acquired in a seconds time scale.

We have performed NMR experiments designed to identify the binding sites on the FNIII2 domain and HuPrP N terminus responsible for the interaction. The implications of our findings are important in prion biology as we provide the first structural evidence that NCAM mediates the interaction with the N-terminal domain of HuPrP through its FNIII1,2 domain. We propose a model where this largely unstructured segment acts as a dynamic element able to recruit NCAM molecules and to mediate physiological processes. We found that the WT HuPrP mediates binding with the FNIII2 domain via its N-terminal segment (residues 23–144). Interestingly, the N terminus contains a glycosaminoglycan (GAG)-binding motif. The binding of GAG is important in prion diseases. This idea is supported by evidence that mutant recombinant PrP binds more GAG, which promotes the aggregation of mutant recombinant PrP more efficiently than HuPrP wild type (43). Thus, our present data corroborate the idea that besides binding metal ions and GAG, the N-terminal domain also mediates cis interactions with the NCAM fibronectin domain.

As opposed to WT HuPrP, the P102L mutant seems to possess an extra binding site localized in the segment 93–114, also denoted as the non-octarepeat copper-binding site (32, 44). Peptides HuPrP(93–114) and HuPrP(93–114, P102L) have the same position in HuPrP N terminus except for the proline-to-leucine substitution at residue 102. Comparison of the ΔG(H,N) values for the FNIII2 domain after titration with both HuPrP(93–114) and HuPrP(93–114, P102L) led to a conclusion that the interaction of the latter with the FNIII2 domain is stronger. Thus, we argue that P102L mutation may affect binding with the FNIII2 domain, presumably leading to a stronger interaction with NCAM, which in turn may lead to abnormal Src family kinase activation.

These findings about the interaction between HuPrP and NCAM may have biological relevance on prion conversion. Different compounds, including antibodies or chemical drugs, with high affinity for PrPC have been employed as candidates for therapeutic approaches aimed at inhibiting prion replication. This approach postulates that any PrPC ligand acts as a molecular chaperone able to stabilize the protein folding, thus limiting the conversion to PrPSc. NCAM does not play a direct role during prion formation as observed in NCAM knock-out mice showing the same incubation period when compared with wild-type mice infected by prions (21). It is plausible that any interference with NCAM-mediated signaling in the diseased brain may favor cell death and inhibit synaptic plasticity. The interactions between PrPC and NCAM could therefore be reduced by accumulation of PrPSc in the diseased nervous system. Thus, it is conceivable that the association of NCAM to PrPC favors functional signaling pathways through FYN, which is also implicated in synaptic functions. Furthermore, PrPC-NCAM crosstalk is crucial for the coordinated regulation of cell cycle progression and the differentiation of neuronal precursors toward different neuronal phenotypes (40). We found that this interaction is mediated by the PrPC N terminus, particularly in the segment from residues 23 to 50. This includes four positively charged residues (i.e. KKRPK) known to play a role in the PrPSc endocytic trafficking and in its localization to lipid rafts (45, 46). Our study suggests that the interaction between residues 23–50 and the NCAM fibronectin domain may determine the fate of PrPC in the cell surface raft domain. Here PrPC participates in the complex molecular networks and signaling mechanisms, both cell-intrinsic and cell-extrinsic, that influence cell fate and differentiation.

The mechanisms that recruit and assemble different transmembrane signaling molecules to distinct membrane subdomains have only started to emerge. In these environments, signaling and scaffolding proteins can self-associate and form dimeric or multimeric assemblies. Protein dimerization has been reported as a common mechanism to cluster downstream signaling components and thereby enhance the signaling cascade (47). According to a recent model, NCAM is present on the cell surface as cis dimers, formed by the interactions between the Ig1 and Ig2 domains (48, 49). The role of cis dimerization in NCAM dimerization is debated, but it is a prerequisite for NCAM clustering on the cell surface, which in turn results in cell-cell adhesion via trans interactions between NCAM clusters.

We described a strong interaction between HuPrP N terminus and FNIII1,2 and the formation of multimeric complexes formed upon the addition of FNIII2 to HuPrP(23–144). This may have important physiological implications as PrPSc may act as a scaffolding protein able to facilitate the FNIII1,2-mediated NCAM self-assembly and clustering in the lipid raft.

In conclusion, we provide structural evidence that NCAM mediates a heterophilic interaction through FNIII1,2 and the N-terminal domain of HuPrP. Importantly, we propose that this largely unstructured segment acts as dynamic element able to recruit NCAM molecules and to mediate physiological processes.

**Experimental Procedures**

**Cell Culture**—P1-P2 FVB WT mice were sacrificed by decapitation in accordance with the guidelines of the Italian Animal Welfare Act.

**Immunostaining**—Cells were fixed in 4% paraformaldehyde containing 0.15% picric acid in PBS, saturated with 0.1 M glycine, permeabilized with 0.1% Triton X-100, saturated with 0.5% BSA (all from Sigma-Aldrich) in PBS, and then incubated for 1 h with primary antibodies followed by a 30-min incubation with secondary antibodies conjugated with STAR580 or STAR635P (Abberior, Göttingen, Germany). All incubations were performed at room temperature (20–22 °C).

**STED Microscopy**—Two-color STED microscopy was performed at the NanoBiophotonics Department (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) (50) equipped with 561- and 640-nm pulsed excitation lasers, a pulsed 775-nm STED laser, and a 100× oil immersion objective lens (NA 1.4).

**Plasmid Construction**—The FNIII1,2 and FNIII2 domains were PCR-amplified from pCEP-Pu vector encoding for FNIII1,2 (kindly provided by Dr. Federico Carafoli, Imperial College London, London, UK) and inserted into a modified pET11a vector (i.e. carrying a C-terminal uncleavable His tag).
PrP$^\text{C}$ N Terminus-mediated Cross-talk with NCAM

### Table 3

| Amino acid sequences of peptides originating from N-terminal HuPrP that were used for characterization of binding properties between HuPrP and NCAM |
|-------------------------------------------------|
| **Names of peptides** | **Amino acid sequences** |
| HuPrP(23–144) | KKRPKPGNNTGSSYRPGDNPAGGNYPFGHGGGQPHGGMQPHGGGGQPQG |
| HuPrP(23–89) | KKRPKPGNNTGSSYRPGDNPAGGNYPFGHGGGQPHGGMQPHGGGGQPQG |
| HuPrP(23–50) | KKRPKPGNNTGSSYRPGDNPAGGNYPFGHGGGQPHGGMQPHGGGGQPQG |
| HuPrP(93–114) | GOTHGQNKPSKPTNKHAG |
| HuPrP(93–114, P102L) | GOTHGQNKPSKPTNKHAG |
| HuPrP(60–68) | PHGGGWQ |

using a restriction-free cloning protocol. Our FNIII1,2 and FNIII2 numbering schemes correspond to Carafoli et al. (42).

Protein Expression and Purification—A freshly transformed overnight culture of *Escherichia coli* BL21 (DE3) cells (Strategene) transformed with pET11a encoding for FNIII2 or FNIII2 was added at 37 °C to 2 liters of minimal medium. For isotopic labeling, 4 g/liter [13C$_6$]glucose and 1 g/liter [15N]ammonium chloride were added. At 0.8 A$_{600}$ expression was induced with isopropyl-$\beta$-d-galactopyranoside to a final concentration of 0.25 mM. Cells were grown in a BIOSTAT B Plus 2-liter vessel (Sartorius) and harvested 18 h after inoculation. Bacterial paste was resuspended in 25 mM Tris-HCl, 0.8% Triton X-100, 1 mM PMSF, pH 8, and lysed by a Panda homogenizer. 15N amide chemical shifts between the protein-peptide complex and the free state of the FNIII2 domain (59).

Modeling the Complex between FNIII2 Domain and HuPrP

Modeling the Complex between FNIII2 Domain and HuPrP (93–114, P102L)—Modeling of the complex between the FNIII2 domain and HuPrP(93–114, P102L) was made with the HADDOCK software (33, 39).
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