1 **Direct binding of the flexible C-terminal segment of periaxin to β4 integrin suggests**
2 **a molecular basis for CMT4F**

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1   **Abstract**

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3   The process of myelination in the nervous system requires coordinated formation of
4   both transient and stable supramolecular complexes. Myelin-specific proteins play
5   key roles in these assemblies, which may link membranes to each other or connect
6   the myelinating cell cytoskeleton to the extracellular matrix. The myelin protein
7   periaxin is known to play an important role in linking the Schwann cell cytoskeleton
8   to the basal lamina through membrane receptors, such as the dystroglycan complex.
9   Mutation that truncate periaxin from the C terminus cause demyelinating peripheral
10   neuropathy, Charcot-Marie-Tooth disease type 4F, indicating a function for the
11   periaxin C-terminal region in myelination. We identified the cytoplasmic domain of
12   $\beta4$ integrin as a specific high-affinity binding partner for periaxin. The C-terminal
13   region of periaxin remains unfolded and flexible when bound to the third fibronectin
14   type III domain of $\beta4$ integrin. Our data suggest that periaxin is able to link the
15   Schwann cell cytoplasm to the basal lamina through a two-pronged interaction *via*
16   different membrane protein complexes, which bind close to the N and C terminus of
17   this elongated, flexible molecule.

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1 **Introduction**

2 Long axonal segments in the vertebrate peripheral nervous system (PNS) are
3 ensheathed by myelin, which accelerates nerve impulse propagation and which
4 supports axons both mechanically and trophically (1). A Schwann cell wraps its
5 plasma membrane, partially excluding its cytosol, several times around a selected
6 axonal process. This results in compact myelin, which has high lipid and protein
7 content and is responsible for axonal insulation. Compact myelin is surrounded by a
8 narrow compartment with higher cytosolic content, non-compact myelin, which acts
9 as a supportive metabolic compartment to ensure long-term myelin stability (2).
10 Both myelin compartments contain a specific selection of proteins that have distinct
11 tasks in ensuring the correct formation and stability of myelin: failure may result in
12 one of several disease states, including the peripheral inherited neuropathies
13 Charcot-Marie-Tooth (CMT) disease and Dejerine-Sottas syndrome (DSS). A large
14 number mutations in different PNS proteins has been linked to these conditions
15 (3,4). On the other hand, only a handful of CMT mutations have been characterized
16 at the molecular structural level in order to understand the fine details of disease
17 mechanisms (5–7).

18 The formation of myelin in the CNS and PNS, as well as its lifelong maintenance,
19 requires an intricate network of molecular interactions that link the myelin
20 membrane, the cytoskeleton of the myelinating glial cell, and the extracellular matrix
21 or the axonal surface together into a large supramolecular complex. A number of
22 proteins, many of which seem specific for myelinating cells, have been pinpointed as
23 playing roles in myelination; however, often the molecular details of the relevant
24 processes and protein-protein interactions remain unknown. Myelin proteins have
25 been specifically highlighted as a knowledge gap in structural biology in the past (8),
26 although more structural data from myelin proteins are becoming available.
27 However, structures of protein-protein complexes of myelin-specific proteins mainly
28 remain uncharacterized to date.

29 Unlike oligodendrocytic myelin in the CNS, Schwann cells in the PNS are surrounded
30 by a carbohydrate-rich basal lamina, which is adhered to the outermost (abaxonal)
31 Schwann cell plasma membrane bilayer *via* dystroglycans and α6β4 integrin,
32 contributing to the mechanical stability of myelinated nerves (9–11). Additionally,
33 non-compact PNS myelin contains tight membrane-apposed structures at the
34 abaxonal layer. These structures surround cytosolic channels of non-compact myelin,
35 referred to as Cajal bands, which contain substantial microtubule-based transport as
36 well as ribosomal activity (12,13). The membrane appositions have tight morphology
37 and are enriched in periaxin (PRX) – the most abundant PNS non-compact myelin
38 protein (14). Cajal bands and the membrane appositions are important in regulating
39 myelin stability. Furthermore, PRX influences the myelin sheath internode distance,
40 and thus influences nerve conduction velocity (15,16). These structures can be
41 disturbed in human demyelinating diseases, as well as in corresponding mouse
42 models (16–19).

43 Two isoforms of PRX are generated through alternative splicing (20). Disease
44 mutations in PRX often truncate the long C-terminal region of the larger L-PRX
45 isoform (21). The molecular mechanism of disease in these cases has remained
46 enigmatic, as the best-characterized protein interactions and functions of L-PRX so

1   far lie very close to the N terminus. These include the PDZ-like domain, which
2   mediates homo- and heterodimerization of PRX (18,22,23), and the segment after it,
3   which is known to bind dystrophin-related protein 2 (DRP2) and link PRX to the
4   dystroglycan complex (18). A conceivable additional mechanism of PRX function and
5   involvement in disease could involve specific protein interaction sites at the C-
6   terminal, isoform-specific end of L-PRX.

7   We wanted to identify novel binding partners for the L-PRX C-terminal region. The
8   third cytoplasmic fibronectin-type III (FNIII) domain of β4 integrin (β4-FNIII-3) was
9   identified as a high-affinity binder, and the complex was characterized using
10 biophysical and structural biology techniques. The observed direct molecular
11 interaction is likely to be important for the function of PRX and β4 integrin in
12 developing and mature myelin, and it provides a molecular basis for PRX mutations
13 in CMT that result in the expression of truncated L-PRX.

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1 **Materials and methods**

2 **Yeast two-hybrid screening**

3 Yeast two-hybrid screening was performed essentially as previously described (18).
4 Briefly, a random-primed rat sciatic nerve cDNA library in λACTII was screened using
5 the C-terminal region of rat L-PRX (residues 681-1383) in the pAS2-1 vector
6 (Clontech) as bait. Three independent clones of β4 integrin each containing the third
7 FNIII domain were found. To identify the domain of PRX, which interacted with β4
8 integrin, deletion contructs of PRX vwere made by PCR and subcloned into pAS2-1.
9 β-galactosidase activity was tested by filter lift assays with one of the β4 integrin
10 clones (62BpACTII).

11 **GST pulldown**

12 The β4 integrin FNIII-3 domain (amino acids 1512-1593) cDNA was amplified by PCR
13 and subcloned into pGEXKG. As a control, the adjacent fourth FNIII domain was
14 cloned into the same vector. The recombinant protein was expressed and purified
15 using a Glutathione-Sepharose 4B column as described (18). GST pulldowns were
16 performed by incubation of the GST fusion protein bound to Glutathione-Sepharose
17 with a sciatic nerve lysate, as previously described (18).

18 **Immunoaffinity chromatography**

19 Immunoaffinity pull-downs were performed essentially as described (18). PRX and β4
20 integrin in the pull-down fractions were identified by Western blot. PRX antibodies
21 have been described (24). Antibodies against β4 integrin were raised in rabbits using
22 a peptide corresponding to amino acids 1756-1772, to which an N-terminal cysteine
23 was attached for coupling to keyhole limpet hemocyanin (CTEPFLIDGLTLGTQRLE), as
24 described (20).

25 **Immunofluorescence**

26 Mice were perfused intravascularly with 4% paraformaldehyde in 0.1 M sodium
27 phosphate buffer (pH 7.3) and sciatic nerve cryosections were prepared and
28 immunostained as described (25). Antibodies against PRX have been described (26),
29 and the monoclonal antibody against β4 integrin was a generous gift from Dr. S.J.
30 Kennel, Biology Division, Oak Ridge National Laboratory.

31 **Transfection and coimmunoprecipitation**

32 Full-length cDNA clones encoding rat β4 integrin and human α integrin were
33 subcloned into the expression vectors pcDNA3.1 and pRC/CMV, respectively, and
34 were generous gifts from Dr M.L. Feltri, Hunter James Kelly Research Institute,
35 University of Buffalo and Dr Arnoud Sonnenberg, Netherlands Cancer Institute. PRX
36 cDNA in the expression vector pCB6 has been described (27). PRX, β4 integrin, and
37 α integrin were expressed in HEK293 cells. After transfection, the proteins were
38 immunoprecipitated as described (18).

39 **Proteomics**

40 Proteomics analyses were carried out exactly as described (24), comparing the PRX-
41 bound proteomes of PRX$^{-/-}$ mice carrying a transgene for either wild-type PRX
42 (PrxTg/PRX$^{-/-}$) or PRX with a C-terminal truncation mutation (mouse L-PRX truncated

1 at residue 1016; ΔCPrxTg/PRX^{-/-}) corresponding to human CMT4F R1070X (28,29).
2 Proteins from mouse nerve lysates were crosslinked followed by
3 immunoprecipitation with a PRX antibody, and then subjected to mass spectrometry
4 (MS) analyses. Normalized abundance of β4 integrin was determined by MS using
5 Progenesis (Nonlinear Dynamics) as described (24).

6 **Recombinant protein production**

7 Synthetic genes encoding the rat L-PRX C-terminal region (UniProt ID: Q63425,
8 amino acids 1036 – 1383; PRX-C) and rat β4-FNIII-3 (UniProt ID: Q64632, amino acids
9 1512 – 1602) were ordered from DNA2.0 (Newark, CA, USA) in the pJ401 bacterial
10 expression vector. An additional sequence encoding for an N-terminal hexahistidine
11 tag, a short linker, and a tobacco etch virus (TEV) protease digestion site
12 (MHHHHHHSSGVDLGTENLYFQS) were included at the start of the protein-coding
13 insert.

14 PRX-C was expressed in *E. coli* BL21(DE3) using 0.4 mM IPTG induction for 1.5 h in LB
15 medium containing 100 μg/ml ampicillin, at 37 °C. After expression, the cells were
16 pelleted by centrifugation and sonicated in Ni-NTA washing buffer (40 mM HEPES,
17 400 mM NaCl, 6 M urea, 20 mM imidazole, 1 mM PMSF, pH 7.5) supplemented with
18 protease inhibitors (Roche). Purification was performed using Ni-NTA affinity
19 chromatography and standard procedures. Elution was done with 32 mM HEPES, 320
20 mM NaCl, 4.8 M urea, 500 mM imidazole, pH 7.5. The eluted fraction was dialyzed at
21 4 °C with constant stirring against 40 mM HEPES, 400 mM NaCl, 1 mM DTT, pH 7.5,
22 before addition of recombinant TEV protease for affinity tag removal (30). The
23 digestion was allowed to proceed overnight while dialyzing, which resulted in
24 cleaved PRX-C with an additional N-terminal Ser residue. The protein was subjected
25 to a 2nd Ni-NTA step, in the absence of urea. The unbound and wash fractions were
26 combined and concentrated, and sequential size exclusion chromatography (SEC) on
27 a HiLoad Superdex 200 pg 16/600 column (GE Healthcare) was used to separate the
28 cleaved protein from contaminants and degradation products. Depending on
29 downstream application, either 20 mM HEPES, 300 mM NaCl, 1% (v/v) glycerol, 0.5
30 mM TCEP, pH 7.5 (SEC buffer) or 20 mM HEPES, 150 mM NaCl, pH 7.5 (HBS) was
31 used as running buffer.

32 β4-FNIII-3 was expressed in *E. coli* BL21(DE3) in LB or TB medium containing 100
33 μg/ml ampicillin, with 0.4 mM IPTG induction for 3 h at 37 °C. The cells were
34 harvested as above and resuspended in 40 mM HEPES, 400 mM NaCl, 20 mM
35 imidazole, pH 7.5. After lysis by sonication, a Ni-NTA chromatography was carried
36 out essentially as described above, omitting urea in all buffers. After Ni-NTA, the
37 eluted protein was directly subjected to SEC using a HiLoad Superdex 75 pg 16/60
38 column (GE Healthcare) using either HBS or SEC buffer.

39 **Pulldown experiment with purified proteins**

40 Purified recombinant β4-FNIII-3 and PRX-C were used in pulldown experiments to
41 confirm the direct interaction. PRX-C with and without the His-tagged β4-FNIII-3 was
42 mixed with Ni-NTA agarose for 1 h at +4 °C, in binding buffer (20 mM HEPES, 150
43 mM NaCl, 20 mM imidazole, pH 7.5). The matrix was collected by centrifugation at
44 150 g for 5 min at +4 °C. Three washes with binding buffer were carried out, and

1   proteins were eluted with 20 mM HEPES, 150 mM NaCl, 500 mM imidazole, pH 7.5.
2   All fractions were analyzed with SDS-PAGE.

3   In addition, a partially degraded PRX-C sample was employed to identify fragments
4   that do and do not bind β4-FNIII-3. The pulldown experiment was carried out exactly
5   as above. Bands were excised from the gel and processed for tryptic peptide
6   mapping.

7   **Synchrotron radiation circular dichroism (SRCD) spectroscopy**

8   SRCD data were collected from $0.15 - 0.6$ mg ml^{-1} protein samples in 20 mM Na-
9   phosphate, 150 mM NaF, pH 7.5 on the AU-CD beamline at the ASTRID2 synchrotron
10   (ISA, Aarhus, Denmark). 100-μm pathlength closed circular cells (Suprasil, Hellma
11   Analytics) were used for the measurements. Spectra were recorded from 170 to 280
12   nm at 20 °C. Temperature scans were performed from 10 to 90 °C in 5 °C intervals,
13   with 5 min incubation per time point prior to spectral acquisition.

14   Buffer spectra were subtracted from the protein samples, and CD units were
15   converted to $\Delta\varepsilon$ (M^{-1} cm^{-1}), using rPRX-C concentration determined using
16   refractometry and/or rFNIII-3 concentration determined using absorbance at 280
17   nm. Deconvolution was performed using DichroWeb (31) with the CDSSTR algorithm
18   (32) and SP175 reference set (33), or using BeStSel (34). Secondary structure
19   prediction was performed using JPred (35).

20   **Small-angle X-ray scattering (SAXS)**

21   Synchrotron SEC-SAXS data for PRX-C, β4-FNIII-3, and their complex were collected
22   on the B21 beamline at Diamond Light Source (Didcot, UK) using an on-line size
23   exclusion setup: the chromatography was performed using an Agilent 1200-series
24   HPLC system and a Superdex 200 increase 3.2/300GL (GE Healthcare) column with
25   20 mM Tris-HCl, 150 mM NaCl, pH 7.4 as mobile phase at an isocratic flow of 0.04
26   ml/min. 45 μl injections of $6.5 - 9.8$ mg/ml total protein were performed for PRX-C,
27   β4-FNIII-3, and their equimolar complex (145 μM each). Scattering was directly
28   recorded from the eluted proteins at 6 s exposure per frame, 591 frames per run.
29   The frames containing a stable R_g within an eluted I_0 peak were selected and
30   combined using ScÅtter (http://www.bioisis.net/tutorial). Data were processed and
31   analyzed using the ATSAS package (36). GNOM was used to calculate distance
32   distribution functions (37), and *ab initio* modeling was performed using GASBOR
33   (38). Multiphase modeling of protein complex data was performed using MONSA
34   (39) and ensemble optimization analysis with EOM (40). See Supplementary Table 1
35   for further details.

36   For IDPs, more accurate values of R_g can be obtained from SAXS data using the
37   Debye formalism. Briefly, by plotting $[I(s)]^{-1}$ *vs.* $s^{2.206}$, in the range $(s\,R_g)^2 < 3$, one can
38   use much more data than in a regular Guinier plot. Here, a linear fit of the plot is
39   used to obtain $R_g = (a/0.359b)^{0.453}$; a = slope, b = y intercept (41). Theoretical values
40   for R_g and D_{max} for a random chain can be calculated from sequence length as
41   follows: $R_g = R_0 N^v$; $R_0 = 1.98$ Å, N = number of residues, v = 0.602 (42) for denatured
42   proteins; $R_0 = 2.54$ Å, v = 0.522 (43) for IDPs. Average end-to-end distance can be
43   estimated as $\langle L^2 \rangle = L_0 N$; $L_0 = 81.8$ Å (42).

44   **Mass spectrometry and covalent crosslinking**

1   The molecular weight of PRX-C and β4-FNIII-3 was verified by mass spectrometry. In
2   short, the undigested masses were determined using ultra-performance liquid
3   chromatography (UPLC) coupled electrospray ionization (ESI) time-of-flight mass
4   spectrometry in positive ion mode using a Waters Acquity UPLC-coupled Synapt G2
5   mass analyzer with a Z-Spray ESI source.

6   Protein crosslinking was carried out to conjugate surface-exposed carboxylate
7   sidechains with lysines with a zero-length crosslinker. All crosslinking reactions were
8   carried out at 40 µM final protein concentration in 100 mM bis-tris methane, 150
9   mM NaCl, 4 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 20 mM *N*-
10 hydroxysuccinimide, pH 6.5. The activation step was allowed to proceed for 15 min
11 at ambient temperature, followed by quenching the reactions through adding 2-
12 mercaptoethanol to 20 mM. After addition of the second protein in selected
13 reactions, incubation was continued for another 3 h at ambient temperature and
14 stopped by adding ethanolamine to 10 mM. The reactions were analyzed using SDS-
15 PAGE.

16 The crosslinked proteins were identified using matrix-assisted laser
17 desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. From stained
18 SDS-PAGE gels, gel bands were cut, staining removed by sequential washing with 50
19 mM NH_4HCO_3 in 40% acetonitrile (ACN). Proteins were subjected to in-gel Cys
20 reduction using 20 mM DTT and subsequent alkylation using 40 mM α-
21 iodoacetamide. After this, all proteins were in-gel digested (20 ng of trypsin (Sigma-
22 Aldrich) per gel piece), followed by peptide extraction from gel pieces using 30%
23 ACN/0.1% trifluoroacetic acid (TFA), and transfer to a Bruker anchor plate. 800 µg/ml
24 α-cyano-4-hydroxy cinnamic acid in 85% ACN/0.1% TFA with 1 mM $NH_4H_2PO_4$ was
25 used as matrix. Peptide mass spectra and MS/MS spectra were measured with a
26 Bruker Ultra fleXtreme MALDI-TOF mass spectrometer and compared to theoretical
27 spectra generated using the known protein sequences.

28 **Multi-angle static and quasielastic light scattering**

29 SEC-MALS and quasielastic light scattering (QELS) data were collected to determine
30 the monodispersity, hydrodynamic radius, and molecular weight of PRX-C, β4-FNIII-
31 3, and their equimolar complex (145 µM each). The chromatography was performed
32 using an Agilent 1200-series HPLC system and a Superdex 200 increase 3.2/300GL
33 (GE Healthcare) column with 20 mM Tris, 150 mM NaCl, pH 7.4 as mobile phase.
34 Protein samples of 160 – 250 µg were injected into the column at an isocratic flow of
35 0.04 ml/min, and light scattering was recorded using a Wyatt miniDAWN HELEOS-II
36 instrument with 18 detectors and a QELS module at ambient temperature. The
37 refractive index was measured using a Wyatt Optilab T-rEX refractometer and used
38 as the concentration source. All data were analyzed using the ASTRA software
39 (Wyatt).

40 **Protein crystallography**

41 β4-FNIII-3 was crystallized using sitting-drop vapor diffusion in drops consisting of
42 150 nl protein solution (12.7 mg/ml in SEC buffer) mixed with 150 nl of reservoir
43 solution. Initially, crystals formed in a wide variety of PEG-based conditions in PACT
44 Premier and JCSG+ (Molecular Dimensions) crystal screens. Optimized crystals used

1   for diffraction data collection were grown at 20 °C in conditions containing 16 – 22%
2   (w/v) PEG 3350 and 180 – 240 mM NH_4NO_3.

3   Prior to diffraction data collection, the crystals were cryoprotected briefly by adding
4   1.5 µl of cryoprotectant solution directly into the sitting drop mix. The
5   cryoprotectant consisted of 75% (v/v) well reservoir and 25% (v/v) PEG 400. After
6   soaking in cryoprotectant, crystals were mounted in loops and snap-frozen in liquid
7   N_2.

8   X-ray diffraction data were collected at 100 K on the P13 beamline, EMBL/DESY,
9   Hamburg, Germany (44) and the ID30A-1 beamline at ESRF (Grenoble, France)
10   (45,46). Data were processed using XDS (47). Phasing was performed with molecular
11   replacement using the human β4-FNIII-3 structure (PDB ID 4wtw) (48) as the search
12   model in Phaser (49). The structure was refined in phenix.refine (50), and model
13   building was done in Coot (51). The low solvent content of 36% (including ordered
14   water) leads to a higher-than-average difference between R_{work} and R_{free} during
15   structure refinement.

16   The structure was validated and analyzed using DSSP (52), MolProbity (53), PyMOL,
17   PDB2PQR (54), APBS (55), and UCSF Chimera (56). The crystal structure was
18   subjected to atomistic molecular dynamics simulations for 550 ns in YASARA (57),
19   essentially as described (58).

20   **Isothermal titration calorimetry (ITC)**

21   PRX-C and β4-FNIII-3 were dialyzed into HBS overnight to ensure matching buffer.
22   The proteins were passed through a 0.22-µm filter, and concentrations were
23   checked. ITC was performed at 25 °C using a Malvern MicroCal iTC200 calorimeter
24   with reference power set to 5 µcal/s. 680 µM β4-FNIII-3 was titrated into 350 µl of
25   68 µM PRX-C under constant stirring. A total of 38 1-µl injections were performed,
26   with a 240-s waiting period between injections. Data were analyzed using Origin. The
27   titration experiment was repeated twice with two separate protein batches and
28   nearly identical values were obtained in each case.

29   **Thermal stability assays**

30   Thermal stability experiments were performed for 0.25 – 2 mg/ml β4-FNIII-3 and a
31   20 µM equimolar complex of PRX-C and β4-FNIII-3 in SEC buffer using a label-free
32   fluorescence-based approach (nanoDSF). The instrument used was a NanoTemper
33   Prometheus NT.48 nanoDSF with a backscattering option to detect aggregation
34   onset. Each 10-µl sample was loaded inside a glass capillary, and a constantly
35   monitored scan from 20 – 95 °C using a 2 °C/min ramp rate was performed.
36   Fluorescence at emission wavelengths 330 nm and 350 nm (F_{330} and F_{350},
37   respectively) was monitored, and a single transition event was observed in the
38   fluorescence ratio (F_{350}/F_{330}) in all samples. Melting temperature midpoint (T_m)
39   values were extracted from the 1st derivative peak maximum.

40

41

1　**Results**

2　**Identification of the cytoplasmic domain of β4 integrin as a potential periaxin**
3　**ligand**

4　In order to identify putative protein ligands for the C-terminal segment of L-PRX,
5　which is missing in the presence of the CMT4F R1070X mutation (24,28), we carried
6　out a yeast 2-hybrid screen of a rat sciatic nerve cDNA library with the C-terminal
7　segment, missing upon the R1070X mutation, as bait. As a result, we obtained three
8　separate clones containing the β4-FNIII-3 domain (Figure 1A). One of these (clone
9　62B) was further used in confirmatory experiments.

10　The interaction was confirmed and the binding site coarsely mapped by using
11　different domains of L-PRX in the screen, with the obtained β4 integrin construct 62B
12　as bait (Figure 1A). The results indicate that the last ~350 residues, containing the
13　acidic region of L-PRX, are crucial for the interaction in this system. Interestingly,
14　both PRX(996-1165) and PRX(1168-1383) gave negative results, suggesting that the
15　binding site may be located close to the point separating these two constructs, or
16　that the site might be a combination of more than one segment required for high-
17　affinity binding.

18　**Evidence for β4 integrin-PRX interaction in tissues and cells**

19　As the next step, GST pulldowns from sciatic nerve lysates were carried out using a
20　GST-β4-FNIII-3 construct. Western blotting identified L-PRX in the fraction bound to
21　the GST fusion protein, but not GST alone (Figure 1B). DRP2 was also identified in the
22　fraction pulled down by GST-β4-FNIII-3, while GST-β4-FNIII-4 was unable to pull
23　down PRX or DRP2 (Supplementary Figure 1). Immunoaffinity pulldowns were
24　further carried out using an anti-PRX antibody, and an analysis of the fractions
25　showed copurification of L-PRX and β4 integrin (Figure 1C), when SDS - breaking up
26　protein complexes - was not used during extraction.

27　The putative interaction between L-PRX and β4 integrin was further studied using co-
28　immunoprecipitation from cultured HEK293 cells overexpressing both proteins. After
29　immunoprecipitation with either β4 integrin or PRX antibodies, both proteins were
30　observed in the eluted fraction, showing they are present in the same complex
31　(Figure 1D).

32　To observe the localization of PRX and β4 integrin in myelinating glial cells,
33　immunofluorescence staining of mouse sciatic nerves was carried out. The result
34　confirms earlier studies (9,59,60), in that both PRX and β4 integrin are localized at
35　the outermost membrane layer of myelin (Figure 1E).

36　**Changes in the L-PRX interactome in a mouse model of CMT4F**

37　Using the mouse model for CMT4F (24), which carries a truncating mutation
38　mimicking human L-PRX with the R1070X mutation, we carried out proteomics
39　experiments to follow the expression level of possible PRX ligand proteins in this
40　model. PRX was immunoprecipitated from mouse nerves after crosslinking, and the
41　bound proteins were analyzed. The levels of DRP2 were decreased in the PRX
42　interactome of these mutant animals (24). In addition, a several-fold drop in the
43　level of β4 integrin could here be observed in the mutant compared to the wild-type

1 animals based on data from the same experiment (Figure 1F). The result provides
2 strong evidence for a functional protein complex involving L-PRX and β4 integrin in
3 mouse peripheral nerve myelin *in vivo*.

4 **Complex of purified recombinant β4 integrin and PRX**

5 The above experiments provided evidence for a direct interaction between L-PRX
6 and β4 integrin in myelinating Schwann cells. We conducted a biophysical
7 characterization of the interaction using recombinant PRX-C and β4-FNIII-3.

8 Pulldown experiments with the purified components confirmed the direct molecular
9 interaction suggested by the data above. His-tagged β4-FNIII-3 pulled down PRX-C
10 (Figure 2A). Pulldown of a partially degraded PRX-C sample showed that essentially
11 all PRX fragments on the SDS-PAGE gel were pulled down by β4-FNIII-3 (Figure 2B);
12 MS analysis indicated that the shortest fragments were missing the very C-terminal
13 regions, while the acidic domain was well-covered in all fragments (data not shown),
14 suggesting that the N-terminal half of the PRX-C construct contained the binding site.

15 SEC-MALS and QELS experiments verified the interaction *in vitro*, whereby the
16 observed complex mass fit to a 1-to-1 complex, with an increased hydrodynamic
17 radius (R_h) compared to the individual interaction partners alone (Figure 2C, Table 1).
18 R_h of the complex was only slightly higher than that for PRX-C, indicating that the
19 complex remained in an extended overall conformation; this is also reflected in the
20 very small change in SEC elution volume. As β4-FNIII-3 is small and tightly folded, this
21 implicates that the PRX-C segment does not become compact upon complex
22 formation, which would be indicated by a decreased R_h.

23 A dissociation constant of 1.7 ± 0.1 µM was obtained for the protein-protein
24 complex using ITC, with a binding stoichiometry of ~1 (Figure 2D), being in good
25 corroboration with the light scattering data above. Hence, the C-terminal region of L-
26 PRX and β4-FNIII-3 bind each other with a low micromolar affinity, in a complex tight
27 enough to survive *e.g.* separation by SEC. The stoichiometry suggests that the
28 interaction site is either distinct from the repeat sequences in L-PRX, or that
29 maximally one β4 integrin molecule can bind to PRX-C even if the binding site would
30 involve the repeats.

31 Covalent crosslinking and mass spectrometry were used in an attempt to map the
32 binding site in more detail. An additional band containing both proteins was
33 apparent on SDS-PAGE after crosslinking (Figure 2E). MS analysis of tryptic peptides
34 from this band confirmed the presence of both PRX-C and β4-FNIII-3.

35 Thermal stability of the proteins was studied using label-free differential scanning
36 fluorimetry (nanoDSF), whereby the signal came from the folded β4-FNIII-3 domain.
37 β4-FNIII-3 has the same melting point (+70°C) in the presence and absence of PRX-C
38 (Table 2), suggesting no large structural changes are induced by PRX-C binding.

39 **Structural insights into the protein-protein complex**

40 While PRX-C is predicted to contain segments of β strand, the purified protein is
41 essentially unfolded, as shown by SRCD spectroscopy (Figure 3A, Table 2). The β
42 strand predictions coincide with repeats in the sequence (Figure 3B), and they could

1 be of functional relevance in ligand protein binding and induction of secondary
2 structures in PRX-C.

3 SRCD was used to shed light on possible changes in secondary structure content
4 accompanying complex formation. The spectra clearly show that the overall
5 secondary structure content remains identical in the complex, compared to the two
6 proteins in isolation (Figure 3A). Together with the SAXS data (see below), SRCD
7 shows that upon β4-FNIII-3 binding, the PRX C-terminal segment does not obtain
8 large amounts of folded structure. Heat denaturation experiments by SRCD
9 confirmed the same melting point for β4-FNIII-3 in the presence and absence of PRX-
10 C (Table 2).

11 To aid in structural modelling and understanding the interactions in the complex, we
12 solved the high-resolution crystal structure of the rat β4-FNIII-3 domain (Figure 4,
13 Table 3). The structure is similar to the corresponding human protein (48), consisting
14 of a β sandwich made of 7 β strands. In two of the four monomers in the asymmetric
15 unit, the His tag could be partially seen, being in different conformations (Figure 4).

16 The purified PRX-C was subjected to SAXS experiments to gain more insight into
17 flexibility, molecular dimensions, and conformational ensembles. These experiments
18 indicate that PRX-C behaves much like a random polymer chain and is highly
19 extended (Figure 5, Table 1).

20 While SAXS indicated a highly disordered nature for PRX-C alone, we wanted to see,
21 whether it becomes more ordered in the complex with β4-FNIII-3. The β4-FNIII-3
22 crystal structure was used to model the solution structure of the complex, which
23 cannot be crystallized due to the flexible nature of PRX and the fact that the exact
24 binding site remains unknown. SAXS analysis of the protein complex, based on a SEC-
25 SAXS experiment, shows that PRX-C remains elongated, and extra density
26 corresponding to the size of β4-FNIII-3 appears close to one end of the complex
27 (Figure 5D,E). This is in line with the results from SEC-MALS (see above).

28 The structure of β4-FNIII-3 can be used to predict the binding site for PRX. A surface
29 analysis of the domain indicates an elongated hydrophobic groove lined by β strands
30 4 and 5 (Figure 4,6A), which could accommodate a linear motif, possibly in a β
31 conformation; this could extend the smaller β sheet from 3 to 4 β strands. MD
32 simulations of the structure further show that this region is the most mobile
33 segment of β4-FNIII-3, and the cavity can open even more (Figure 6B-D).
34 Furthermore, although the FNIII-3 and -4 domains of β4 integrin are rather
35 homologous, sequence conservation in the possible binding site is very low - in line
36 with the observation that FNIII-4 does not bind to PRX (Supplementary Figure 1).

37

1 **Discussion**

2 At the cellular level, myelin formation involves a substantial amount of lipid and
3 protein synthesis and their subsequent assembly into a multilayered, tightly packed
4 proteolipid multilayer. The molecular interactions involved in this process can be
5 roughly divided into those involved in the formation of the compact myelin
6 compartment, and into those relevant for non-compact myelin. This division does
7 not imply that a single protein cannot take part in both aspects; for example, MBP
8 functions both in membrane packing and in the regulation of cytoplasmic channels.

9 Both PRX and β4 integrin play important roles in peripheral nerve myelination
10 (11,15,25). Recently, it was suggested that PRX may be a ligand for β4 integrin (61),
11 and the interaction has been highlighted before (16), but not studied in detail. We
12 set out to confirm this putative direct molecular interaction, and to obtain 3D
13 structural data on the protein-protein complex formed by PRX and β4 integrin.

14 **L-PRX binds β4 integrin directly with high affinity**

15 Integrins are involved in myelination by both oligodendrocytes (CNS) and Schwann
16 cells (PNS). In Schwann cells, the two main isoforms of integrin are α6β1 and α6β4.
17 While α6β1 is expressed in the early stages of myelination and is crucial for the radial
18 sorting of axons (62,63), α6β4 is predominant in the late stages and mature myelin
19 (9,59). This indicates a developmental switch during myelination, and suggests β4
20 integrin is important for myelin maturation and maintenance. The levels of the
21 Schwann cell laminin receptors integrin α6β1, α6β4 and dystroglycan are all
22 regulated by the transcriptional co-activators Yap and Taz (63). The two basal lamina
23 receptors of mature myelin, α6β4 integrin and dystroglycan, known to be together
24 responsible for myelin stability (11), are according to our results bound to the same
25 scaffold protein, L-PRX, in cytoplasmic membrane appositions of the Schwann cell
26 abaxonal plasma membrane.

27 We have shown here with a collection of methods that L-PRX binds, through its C-
28 terminal region, directly to β4-FNIII-3 with high affinity. Considering the presence of
29 both proteins in a large protein scaffold at the Schwann cell outermost membrane, it
30 is likely that the affinity is even higher, when full-length proteins interact and avidity
31 is increased by *e.g.* protein clustering and oligomerization. PRX could function as a
32 ruler between integrins and dystroglycans on the cell surface; on the other hand,
33 changes in the clustering of these molecules, *e.g.* through changes in the basal
34 lamina, might affect the conformation of PRX as well as its cytosolic interactions.

35 Similarly to the reduction seen here in the PRX interactome of β4 integrin in mice
36 with truncated L-PRX, corresponding to human CMT4F mutation R1070X (24), a
37 recent study (61) showed the loss of PRX from the β4 integrin interactome in mice
38 with β4 integrin deficiency. It was thereby suggested that PRX might link β4 integrin
39 functionally to PMP22 (61), but a direct or indirect interaction between PRX and
40 PMP22 remains to be detected.

41 **L-PRX is intrinsically disordered**

42 The C-terminal segment of L-PRX is highly flexible and intrinsically disordered, as
43 shown by structure predictions, SRCD spectroscopy, and SAXS. The potential of
44 intrinsically disordered regions in mediating specific protein interactions has been

13

1 recently highlighted by a number studies (64–66). Such complexes involve usually
2 the recognition of short linear motifs by a folded binding partner. In the current case,
3 PRX-C remains highly flexible even upon β4 integrin binding. Considering the ratio
4 R_g/R_h, the complex, having a lower ratio, is likely to be somewhat less flexible than
5 PRX-C alone. Molecular flexibility may be important for the supramolecular
6 organization of the protein scaffold at the outermost layer of PNS myelin, whereby
7 PRX plays a central role.

8 Intriguingly, many of the myelin-specific proteins have a high degree of intrinsic
9 disorder, as well as specific interactions, which makes myelin an interesting case for
10 studying biomedically relevant intrinsically disordered proteins and their
11 interactions. A well-studied example is the myelin basic protein, which is intrinsically
12 disordered in solution, but partially folds upon membrane binding (67,68). MOBP
13 and the cytoplasmic domain of P0 are predicteded to be disordered (69), and the
14 latter has been experimentally shown to be intrinsically disordered in solution
15 (70,71). The cytoplasmic domain of the large myelin-associated glycoprotein is an
16 intrinsically disordered protein, but forms a specific heterotetramer upon dynein
17 light chain binding, which is likely to help in dimerization and affect binding to the
18 axonal surface (66).

19 In addition to the C-terminal segment studied here, most of the L-PRX-specific region
20 is predicted to be intrinsically disordered. Hence, a monomer of L-PRX, assumed to
21 be mainly in random coil conformation, could reach a length of >30 nm (72), which
22 would be nearly doubled in the case of an L-PRX dimer formed through domain
23 swapping at the N-terminal PDZ-like domain (22). PRX may thus be able to act as a
24 molecular ruler and mediate protein interactions across partners distributed widely
25 across the Schwann cell abaxonal membrane. The distance between bound DRP2
26 and β4 integrin can be estimated to be up to ~25-30 nm, depending on the exact
27 binding site and the conformation of L-PRX between the binding sites. The flexibility
28 of L-PRX should allow it to stay bound to both protein complexes, even in the case
29 they rearrange on the membrane.

30 **Insights from structure of the protein complex**

31 The binding site for β4 integrin in L-PRX remains unknown, although the mapping in
32 this study has suggested that the acidic region of L-PRX is involved. A sequence
33 alignment of this region (Figure 3B) from different species shows that only a few
34 segments are highly conserved; it is conceivable that these regions have functional
35 relevance and they could form the binding site. Further studies will be required to
36 obtain higher-resolution structural data to fully understand the binding interactions.

37 Typical modes of target protein binding by FNIII domains include transient opening
38 and domain swapping (73). It is possible that some of the conserved segments in L-
39 PRX are involved in a domain-swapping rearrangement of β4-FNIII-3. Such a
40 mechanism would be compatible with the lack of change in secondary structure
41 content upon complex formation, which we observed in SRCD experiments. High-
42 resolution structural studies should answer many of the currently open questions,
43 which will require the identification of the exact binding site(s).

44 Considering the structures of both L-PRX and β4 integrin, it is possible that additional
45 binding surfaces are present in both proteins. β4 integrin has four FNIII domains in

1   its cytoplasmic domain, and the C-terminal region of L-PRX is large and flexible,
2   containing repetitive sequences. The fourth FNIII domain of β4 integrin does not
3   bind to PRX-C (Supplementary Figure 1), however, and it is important to recapitulate
4   that the stoichiometry of the interaction between PRX-C and β4-FNIII-3 was
5   observed here to be 1:1.

6   **Relevance for understanding demyelinating disease**

7   A number of proteins have been characterized as targets for mutations in human
8   hereditary neuropathies, whereby the structure of the myelin sheath is
9   compromised. Detailed knowledge about protein interactions in myelin is required
10   to accurately understand the molecular bases of such diseases. One such protein is
11   PRX, which is expressed as two isoforms, S- and L-PRX. While S-PRX consists of only a
12   dimeric PDZ-like domain and a short tail, L-PRX dimerizes through the N terminus
13   similarly to S-PRX (22), but has in total ~1400 residues. Little is known about the
14   structure and function of these long, repetitive L-PRX-specific segments. Expression
15   of S-PRX may result in PRX heterodimerization (23) and affect the regulation of the
16   PRX-containing protein scaffold, as S-PRX interacts with neither DRP2 nor β4
17   integrin.

18   Most PRX mutations causing human hereditary neuropathy introduce a premature
19   stop codon into the sequence (74–76). In line with this, patients with such mutations
20   have PRX expressed, but its size is smaller than in normal individuals (29,77). These
21   observations hint at the possibility that an important function of L-PRX during myelin
22   formation and maintenance lies at its C-terminal region. This is intriguing, given our
23   observation that this region of L-PRX is intrinsically disordered. Here, we have shown
24   a tight interaction between this C-terminal region and the third FNIII domain in the
25   β4 integrin cytoplasmic domain – in the case of PRX truncations, such an interaction
26   would be abolished. This is expected to cause larger-scale disturbances in the PRX-
27   related protein scaffold reaching to the basal lamina and may translate to defects in
28   myelin maintenance. Interactions with DRP2 are important for PRX function, but for
29   a stable myelin sheath, links between the Schwann cell cytoplasm and the basal
30   lamina through β4 integrin may be crucial. L-PRX, hence, mediates a two-pronged
31   interaction from the cytoplasmic side through the Schwann cell plasma membrane
32   to the basal lamina (Figure 7), and might have a role in sequestering different types
33   of laminin receptors.

34   Loss of O-glycosylation causes similar phenotypic effects as L-PRX truncation in
35   mouse models of CMT4F (78). Several O-glycosylation sites in PRX were identified,
36   and some of them reside in the C-terminal region, which binds β4 integrin. It is
37   possible that PTMs, including O-glycosylation, regulate PRX interactions with β4
38   integrin in a dynamic manner.

39   Integrin β4 has been highlighted both as a biomarker for Guillain-Barré syndrome
40   (79–81), an autoimmune demyelinating disease of peripheral nerves, and as a
41   central molecule in the entry of *Mycobacterium leprae* into Schwann cells, *via* the
42   basal lamina (82). Leprosy is characterized by peripheral nerve damage initiated by
43   mycobacterial infection of the Schwann cells. It is currently not known, how L-PRX
44   binding to β4 integrin might modulate this process. Our data provide starting points
45   for such studies in the future.

1 **Concluding remarks**

2 The dimeric, highly elongated, flexible L-PRX is capable of acting as a central protein
3 scaffold within non-compact myelin, linking integrins and dystroglycans together,
4 thus bridging together two major protein complexes linking Schwann cells to the
5 extracellular matrix. This could have high relevance in ensuring the necessary
6 stability of membrane appositions that drive the formation of Cajal bands. The PRX-
7 β4 integrin complex is likely to be important in both normal myelination, myelin
8 maintenance, as well as the pathophysiology of neurodegenerative disease.

9

1 **Figure legends**

2 **Figure 1. Identification of an interaction between periaxin and β4 integrin**

3 A. Top: structure of full-length L-PRX and the bait used in a yeast two-hybrid screen
4 using the C terminus of L-PRX (aa 681-1383) fused to the GAL4 DNA-binding domain.
5 Bottom: structure of the intracellular domain of β4 integrin and one (62B) of the
6 three clones recovered from the screen, all of which included the third FNIII domain
7 of β4 integrin.

8 B. Identification of the region of PRX, which binds to β4 integrin. The strength of
9 interaction between the 62B clone and a series of PRX constructs in a yeast two-
10 hybrid β-galactosidase assay was assessed semiquantitatively by the time taken for
11 colonies to turn blue (+++, 30 min; ++, 30–60 min; +, 60–180 min).

12 C. Interaction of β4-FNIII-3 with PRX *in vitro*. GST or a GST-β4-FNIII-3 fusion protein
13 were incubated with a sciatic nerve lysate *in vitro* and any bound L-PRX was detected
14 by Western blotting (IB) using a PRX antibody after SDS-PAGE. The input lane
15 confirms the presence of PRX in the lysate. Coomassie blue staining shows that
16 equivalent amounts of GST and the GST fusion proteins were used.

17 D. Immunoaffinity copurification of β4 integrin and PRX. Detergent extracts of mouse
18 sciatic nerve in the non-ionic detergent Igepal were incubated with beads to which
19 affinity-purified sheep anti-PRX antibodies had been covalently coupled. After
20 extensive washing, bound proteins were eluted and analyzed by SDS-PAGE and
21 Western blotting. The SDS control lane contains proteins that bound to the beads
22 after first solubilizing the nerves in SDS to disrupt protein-protein interactions,
23 followed by dilution of the SDS with Triton X-100.

24 E. Coimmunoprecipitation of β4 integrin and PRX from transfected HEK293 cells. PRX
25 and β4 integrin were detected by Western blot (IB) after immunoprecipitation (IP)
26 with β4 integrin antibodies when β4 integrin, α6 integrin, and PRX were
27 coexpressed. Preimmune serum (PI) did not precipitate either protein. Reciprocally,
28 β4 integrin and PRX were detected by Western blot after immunoprecipitation (IP)
29 with anti-PRX antibodies, when β4 integrin, α6 integrin, and PRX were coexpressed.

30 F. Immunohistochemistry. Both PRX (green) and β4 integrin (red) localize at the
31 abaxonal membrane in mature myelin.

32 G. Quantification of β4 integrin from the immunoprecipitation of crosslinked sciatic
33 nerves from both the full-length PRX transgenic mouse on a PRX null background
34 (n=3) versus C-terminally truncated PRX transgene on a PRX null background (n=3).

35

36 **Figure 2. Direct molecular interaction assays using purified recombinant proteins.**

37 A. Pulldown with pure recombinant proteins on a Ni-NTA affinity matrix. Top, PRX-C
38 and His-tagged β4-FNIII-3; middle, PRX-C alone; bottom; partially degraded PRX-C
39 and His-tagged β4-FNIII-3. Samples: 1, input sample; 2, unbound fraction; 3-5,
40 washes; 6, elution. PRX-C is indicated in red and β4-FNIII-3 in blue. Sizes of molecular
41 weight markers are indicated on the left (kDa).

1   B. Sequence of the PRX-C construct, indicating the presence of acidic and basic
2   repeats. The underlined segment (the acidic domain) can be detected in all the PRX-
3   C bands in the bottom panel of (A).

4   C. SEC-MALS analysis shows molecular mass expected for a 1:1 complex. PRX-C, red;
5   β4-FNIII-3, blue; complex, black.

6   D. ITC titration of the complex indicates 1:1 stoichimetry. ΔH = 6.2±0.06 kcal/mol, ΔS
7   = 5.8 cal/mol°.

8   E. Covalent crosslinking. The complex is only visible, when both proteins have been
9   activated (Lane 1). The magenta arrowhead indicates the 1:1 complex. PRX-C is
10   indicated in red and β4-FNIII-3 in blue. Samples: 1, both proteins activated; 2, PRX-C
11   activated; 3, PRX-C activated, β4-FNIII-3 added; 4, no activation; 5, no activation,
12   PRX-C added; 6, no activation, β4-FNIII-3 added; 7, β4-FNIII-3 activated; 8, β4-FNIII-3
13   activated, PRX-C added.

14

15   **Figure 3. L-PRX remains disordered in the complex.**

16   A. SRCD spectroscopy. PRX-C, red; β4-FNIII-3, blue; complex, black; sum of individual
17   protein spectra, magenta.

18   B. Secondary structure predictions of the PRX-C acidic region, aligned from selected
19   species.

20

21   **Figure 4. Crystal structure of rat β4-FNIII-3.** Two of the 4 individual chains in the
22   asymmetric unit are shown. The chain is coloured from the N (blue) to the C (red)
23   terminus. The His tag is partially resolved at the N terminus, and takes different
24   conformations in different monomers. Secondary structure elements are labelled.

25

26   **Figure 5. Structure of the complex in solution.**

27   A. SAXS scattering curve. PRX-C, red; β4-FNIII-3, blue; complex, black.

28   B. Dimensionless Kratky plot. The black cross indicates the theoretical peak position
29   for a folded, perfectly globular protein.

30   C. Distance distribution function.

31   D. Multi-phase model from MONSA. PRX-C, pink; β4-FNIII-3, blue.

32   E. Rigid-body fit between a chain-like model of PRX-C (pink) and the integrin crystal
33   structure (blue).

34   F. EOM analysis for PRX-C. Dashed lines, theoretical distribution for a random coil;
35   solid lines, the ensemble for PRX-C. Maximum dimension is shown in red and radius
36   of gyration in black. PRX-C is slightly more elongated than a random coil.

37

38   **Figure 6. Possible binding site for L-PRX.**

1   A. Surface of the β4-FNIII-3 crystal structure, coloured by electrostatic potential. The
2   predicted binding site is shown by a green dashed line.

3   B. MD simulation indicates the possible binding site is flexible (arrow) and may open
4   up more.

5   C. RMSF during the 550-ns simulation.

6   D. Comparison of the start (magenta) and end (yellow/green) points of the
7   simulation. The loop at the potential binding site (arrow) is the most mobile region.
8   The largest peak in the RMSF plot (panel C) is coloured green in the post-simulation
9   structure.

10

11   **Figure 7. Schematic view of the PRX-linked protein scaffold in PNS myelin.**

12

13

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15 Conflicts of interest

16 The authors declare that the research was conducted in the absence of any
17 commercial or financial relationships that could be construed as a potential conflict
18 of interest.

19 Author contributions

20 PB, DS, and PK conceived of the project. DS and PB carried out and analyzed yeast
21 two-hybrid, immunochemical, and proteomics experiments. AR and HL performed
22 experiments with purified proteins. AR and PK performed analysis of protein
23 structure. AR, DS, and PK drafted the manuscript. All authors contributed to
24 manuscript revision.

25 Ethical statement

26 All animal work conformed to United Kingdom legislation (Scientific Procedures) Act
27 1986, and to the University of Edinburgh Ethical Review Committee policy.

28 Data availability statement

29 Crystallographic data are available at the Protein Data Bank, under the accession
30 code 6HYF. The raw data supporting the conclusions of this manuscript will be made
31 available by the authors, without undue reservation, to any qualified researcher.

32

1 **Table 1. Characterization of the PRX-C/β4-FNIII-3 complex mass and size.**
2

	MS	SEC-MALS			QELS	SEC-SAXS	
	M_r (measured), Da	M_r (measured), kDa	M_r (theoretical), kDa	% of expected	R_h (nm)	R_g (nm) Guinier/ Debye/ Theoretical denatured,IDP/EOM	D_{max} (nm) GNOM/ Theoretical/EOM
PRX-C	35869	41.00 ± 0.02	35.87	114	4.79 ± 0.08	5.68 ± 0.04/ 6.21/ 6.75,5.39/6.14	23.0/ 16.9/ 19.6
β4-FNIII-3	12756	14.30 ± 0.02	12.76	112	2.15 ± 0.04	2.11 ± 0.01/- /-/-	9.8/-/-
complex	-	48.27 ± 0.04	48.63	99	5.22 ± 0.08	5.69 ± 0.06/ 6.11/-,-/-	25.2/-/-

3
4
5
6
7 **Table 2. Folding and thermal stability of PRX-C and β4-FNIII-3.**
8

	SRCD deconvolution*			Secondary structure prediction**			T_m, °C	
	% helix	% strand	% other	% helix	% strand	% other	SRCD	nanoDSF
PRX-C	3 3	38 31	58 66	0	7.7	92.3	-	-
β4-FNIII-3	1 2	45 50	51 49	0 (0)	37.2 (34.5)	62.8 (65.5)	59.5 ± 0.6	69.8 ± 0.1
complex	2 3	39 35	56 61	0	14.9	85.1	58.8 ± 0.7	70.3 ± 0.1

9 * Deconvolved using DichroWeb (values above), or with BeStSel (values below).
10 ** Predicted from primary structures using JPred. Values in brackets were extracted
11 from the crystal structure using DSSP. The complex values were calculated from the
12 JPred predictions, assuming a 1:1 complex.
13

14

1　　**Table 3. Crystallographic data collection and refinement statistics.**

2　　Values in parentheses correspond to the highest-resolution shell.

Data collection statistics	
Beamline	ID30A-1 (ESRF)
Space group	$P2_12_12_1$
Unit cell a,b,c (Å)	51.22, 52.84, 144.53
Wavelength (Å)	0.966
Resolution (Å)	50-1.60 (1.64-1.60)
Unique reflections	51757 (3746)
Multiplicity	3.3 (3.3)
Completeness (%)	98.3 (98.6)
R_{merge} (%)	6.1 (239.2)
R_{meas} (%)	7.3 (283.9)
<I/σ(I)>	8.8 (0.6)
$CC_{½}$ (%)	99.8 (16.6)
Wilson B (Å^2)	39.2
Refinement statistics	
R_{cryst} (%)	19.0
R_{free} (%)	23.9
RMSD bond length (Å)	0.013
RMSD bond angle (°)	1.1
Mean B value (Å^2)	49.7
Ramachandran plot	
Res. in favoured regions (%)	95.9
Outlier residues (%)	0.8
MolProbity score/percentile	2.05 / 44th
PDB entry	6HYF

3

4

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45
46
47

A

Periaxin: PDZ, B, Repeat, Acidic

Prx 681-1383

β4 integrin intracellular domain: Calx-β, FNIII-1, FNIII-2, FNIII-3, FNIII-4

62B
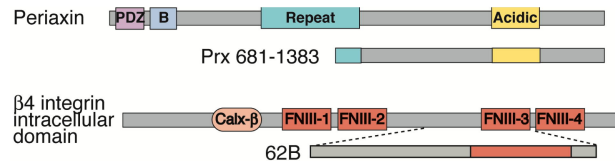
	βgal
Prx 762-1383	+++
Prx 996-1383	+++
Prx 1036-1383	+++
Prx 996-1165	−
Prx 1168-1383	−

B

Input, GST, FN3/GST

150—

IB: Periaxin

37—
25—

Coomassie blue

C

Input, SDS, IAP

250—
150—

IB: β4 integrin

250—
150—

IB: Periaxin

D

Input | IP: β4 PI β4

α6 integrin
β4 integrin
Periaxin

250—
150—

IB: Periaxin

Input | IP: Periaxin

250—
150—

IB: β4 integrin

250—
150—

IB: β4 integrin

250—
150—

Periaxin

E

PRX
β4

F

normalized abundance

3×10⁸
2×10⁸
1×10⁸

PrxTg/Prx⁻/⁻   ΔCPrxTg/Prx⁻/⁻

A

Lanes 1 2 3 4 5 6

B

```
LKIPEVELVTPGAQETEKVTSGVKPSGLQVSTTRQVVAEGQEGAQRVSSLG
ISLPQVELASFGEAGPEIAAPSAEGTVGSR-------------FKMPT
IQVPQVMLELPGTQVAGGDLLVGEGI----------LKLKLPTLGAGGKGEGAEAQSPEAQHTFH
VTVPQLELDVGLGHEAQAGETAKSEGG---------LKVRLPLFGLARAKEGIETGEKVKS
ISLPDVELTSPVSSHAEYQVVEGDGDGGHK---------PKLRLPRVGLSQSESASGEGSPSPEEEEEGSGEGASGRRGR
---------------------VRVRLPRVGLASPSKGSKGGQDAASKSPVGEKS
---------------------PKFRFPRVSLSPKARSGSKDREEGG
---------------------FRVRLPSVGFSETAAPGSARIEGTQAAAI
```

acidic   basic

C

light scattering (relative) — MW (kDa)
elution volume (ml)

D

heat (kcal/mol)
molar ratio

E

Lanes 1 2 3 4 5 6 7 8

A

B

A

B

C

D