Published in final edited form as:

Matrix Biol. 2010 June ; 29(5): 357–368. doi:10.1016/j.matbio.2010.02.006.

Fibrillin-2 is dispensable for peripheral nerve development, myelination and regeneration

Michael A. Chernousov^{a,1}, Kelly Baylor^{a,2}, Richard C. Stahl^a, Mark M. Stecker^{a,2}, Lynn Y. Sakai^b, Sui Lee-Arteaga^c, Francesco Ramirez^c, and David J Carey^a

^a Weis Center for Research, Geisinger Clinic, 100 North Academy Avenue, Danville, PA 17822-2613, USA

^b Shriners Hospital for Children, Oregon Health and Science University, Portland, Oregon 97239, USA

^c Department of Pharmacology and Systems Therapeutics; Box 1603 Mount Sinai School of Medicine; One Gustave Levy Place; New York, NY, 10029, USA

Abstract

The extracellular matrix of peripheral nerve is formed from a diverse set of macromolecules, including glycoproteins, collagens and proteoglycans. Recent studies using knockout animal models have demonstrated that individual components of the extracellular matrix play a vital role in peripheral nerve development and regeneration. In this study we identified fibrillin-1 and fibrillin-2, large modular structural glycoproteins, as components of the extracellular matrix of peripheral nerve. Previously it was found that fibrillin-2 null mice display joint contractures, suggesting a possible defect of the peripheral nervous system in these animals. Close examination of the peripheral nerves of fibrillin-2 deficient animals described here revealed some structural abnormalities in the perineurium, while general structure of the nerve and molecular composition of nerve extracellular matrix remained unchanged. We also found that in spite of the obvious motor function impairment, fibrillin-2 null mice failed to display changes of nerve conduction properties or nerve regeneration capacity. Based on the data obtained we can conclude that peripheral neuropathy should be excluded as the cause of the impairment of locomotory function and joint contractures observed in fibrillin-2 deficient animals.

Keywords

fibrillin; extracellular matrix; peripheral nerve

1. INTRODUCTION

Fibrillin-1 and -2 are large multidomain glycoproteins and the structural components of extracellular microfibrils that impart physical properties to a large variety of tissues, alone or together with elastin as elastic fibers, and that modulate cell performance during organ growth

¹Corresponding author: Phone: 570-271-5361, FAX: 570-271-6701, mchernousov@geisinger.edu.

²Present address: Department of Neuroscience, Joan C. Edwards Marshall University School of Medicine, 1600 Medical Center Drive, Suite G500, Huntington, WV 25705, USA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

and tissue remodeling by sequestering TGF β and BMP complexes and interacting with integrins and cells surface proteoglycans (Ramirez and Dietz, 2009). In spite of being part of the same extracellular macro-aggregates, structural mutations in fibrillin-1 and fibrillin-2 are causally associated with distinct human conditions (Marfan syndrome (MFS) and congenital contractual arachnodactyly (CCA), respectively) that are in part replicated in the discrete phenotypes of mice lacking fibrillin-1 (*Fbn1*) or fibrillin-2 (*Fbn2*) gene expression (Ramirez and Dietz, 2009). Like CCA patients, *Fbn2*^{-/-} mice display transient contractures of small joints, persistent contractures of large joints and reduced bone mass, in addition to a bone-patterning defect, bilateral syndactyly, which was genetically linked with impaired BMP signaling and is observed in neither CCA patients nor *Fbn1*^{-/-} mice (Arteaga-Solis et al. 2001; Carta et al. 2006; Viljoen, 1994;). Whereas syndactyly is in line with the broad profile of *Fbn2* activity in embryonic tissues, bone and joint manifestations are consistent with histological evidence that fetal and early postnatal expression of the protein is predominantly restricted to tendon/ligament, perichondrium, bone and peripheral nerves (Boregowda et al. 2008; Charbonneau et al. 2003; Quondamatteo et al. 2002; Ritty et al. 2003; Zhang et al. 1995).

The present study was designed to investigate the formal possibility that joint contractures in *Fbn2*^{-/-} mice (and by extrapolation in CCA patients) may be accounted for by neurological abnormalities that affect peripheral nerve development and/or myelination. To this end, *Fbn2*^{-/-} mice were subjected to a series of behavioral and electrophysiological tests, as well as extensive immunohistological analyses. Although architectural abnormalities were identified in the mutant perineurium, these experiments revealed no major defects in peripheral nerves, neuromuscular junctions (NMJ) or nerve conduction properties that could explain the large joint contractures and severe locomotory impairment of *Fbn2*^{-/-} mice. Hence, mechanism(s) other than peripheral neuropathy is (are) responsible for these manifestations in CCA and mouse models of the disease.

2. RESULTS AND DISCUSSION

Like CCA patients, newborn *Fbn2*^{-/-} mice display small joint contractures that disappear within the first few days of postnatal life and large joint contractures that persist throughout adulthood (Arteaga-Solis et al. 2001). While being lifted by the tail, *Fbn2*^{-/-} mice retracted the hindlimbs toward their body whereas wild type (WT) and *Fbn2*^{+/-} littermates extended their limbs downward (Fig. 1A and B). We have also occasionally seen forelimb flexion in tail-suspended *Fbn2*^{-/-} mice. To assess possible functional deficits in forelimbs of *Fbn2*^{-/-} animals, a wire hanging test was performed. We found that *Fbn2*^{-/-} mice exhibited a significant deficit in the ability to hang on the wire relative to WT or *Fbn2*^{+/-} littermates (Fig. 1C; $p < 0.0001$). These data suggest that a possible neurological defect, likely but not exclusively affecting the peripheral nervous system, is involved in the phenotype of fibrillin-2 deficient animals. To further explore this possibility, mice were subjected to a Rotarod test that measures balance and motor coordination. Consistent with motor function impairment, *Fbn2*^{-/-} mice performed significantly worse than WT or *Fbn2*^{+/-} littermates (Fig. 1D; $p < 0.0001$). By contrast, a Hot plate analgesia test did not reveal appreciable differences between mice of the three different genotypes, indicating that the nociceptive sensory function was not altered in *Fbn2*^{-/-} animals (Fig. 1E). To analyze whether *Fbn2*-deficient mice have a defect in proprioceptive sensory organs, cross-sections of soleus muscle of adult *Fbn2*^{-/-} and WT mice were dual stained using antibodies to slow-tonic myosin heavy chain, a marker of muscle spindles, and to collagen type IV, which encapsulates muscle spindles. Muscle spindles could readily be detected in the muscles of both WT and *Fbn2*^{-/-} animals, and no apparent differences in the amount or distribution of the spindles were found (Fig. 2, A–D). Consistent with that, no differences were also detected when sections of cervical (Fig. 2, E, F) or lumbar (data not shown) dorsal root ganglia of WT and *Fbn2*^{-/-} mice were stained using antibody to

proprioceptive neuronal marker parvalbumin. Collectively, these data suggest the absence of both nociceptive and proprioceptive sensory defects in fibrillin-2 deficient animals.

Based on the above evidence, the profiles of fibrillin-1 and fibrillin-2 immunostaining were compared in the hindlimbs obtained from newborn (P4) *WT* and *Fbn2*^{-/-} mice. The analyses revealed strong fibrillin-1 immunoreactivity in the nerves as well as surrounding muscle and connective tissues of both *WT* and *Fbn2*^{-/-} animals. By contrast, fibrillin-2 was almost exclusively expressed in the nerves of newborn *WT*, but not *Fbn2*^{-/-} mice (Fig. 3, A–D). Expression of fibrillins in postnatal (P7) and adult sciatic nerve of *WT* mice was also examined. We found that the expression of fibrillin-1 in the nerve persisted throughout development and in adulthood. In particular, a substantial amount of fibrillin-1 was noted in the perineurium and in cable-like structures located in the endoneurium of P7 and adult nerves (Fig. 3E and G, see also Fig. 7I and J). By contrast, fibrillin-2 was not detectable either in P7 or in adult nerves (Fig. 3F and H). These results were consistent with previous data on the expression of *Fbn2* in fetal and postnatal human nerves and with the earlier suggestion that continued and robust *Fbn1* expression during postnatal and adult life may bury fibrillin-2 molecules (which are mostly produced during fetal/early postnatal life) within the growing microfibrils (Charbonneau et al. 2003; Zhang et al. 1995).

To learn more about the nature of fibrillin-1-positive cable-like structures in the endoneurium, dual staining of sciatic nerve sections using anti-fibrillin-1 and anti-neurofilament antibodies was carried out. No co-localization of these proteins was detected, suggesting that fibrillin-1 containing structures in the nerve are not part of, or directly associated with axons (Fig. 3, I–K). To characterize longitudinal distribution of fibrillin-1 in the nerve, teased nerves obtained from adult *WT* mice were dual stained using antibodies to fibrillin-1 and $\alpha 2$ chain of laminin, which is present in basal lamina tubes surrounding axon-Schwann cells units in the nerve (Occhi et al. 2005). The staining revealed fibrillin-1-positive, laminin-negative fibrillar structures that were loosely associated with basal lamina tubes. On the contrary, the basal lamina tubes, which were strongly laminin-positive, showed weak fibrillin-1 staining. We could not detect any appreciable accumulation of fibrillin-1 in the areas surrounding nodes of Ranvier, although it was not excluded from these regions (Fig. 4, A–D).

Synaptic basal lamina is another example of specialized extracellular matrix (ECM) associated with the peripheral nervous system. We examined a possible presence of fibrillin-1 in the synaptic basal lamina by dual staining of cross-sections of skeletal muscle of adult *WT* mice using antibodies to fibrillin-1 together with Alexa 594-labeled α -bungarotoxin (rBTX), which binds specifically to acetylcholine receptor (AChR) at NMJs. Although fibrillin-1 was readily detected in extracellular matrix surrounding myofibers, it was not enriched in the synaptic basal lamina adjacent to NMJ (Fig. 4, E and G). That was in contrast with $\alpha 2$ chain of laminin, which was previously shown to be present at synaptic sites (Patton et al. 1997) (Fig. 4, F and H). When the staining of adult teased nerves and skeletal muscle sections was repeated using antibodies to fibrillin-2, no appreciable immunoreactivity was detected (data not shown).

Genetic ablation of some components of the nerve ECM, such as certain laminin isoforms, has been found to cause demyelination of proximal and distal portions of the peripheral nervous system (Wallquist et al. 2005; Yang et al. 2005). Accordingly, Toluidine blue-stained semithin sections of sciatic nerve were examined to assess peripheral nerve myelination of *Fbn2*^{-/-} mice. Thickness of myelin sheaths appeared to be the same in mutant and *WT* animals, and no obvious signs of dysmyelination were found (Fig. 5A and B). Likewise, ultrastructural analysis of ultrathin nerve sections also failed to reveal myelin sheath abnormalities in *Fbn2*^{-/-} mice (data not shown). Dual staining of sciatic nerve sections with anti-neurofilament antibody and antibody to peripheral myelin specific protein P₀ was also carried out. This revealed no obvious peripheral axonal dystrophy or bundles of unmyelinated axons in the adult (Fig. 5G and H, and

inserts) or P13 (data not shown) mutant nerves. Furthermore, neither myelin nor axonal abnormalities were found in immunostained spinal roots of *Fbn2*^{-/-} mice (data not shown). To assess the longitudinal structure of myelin, teased nerves of adult *WT* and *Fbn2*^{-/-} mice were stained with a panel of antibodies to nodal (Na⁺ channel, ankyrin G), paranodal (E-cadherin) and juxtaparanodal (Kv1.1 channel) markers. No differences in the level or organization of the nodal proteins between *WT* and *Fbn2*^{-/-} animals were found (Fig. 6). Collectively, these data ruled out the possibility that loss of fibrillin-2 deposition in the nerve ECM negatively affects the myelination process.

Fbn2 inactivation has been shown to cause disorganization of the digital ECM during autopod development (Arteaga-Solis et al. 2001). To investigate whether a comparable defect was observed in the mutant peripheral nerve system, cross-sections of sciatic nerves of *WT* and *Fbn2*^{-/-} mice were stained with a panel of antibodies to various ECM proteins, including collagens type IV and V, fibronectin, laminin and fibrillin-1. Once again, at the light microscopic level these analyses showed no relationship between loss of fibrillin-2 deposition and changes in the levels, expression patterns or organization of these major components of the endoneurial matrix (Fig. 7). However, closer examination of the perineurial ECM did reveal subtle differences between *WT* and *Fbn2*^{-/-} nerves. These differences included a less compact perineurium in the mutant with the occasional presence of small cavities between the perineurium and endoneurium (Fig. 8, arrows in B and F). Electron microscopy supported these findings and showed appreciable ultrastructural abnormalities in the mutant perineurium. In contrast to the highly organized lamellar structure of the *WT* perineurium, layers of perineurial cells in the mutant nerves were often of uneven thickness and ruffled (Fig. 8, G and H). These observations suggest that fibrillin-2 participates in the organization of perineurial cells and matrix. That is consistent with the enriched staining of the perineurium of neonatal nerves with anti-fibrillin-2 antibodies (see Fig. 3B).

Other possible causes for the loss of motor coordination in *Fbn2*^{-/-} mice include deficits in NMJs as suggested by the phenotype of mice lacking $\beta 2$ chain of laminin (Noakes et al. 1995). To explore this possibility, cross-sections of gastrocnemius muscle of adult *WT* and *Fbn2*^{-/-} mice were stained with rBTX. We found no appreciable differences in the amount or distribution of rBTX-labeled NMJs in the muscles of *WT* and *Fbn2*^{-/-} mice (Fig. 9A and B). To further characterize the NMJ structure, en face dual staining of the muscle with antibody to SV2 and rBTX, that label pre- and postsynaptic sites, respectively, was carried out. That also failed to reveal overt structural abnormalities in mutant compared with *WT* NMJs (Fig. 9, C–J). These data are consistent with the apparent lack of accumulation of fibrillins in synaptic basal lamina described above.

The last set of experiments addressed whether or not loss of fibrillin-2 negatively impacted nerve function and/or nerve regeneration. Accordingly, compound motor action potentials (CMAP) were measured in *WT* and *Fbn2*^{-/-} mice of different ages. The results showed no effect of *Fbn2* ablation on CMAP conduction velocity or CMAP amplitude (Table 1). However, the CMAP duration was slightly elevated in *Fbn2*^{-/-} mice. In the absence of other conduction abnormalities that suggests that fibrillin-2 has a minor effect on the response of the muscle to stimulation. It was also demonstrated that although conduction velocities increased in the older animals at a rate of roughly 0.11 m/sec/week, there were no differences between the *WT* and *Fbn2*^{-/-} mice. Next, the potential effect *Fbn2* inactivation on peripheral nerve regeneration was examined using the well-established nerve crush injury model. Sciatic nerves of adult *Fbn2*^{-/-} mice and *WT* littermates were crushed and nerve stumps distal to the injury site were examined 7 and 21 days after the injury. Axonal regeneration and remyelination were assessed by staining cross sections of the distal stump using antibodies to neurofilament and P₀. No difference in nerve regeneration and remyelination were detected between *Fbn2*^{-/-} and *WT* mice (Fig. 10).

In summary, our study indicates that motor function abnormalities and joint contractures in *Fbn2*^{-/-} mice can not be accounted for by peripheral neuropathological abnormalities, such as hypomyelination, nodal/paranodal deficits or altered NMJs. Furthermore, the peripheral nerves of mutant animals did not display reduced conduction velocity or impaired regeneration capacity. Therefore, peripheral neuropathy should be excluded as the underlying cause of these particular phenotypes in *Fbn2*^{-/-} mice and CCA patients. Despite this negative outcome, our study has nonetheless provided two new insights into microfibril function. First, it has shown that fibrillins are expressed in postnatal and adult peripheral nerves; second, it has implicated fibrillin-2 in guiding the organization of the perineurial matrix. Lastly this study, together with earlier and ongoing investigations (Charbonneau et al. 2003; Zhang et al. 1995), strongly suggest that fibrillin-1 and fibrillin-2 are differently distributed within mature microfibrils and elastic fibers as a result of discrete expression patterns of these genes during and after fetal development. This postulate has important implications with regard to the timely release of discrete matrix-bound TGFβ and BMP ligands during tissue remodeling and repair.

3. EXPERIMENTAL PROCEDURES

3.1. Animals

Generation and genotyping of *Fbn2*-null mice have been described earlier (Arteaga-Solis et al. 2001). All animal protocols were approved by Institutional Animal Care and Use Committee of Weis Center for Research, Geisinger Health System.

3.2. Wire hanging test

Six *Fbn2*^{-/-} mice, seven *Fbn2*^{+/-} and seven *WT* littermates ranging in age between 8 to 22 weeks were tested in wire hanging performance. Animals were made to grasp the wire suspended above the ground with their forepaws, and the time (in seconds) until the animal fell was recorded. An arbitrary cutoff time of 60 sec was adopted. The test was repeated three times for each animal with 5 min rest between trials. The mean values of three trials were used for analysis.

3.3. Rotarod test

Twenty-four *Fbn2*^{-/-} mice, twenty-two *Fbn2*^{+/-} and fourteen *WT* littermates ranging in age between 20 to 27 weeks were tested using Rotarod and Hot plate tests as described below. Locomotor function was tested on an accelerating Rotarod (Med Associates Inc., St. Albans, VT). The rod was set to accelerate from 3 to 30 rpm over a period of 5 min. A 300 sec cutoff was employed. Performance was measured by the duration time (sec) on the rotating drum. The mice were acclimatized by three training runs 24 hrs before testing. For the testing, the animals were given one warm-up trial followed by two final trials. The animals were allowed to rest for 5–7 min between trials. The mean values on the time spent on the Rotarod during the two final trials were used for analysis.

3.4. Hot plate test

Nociceptive sensory function was tested on a Hot Plate Analgesia Meter (Columbus Instruments, Columbus, OH). Mice were placed on the plate that was maintained at 55°C. The plate was enclosed by plexiglass walls to restrict movement of the mice off the plate surface. Mice were observed until they displayed signs of discomfort characterized by lifting and licking of their paws. A foot-pedal operated timer was used to measure the time from contact with the warm surface to first sign of discomfort. An arbitrary cutoff time of 30 sec was adopted to minimize tissue injury.

3.5. Immunofluorescence

For immunofluorescent staining, sciatic nerves, spinal roots or dorsal root ganglia were isolated, embedded in Tissue-Tek freezing medium, and frozen at -20°C . Cryosections ($8\text{ }\mu\text{m}$ thick) were cut using a Leica CM 1950 cryostat, placed on glass microscope slides, and fixed with 3% paraformaldehyde in PBS for 30 min. For myelin staining, slides were additionally incubated in ice-cold methanol for 15 min. Following a rinse with PBS, sections were blocked for 1 hr in blocking buffer (5% dry milk, 100 mM NaCl, 50 mM Tris-HCl, pH 7.5), and then additionally blocked with PBS containing 10% donkey serum for 1 hr. After that sections were incubated with primary antibodies diluted in blocking buffer for 1.5 hrs in humid chamber at room temperature. The slides were rinsed and incubated with fluorescein isothiocyanate (FITC)- or Rhodamine Red-X(RRx)-conjugated affinity-purified donkey secondary antibodies from Jackson ImmunoResearch for 1 hr. After a final rinsing with blocking solution and then PBS, the sections were overlaid with coverslips using Vectashield mounting solution (Vector Laboratories) and imaged with a Nikon fluorescent microscope.

To visualize neuromuscular junctions, gastrocnemius muscles were dissected from the animals, and $8\text{ }\mu\text{m}$ thick muscle cross sections were prepared as described above. For longitudinal sections, the whole muscles were first fixed for 20 min in 1% paraformaldehyde in PBS at 4°C , rinsed with PBS, sunk in 25% sucrose/PBS for 2.5 hrs at 4°C , frozen and sectioned at $8\text{--}20\text{ }\mu\text{m}$. Muscle sections were stained with Alexa Fluor 594-conjugated α -bungarotoxin (Molecular Probes) alone or in combination with anti-SV2 antibody using the procedure described above.

For teased nerve staining, dissected sciatic nerves were placed in 4% paraformaldehyde in PBS for 10 min and then rinsed with PBS. Teased nerve fibers were prepared, air dried on glass slides for 2 hrs at room temperature and stored at -20°C . The preparations were postfixed/permeabilized by immersion in -20°C acetone for 10 min, blocked at room temperature for 1.5 hrs in blocking buffer containing 10% donkey serum, 0.5% Triton X-100 in PBS, and incubated overnight at 4°C with primary antibodies diluted in blocking buffer. After extensive washing with PBS, nerve preparations were incubated with secondary antibodies, mounted and imaged as described above for the tissue section staining.

The following primary antibodies were used for immunofluorescent staining: rabbit polyclonal antibodies to fibrillin-1 and fibrillin-2 (Charbonneau et al. 2003), heavy chain of neurofilament (Millipore/Chemicon), collagen type IV (a gift from Hynda Kleinman, NIH), fibronectin (Chernousov et al. 1998), collagen type V (Chernousov et al. 2000), Na^{+} channel (Millipore/Upstate), parvalbumin (Swant); chicken polyclonal antibodies to P_0 (Millipore/Chemicon); mouse monoclonal antibodies to ankyrin G (Invitrogen/Zymed Laboratories), E-cadherin (BD Transduction Laboratories), K^{+} channel Kv1.1 (Millipore/Upstate), SV2 and slow-tonic myosin heavy chain (S46) (both from Developmental Studies Hybridoma Bank); rat monoclonal antibody to $\alpha 2$ chain of laminin (Sigma).

3.6. Electron microscopy

Isolated sciatic nerves were perfused with 100 mM sodium cacodylate, pH 7.4 on ice and then fixed by perfusion with 2.5% glutaraldehyde, 2% paraformaldehyde in 100 mM sodium cacodylate, pH 7.4 on ice. Nerves were then postfixed in 2% osmium tetroxide in 100 mM phosphate buffer, dehydrated in ethanol and embedded in Epox812. (Fullam, Latham, NY). To reveal general structure of myelin, semi-thin cross-sections of sciatic nerves were prepared and stained with Toluidine blue and visualized by brightfield light microscopy. For ultrastructural analysis, ultrathin section were cut, placed on copper grids, stained with lead citrate and uranyl magnesium acetate, and examined in JEOL (Peabody, MA) JEM-1200EX transmission/scanning electron microscope.

3.7. Nerve conduction studies

CMAP were generated using platinum needle electrodes (Model E2-48, Astro-Med, Inc., West Warwick, RI) placed into the muscles of the hindpaw. The sciatic nerve was stimulated at multiple locations using similar needle electrodes placed in a tripolar fashion with roughly 2 mm between the electrodes. Stimulation was accomplished with a Grass S88 stimulator connected to a Grass PSIU6 current isolation unit. Stimulation intensity was in the range of 2–15 mA to assure supramaximal stimulation with duration of 0.01 msec (Stecker et al. 2008). All data were recorded and analyzed using software created specially for this purpose using LabWindows/CVI (National Instruments, Austin, TX). The main elements abstracted from each CMAP was the peak to peak amplitude, the onset latency, the latency of the highest peak and an index of CMAP duration taken as the time difference between the highest and lowest CMAP amplitudes. Conduction velocities were estimated from the distance and the onset latency for the CMAP using a synaptic delay of 0.5 msec. These values were confirmed by linear regression of onset latency vs. distance. A total of 162 CMAP's were obtained in 15 *WT* and 15 *Fbn2*^{-/-} mice. Linear regression analysis was carried out to identify effects of age and genotype. ANOVA was also carried out using three age ranges 6–12 weeks, 12–29 weeks, and 30–55 weeks and genotype as independent variables.

3.8. Sciatic nerve crush injury

Adult mice were anaesthetized and the sciatic nerves on both sides were exposed in the mid-thighs. Nerve crush was produced on the one side by tightly compressing the sciatic nerve with small forceps twice, each time for 60 sec, with 30 sec interval. The forceps tips were previously dipped into India ink to mark the crush site. The sciatic nerve on the other side was exposed but not injured and served as a control. Skin incisions were closed with sutures and the animals were allowed to recover for 7 or 21 days. At these times the animals were euthanized and the nerves were harvested and sectioned at the point that was 6 mm distal to the injury site. To evaluate the extent of nerve regeneration and remyelination, the sections were stained with anti-neurofilament and anti-P₀ antibodies, respectively, as described above.

Acknowledgments

We are indebted to Cindy Rhone (Weis Center for Research) for help with laboratory animals. We also thank John Shaw (Weis Center for Research) for assistance with electron microscopy, Noe Charbonneau (Oregon Health and Science University) for help with immunofluorescence and Tanya Orleanskaya (Geisinger Health Sciences Libraries) for editorial assistance. This work was supported by NIH grant AR42044 to F.R.

References

- Arteaga-Solis E, Gayraud B, Lee SY, Shum L, Sakai L, Ramirez F. Regulation of limb patterning by extracellular microfibrils. *J Cell Biol* 2001;154:275–281. [PubMed: 11470817]
- Boregowda R, Paul E, White J, Ritty TM. Bone and soft connective tissue alterations result from loss of fibrillin-2 expression. *Matrix Biol* 2008;278:661–666. [PubMed: 18838118]
- Carta L, Pereira L, Arteaga-Solis E, Lee-Arteaga SY, Lenart B, Starcher B, Merkel CA, Sukoyan M, Kerkis A, Hazeki N, Keene DR, Sakai LY, Ramirez F. Fibrillins 1 and 2 perform partially overlapping functions during aortic development. *J Biol Chem* 2006;281:8016–8023. [PubMed: 16407178]
- Charbonneau NL, Dzamba BJ, Ono RN, Keene DR, Corson GM, Reinhardt DP, Sakai LY. Fibrillins can co-assemble in fibrils, but fibrillin fibril composition displays cell-specific differences. *J Biol Chem* 2003;278:2740–2749. [PubMed: 12429739]
- Chernousov MA, Rothblum K, Tyler WA, Stahl RC, Carey DJ. Schwann cells synthesize type V collagen that contains a novel alpha 4 chain. Molecular cloning, biochemical characterization, and high affinity heparin binding of alpha 4(V) collagen. *J Biol Chem* 2000;275:28208–28215. [PubMed: 10852920]
- Chernousov MA, Stahl RC, Carey DJ. Schwann cells use a novel collagen-dependent mechanism for fibronectin fibril assembly. *J Cell Sci* 1998;111:2763–2777. [PubMed: 9718369]

- Noakes PG, Gautam M, Mudd J, Sanes JR, Merlie JP. Aberrant differentiation of neuromuscular junctions in mice lacking s-laminin/laminin beta 2. *Nature* 1995;374:258–262. [PubMed: 7885444]
- Occhi S, Zambroni D, Del Carro U, Amadio S, Sirkowski EE, Scherer SS, Campbell KP, Moore SA, Chen ZL, Strickland S, Di Muzio A, Uncini A, Wrabetz L, Feltri ML. Both laminin and Schwann cell dystroglycan are necessary for proper clustering of sodium channels at nodes of Ranvier. *J Neurosci* 2005;25:9418–9427. [PubMed: 16221851]
- Patton BL, Miner JH, Chiu AY, Sanes JR. Distribution and function of laminins in the neuromuscular system of developing, adult, and mutant mice. *J Cell Biol* 1997;139:1507–1521. [PubMed: 9396756]
- Quondamatteo F, Reinhardt DP, Charbonneau NL, Pophal G, Sakai LY, Herken R. Fibrillin-1 and fibrillin-2 in human embryonic and early fetal development. *Matrix Biol* 2002;21:637–646. [PubMed: 12524050]
- Ramirez F, Dietz HC. Extracellular microfibrils in vertebrate development and disease processes. *J Biol Chem* 2009;284:14677–14681. [PubMed: 19188363]
- Ritty TM, Roth R, Heuser JE. Tendon cell array isolation reveals a previously unknown fibrillin-2-containing macromolecular assembly. *Structure* 2003;11:1179–1188. [PubMed: 12962636]
- Stecker MM, Baylor K, Chan YM. Acute nerve compression and the compound muscle action potential. *J Brachial Plex Peripher Nerve Inj* 2008;3:1. [PubMed: 18211681]
- Viljoen D. Congenital contractural arachnodactyly (Beals syndrome). *J Med Genet* 1994;31:640–643. [PubMed: 7815423]
- Wallquist W, Plantman S, Thams S, Thyboll J, Kortessmaa J, Lannergren J, Domogatskaya A, Ogren SO, Risling M, Hammarberg H, Tryggvason K, Cullheim S. Impeded interaction between Schwann cells and axons in the absence of laminin alpha4. *J Neurosci* 2005;25:3692–3700. [PubMed: 15814800]
- Yang D, Bierman J, Tarumi YS, Zhong YP, Rangwala R, Proctor TM, Miyagoe-Suzuki Y, Takeda S, Miner JH, Sherman LS, Gold BG, Patton BL. Coordinate control of axon defasciculation and myelination by laminin-2 and -8. *J Cell Biol* 2005;168:655–666. [PubMed: 15699217]
- Zhang H, Hu W, Ramirez F. Developmental expression of fibrillin genes suggests heterogeneity of extracellular microfibrils. *J Cell Biol* 1995;129:1165–1176. [PubMed: 7744963]

Fig. 1. Ablation of fibrillin-2 causes motor dysfunction

When suspended, *Fbn2*^{-/-} mice retracted their hindlimbs toward the body (B). This was never observed with WT mice (A). C: Performance in wire hanging test. Latency to fall was recorded. D: Analysis of motor function by Rotarod test. The duration time for which the mice could stay on accelerating rotarod was measured. Means of the two final trials for each of the tested animals are shown. E: Hot plate test. Mice were placed on the hot plate and latency to first paw lift and lick was recorded. In C and E, each bar represents the mean $\pm$ S.E. of each genotype group.

Fig. 2. Muscle spindles and proprioceptive neurons are unchanged in $Fbn2^{-/-}$ mice

A–D: Cross-sections of soleus muscle of adult $Fbn2^{+/+}$ (A, C) and $Fbn2^{-/-}$ (B, D) mice were dual stained with S46 (green) and anti-collagen type IV (red) antibodies. Arrows in A and B mark S46-positive muscle spindles. High power images show that the size of muscle spindles remained unchanged in $Fbn2^{-/-}$ muscle (D) as compared to control muscle (C). E–F: Cryosections of cervical DRG of adult WT (E) and $Fbn2^{-/-}$ (F) mice were stained with antibodies to parvalbumin. Arrows indicate parvalbumin-positive proprioceptive neurons. Scale bar: A, B, E, F, 100 μ m; C, D, 50 μ m.

Fig. 3. Expression of fibrillin-1 and fibrillin-2 in mouse peripheral nerve during development
A–D: Cryosections of hind limbs of 4d old *Fbn2*^{+/+} (A, B) and *Fbn2*^{-/-} (C, D) mice were stained with anti-fibrillin-1 (A and C) or anti-fibrillin-2 (B and D) antibodies. The peripheral nerves are marked with asterisks. E–K: Cryosections of the sciatic nerves of 7d old (E and F) and adult (G–K) WT mice were stained with anti-fibrillin-1 (E and G), anti-fibrillin-2 (F and H) antibodies, or dual stained with anti-fibrillin-1 (I) and anti-neurofilament (J) antibodies. K represents a merged image of I and J. Scale bars, A–H, 100 μm; I–K, 25 μm.

Fig. 4. Distribution of fibrillin-1 in adult mouse tissues

A–D: Teased sciatic nerves from adult *WT* mice were dual stained with antibodies to fibrillin-1 (A and B) and $\alpha 2$ chain of laminin (C and D). The position of the node of Ranvier is labeled with arrows. E–H: Cross-sections of the skeletal muscle were dual stained with anti-fibrillin-1 antibodies and rBTX (E and G), or anti-laminin $\alpha 2$ antibodies and rBTX (F and H). Positions of NMJs are labeled with arrows. Scale bars, A–D, 25 μm ; E–H, 40 μm .

Fig. 5. Axonal myelination is normal in fibrillin-2 deficient mice

Semithin (A and B) or 8 μm -thick (C–H) sections of the sciatic nerves of adult *Fbn2*^{+/+} (A–G) and *Fbn2*^{-/-} (B–H) mice were stained with Toluidine blue (A and B), or dual stained using antibodies to P₀ (C and D) and neurofilament (E and F). G represents a merged image of C and E; H is merged image of D and F. Scale bars, 50 μm .

Fig. 6. Nodal structure is normal in *Fbn2*^{-/-} mice

Teased sciatic nerves from adult WT (A–G) or *Fbn2*^{-/-} (B–H) mice were stained with antibodies to Na⁺ channels (A and B), ankyrin G (C and D), E-cadherin (E and F) and K v1.1 channel (G and H). The positions of the nodes are labeled with arrowheads; E-cadherin staining of Schmidt-Lanterman incisures marked with arrows (E and F). Scale bar, 25 μ m.

Fig. 7. Loss of fibrillin-2 does not affect ECM composition of the nerve

Sciatic nerve sections obtained from adult *WT* (A–I) and *Fbn2*^{-/-} (B–J) mice were stained with antibodies to collagen type IV (A and B), fibronectin (C and D), $\alpha 2$ chain of laminin (E and F), collagen type V (G and H) and Fbn1 (I and J). Scale bar, 50 μ m.

Fig. 8. Perineurium is altered in *Fbn2*^{-/-} mice

High power images of the sciatic nerve sections from *Fbn2*^{+/+} (A–E) and *Fbn2*^{-/-} (B–F) mice stained with antibodies to collagen type IV (A and B), fibronectin (C and D) and $\alpha 2$ chain of laminin (E and F) revealed less compact and less organized structure of the perineurium of *Fbn2*^{-/-} animals. Occasionally, the perineurial layer was detached from endoneurium in *Fbn2*^{-/-} mice, forming small cavities (arrows in B and F). G–H: Electron micrographs of the perineurium of WT (G) and *Fbn2*^{-/-} (H) mice. Perineurial structure was disorganized in mutant nerves. Scale bar: A–F, 25 μ m; G–H, 2 μ m.

Fig. 9. NMJs are normal in the absence of fibrillin-2

Labeling AchRs with rBTX in *WT* (A) or fibrillin-2 deficient (B) skeletal muscles revealed normal distribution of NMJs. Dual staining of longitudinal sections of the muscles with antibody to SV2 (C, G, E and I) and rBTX (D, H, F and J) did not revealed differences in overall size or architecture of NMJs between *WT* (C, D, G and H) and the mutant (E, F, I and J) animals. Scale bar: A–B, 100 μ m; C–J, 25 μ m.

Fig. 10. Ablation of *Fbn2* does not impair nerve regeneration

Sciatic nerves of *Fbn2*^{+/+} and *Fbn2*^{-/-} mice were crushed and sections of the nerves 6 mm distal to the injury site were obtained 7 or 21 days after the surgery. Uninjured nerves were used as controls. Sections were dual stained using antibodies to P₀ and neurofilament. Representative images were collected and total amount of axons (A) or amount of myelinated axons (B) were counted. Mean values of axonal counts of three animals per time point for each genotype are shown, error bars indicate SEM.

Table 1Nerve conduction in *WT* and *Fbn2*^{-/-} mice

parameter	genotype	
	<i>Fbn2</i> ^{+/+}	<i>Fbn2</i> ^{-/-}
CMAP amplitude (mV)	2.08 ± 0.12 ^a	2.05 ± 0.16
CMAP conduction velocity (m/sec)	28 ± 0.8	26 ± 0.7
CMAP duration (msec)	2.5 ± 0.1	2.7 ± 0.1

^aThe results are expressed as mean ± SEM