

Chapter 2

Neurofibromin in Skeletal Development

Mateusz Kolanczyk and David A. Stevenson

Abstract Skeletal complications are frequent in neurofibromatosis type 1 (NF1). Various skeletal manifestations are observed including generalized osteopenia (low bone mass), scoliosis, long bone bowing, pseudarthrosis, bone lytic lesions, and sphenoid wing dysplasia. Historically, focal skeletal lesions in NF1 were suspected to be caused by nearby localized tumors. However, genetic experiments have brought an entirely new view of NF1 musculoskeletal pathology, illustrating the power of gene discovery and genetic model systems. We now appreciate that neurofibromin is required for normal musculoskeletal system development. We now understand that NF1 bone dysplasia can be caused by loss of function mutations in *NF1*, occurring in the mesenchymal lineage, rather than through tumor influence as previously thought. This is not to say that the presence of tumors has no impact on the bone. It is likely that tumors can impact bone tissue and further exacerbate pathological processes. Here we review the current state of knowledge of neurofibromin in bone and skeletal muscle. We outline mechanisms underlining NF1 musculoskeletal dysplasias derived from both mouse disease models and observations of human NF1 pathology. We also discuss pathways regulated downstream of *NF1* and their potential impact on the musculoskeleton.

Keywords NF1 · Nf1Prx1–f1 · Neurofibromatosis · Tibial dysplasia · Bone · Muscle · Cartilage · Low bone mass · Scoliosis

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Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant disease caused by loss of function mutations in the *NF1* gene that encodes neurofibromin, a GTPase-activating protein. NF1 is a relatively common disorder (1/3000) and is typically diagnosed clinically [1]. Individuals who have two out of the following seven criteria meet clinical diagnostic criteria for NF1: (1.) Six or more café au lait macules (1.5 cm or larger in postpubertal individuals; 0.5 cm or larger in prepubertal individuals); (2.) Two or more neurofibromas of any type or one or more plexiform neurofibromas; (3.) Freckling in the axillary or inguinal region; (4.) Optic glioma; (5.) Two or more Lisch nodules; (6.) A distinctive osseous lesion (e.g. sphenoid bone dysplasia, pseudarthrosis); (7.) First-degree relative with neurofibromatosis type 1 [2]. NF1 can cause various degrees of morbidity. This is especially true in respect to tumor load, which can vary significantly from patient to patient. Although there is significant clinical variability there are relatively few genotype-phenotype correlations [3, 4], and modifier genes are suspected to exist. However, it is likely that somatic second hit mutations or epigenetic factors also contribute to the high clinical variability [5]. The main morbid symptoms of NF1 are related to an increased susceptibility towards neoplastic transformation (e.g. benign cutaneous neurofibromas, plexiform neurofibromas, optic pathway tumors, and aggressive malignant peripheral nerve sheath tumors). Although primarily considered a neurocristopathy, that is, a defect in neural crest development, multiple organ systems are affected in individuals with NF1, including the musculoskeleton. Here we describe the significant musculoskeletal manifestations of NF1 and the underlying role of neurofibromin.

Bone Development

There are two types of bone development; endochondral and intramembranous ossification (Fig. 2.1). The development of the skeleton is initiated through the process of patterning which is followed by subsequent organogenesis, commencing in the growth of the skeletal elements [6]. Patterning determines spatial arrangement, number and shape of the bone elements. During organogenesis, mesenchymal precursor cells differentiate into chondrocytes (cartilage cells) or osteoblasts (bone cells). During desmal ossification, osteoblasts differentiate directly from mesenchymal cells. Desmal ossification takes place in the bones of skull, parts of the facial skeleton and the collar bone. All other bones develop through the process of endochondral ossification. Here, a cartilaginous system is first formed, which is later replaced by bone. The growth of skeletal elements takes place in a specialized area of long bones called the epiphyseal plate or growth plate. Cartilage differentiation takes place within the growth plate where morphologically distinguished zones are being formed: resting zone, proliferative zone, pre-hypertrophic zone and hypertrophic zone. In the hypertrophic zone, chondrocytes undergo enlargement and eventually apoptosis. In this zone, blood vessels invade the growth plate

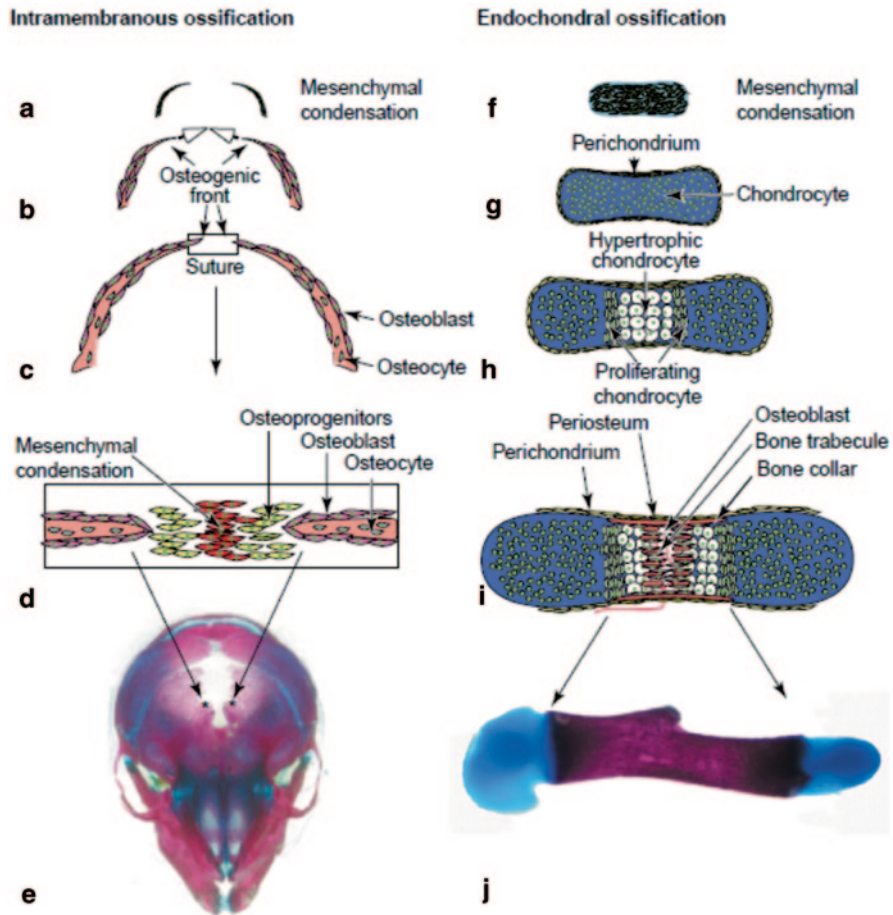


Fig. 2.1 Bone development cartoon (reprinted from publication title: Transcriptional mechanisms in osteoblast differentiation and bone formation. Trends in Genetics Volume 19, Issue 8, August 2003, Page 459, with permission from Elsevier) Intramembranous ossification and endochondral ossification. **a–d** Craniofacial bones are formed directly from condensations of mesenchymal cells without the formation of a cartilage intermediate, a process described as intramembranous ossification. **a** In mice, formation of frontal bones starts at embryonic day 12.5 (E12.5) in mesenchymal condensations on the lateral side of the head. **b** From the mesenchymal condensation, the cellular mass with osteogenic activity spreads upward toward the top of the skull (arrows). Osteoblasts differentiate from mesenchymal condensation and produce bone matrix at E14.5. **c** At E15.5, the ends of the cellular masses that originate on each side meet at the midline where a suture is formed. **d** In the suture, cells in the mesenchymal condensations differentiate into osteoprogenitor cells. Osteoprogenitor cells then differentiate into osteoblasts that produce bone-matrix molecules. Osteoblasts differentiate into osteocytes, which become embedded within the bone matrix. **e** A schematic frontal view of a mouse skull (E18.5) stained with alizarin red and alcian blue. Asterisks indicate osteogenic fronts of frontal bones. **f–j** By contrast, long bones are formed by endochondral ossification. **f** Development of endochondral bones starts with the formation of mesenchymal condensations. **g** Cells in the mesenchymal condensations then give rise to chondrocytes whereas cells at the periphery of the condensations form a perichondrium. **h** Centrally localized chondrocytes proliferate actively before exiting the cell cycle and differentiating into hypertrophic

and osteoblasts initiate new bone formation in the primary spongiosa. Bone is constantly remodeled through the action of osteoclasts, which are derived from the hematopoietic lineage. In mouse bone the *Nf1* gene is most strongly expressed in the prehypertrophic and hypertrophic zone of the growth plate. It is also expressed in osteoblasts and osteoclasts.

Bone Complications in NF1

Short Growth and Generalized Low Bone Mineral Density

The most frequent types of skeletal manifestations in NF1 are short stature and generalized osteopenia or osteoporosis. In adults with NF1, 20–30% exhibit height below the 3rd centile [7, 8]. How *NF1* haploinsufficiency causes short stature is currently unknown. It is interesting that *Nf1* heterozygous mice do not show shortening of skeletal elements or reduced bone volume. However, shortening of skeletal elements was observed in conditional mouse mutants that are deficient for *Nf1* in the central nervous system (CNS), including the hypothalamus [9]. In these mice, reduction in pituitary growth hormone synthesis presumably caused a decrease in growth. Reduced growth was also observed in mice in which *Nf1* was doubly inactivated in cartilage [10, 11]. Thus, short stature in NF1 may be related to deficiency in both endocrine and bone growth plate systems. Short stature itself likely does not cause significant morbidity in NF1, and growth remains proportional. However, functional and societal impact of short stature in NF1 has not been adequately investigated.

Decreased bone mineral density (BMD) has been observed in as much as 50% of individuals with NF1 [12–19]. Generalized low bone mass and osteoporosis may be more problematic in these patients, potentially increasing risk of fractures. While literature suggests that children with NF1 have generally low BMD, the sizes of the studied patient groups are small and the clinical consequences are not well known [19]. In one study of 460 NF1 individuals there was a 5.2-fold increased risk of fractures in individuals with NF1 above 41 years of age, a 3.4-fold increased fracture

chondrocytes. The sequential differentiation of chondrocytes establishes a unique cellular organization that constitutes the epiphyseal growth plate. At the distal end of the epiphyseal growth plate, hypertrophic chondrocytes further mineralize their own cartilaginous matrix. In parallel to this multistep differentiation pathway, cells from a thin layer of mesenchymal cells, called the periosteum, that envelops the cartilage matrix and invades the zone of hypertrophic chondrocytes in addition to blood vessels and osteoclasts. Whereas osteoclasts degrade the matrix of the zone of hypertrophic chondrocytes, osteoblasts deposit a bone-specific matrix. (i) The partially degraded, mineralized cartilage matrix forms a template for deposition of bone matrix by osteoblasts. Endochondral bone contains an outer envelop of cortical bone (the bone collar) that surrounds the marrow cavity and growth plate. The cortical bone is contiguous with the perichondrium. j A mouse humerus stained with alizarin red and alcian blue

risk in NF1 children, and no increased fracture risk in the age group 17–40 years [20]. In another study of 256 children with NF1 ages 5–20 years, the NF1 individuals without a focal long bone dysplasia did not have a difference in the prevalence of ever having a fracture compared to healthy controls [21]. Thus, it appears that individuals with NF1 have an age-dependent increase of fracture risk. It appears also that individuals with NF1 have generally low vitamin-D concentrations, which could be one of the factors leading to decreased BMD [22, 23]. A small study of bone biopsies from 14 NF1 individuals showed decreased trabecular bone volume and osteoidosis, which coincided with increased osteoblasts and osteoclast number [24]. These observations are similar to the findings in *Nf1Col1^{-/-}* mice in which *Nf1* was inactivated in the osteoblast lineage [25]. Inactivation of *Nf1* in osteoblasts causes increased collagen synthesis but inhibits bone mineralization, resulting in the profound osteoidosis. *Nf1Col1^{-/-}* mice show osteoblast-mediated increase of osteoclast number. More precisely the *Nf1^{-/-}* osteoblasts overproduce RANKL, the receptor activator for nuclear factor kappa-B ligand that triggers osteoclastogenesis. It has been shown that in the *Nf1* deficient osteoblasts, activation of Ras triggers downstream activators ERK1/2, RSK2, PKA as well as ATF4, accelerating collagen synthesis and causing overproduction of RANKL [25]. Defective osteoblast differentiation therefore appears to be one of the main causes of bone pathology in NF1.

FGF Signaling and c-Type Natriuretic Peptide

Recent research has established a link between short growth in *Nf1Col2^{-/-}* mice and increased signaling mediated by the fibroblast growth factor receptor (Fgfr) [26]. These mice lack neurofibromin (*Nf1*) in type II collagen-expressing cells and show a number of phenotypes reminiscent of the ones observed in mice characterized by FGFR gain-of-function mutations. In chondrocyte cultures in-vitro, Fgfr activation by FGF2 pulse-stimulation triggered rapid ERK1/2 phosphorylation in wild type (wt) and *Nf1^{-/-}* chondrocytes. However, return to the basal level of ERK activation was delayed in *Nf1^{-/-}* cells, suggesting that prolonged ERK signaling is one factor in deficient chondrocyte growth. In separate experiments, *Nf1Col2^{-/-}* mice were treated with the NC-2 compound, which is a derivative of c-natriuretic peptide (CNP) [26]. CNP is known to bind to the natriuretic peptide receptor B (N-PRB) and stimulate cGMP synthesis, thus suppressing MAPK activation (Fig. 2.2) [27]. The subcutaneous injections of NC-2 compound at 300 mg/kg per day throughout 18 days from birth on resulted in almost complete rescue of the growth defect in *Nf1Col2* mice, suggesting CNP is a valid candidate compound for the treatment of growth deficiency in NF1. Further studies of CNP in other NF1 mouse models will be helpful to access its potential side effects and determine its suitability for the treatment of other bone manifestations.

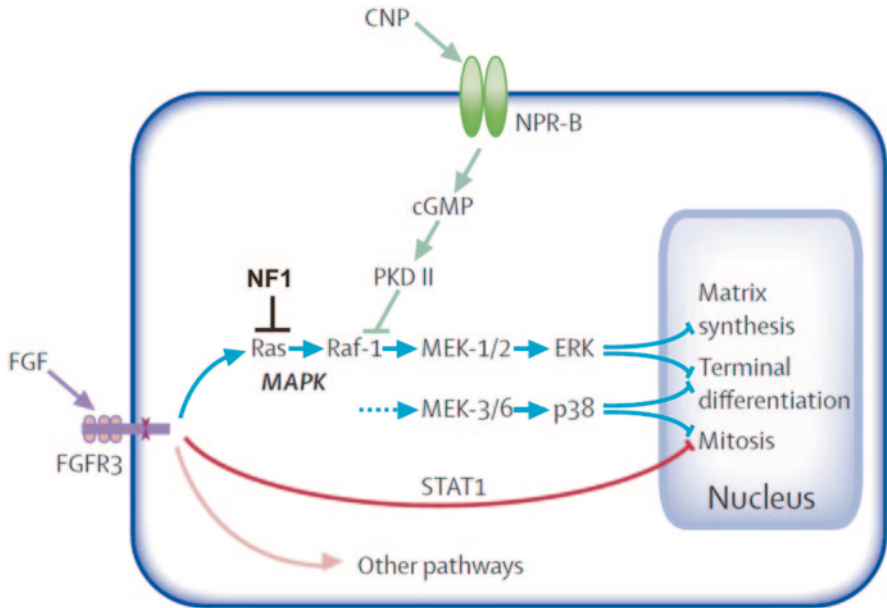


Fig. 2.2 CNP—MAPK pathway (reprinted with modification from publication title: Achondroplasia, The Lancet Vol. 370 July 14, 2007 page 163, with permission from Elsevier). Signalling pathways of FGFR3 most relevant to growth plate chondrocytes FGFR3 signals propagated through STAT1, MAPK-ERK, MAPK-p38, and other pathways inhibit chondrocyte proliferation, post-mitotic matrix synthesis, and terminal (hypertrophic) differentiation. The CNP-NPR-B pathway and NF1 inhibit the MAPK pathways. (Reprinted from The Lancet, 370, Horton WA, Hall JG, Hecht JT, Achondroplasia, pp. 162–172, 2007, with permission from Elsevier)

Long Bone Dysplasia

Apart from generalized bone disease, about 3–5 % of individuals with NF1 develop a focal skeletal dysplasia of the long bones, predominantly the tibia (Fig. 2.3) [28]. It is currently unknown why the tibia is most often affected. The anterolateral bowing typically is identified in infancy, or even prenatally, and is usually unilateral [28]. This suggests that long bone dysplasia has a prenatal origin. Radiographically, tibial bowing in NF1 prior to fracture usually appears as cortical thickening and medullary canal narrowing at the apex of the convexity, typically near the junction of the middle and distal thirds of the tibia (Fig. 2.3b) [29]. The bowed long bone frequently sustains a fracture that is recalcitrant to union resulting in pseudarthrosis (Fig. 2.3c). Mouse experiments suggest that dystrophic bone changes are caused by complete *NF1* loss-of-function in dividing and multipotent bone cells. Supporting this hypothesis, somatic (i.e. genetic alteration occurring after conception) homozygous inactivation of *NF1* was demonstrated in the pseudarthrosis tissue of one NF1 patient with tibial pseudarthrosis [30]. A mouse model that recapitulates most features of NF1 tibial dysplasia is the *Nf1Prx1^{-/-}* mouse (Fig. 2.4), in which *Nf1* was inactivated in the limb bud mesenchyme and head mesenchyme [10]. In these



Fig. 2.3 Long bone dysplasia in neurofibromatosis type 1 (NF1). **a** Photograph of patient. **b** Radiograph of individual with NF1 with tibial bowing showing characteristic cortical thickening with medullary canal narrowing. **c** Radiograph of pseudarthrosis after long standing fracture of the tibia in an individual with NF1

mice, all cells of mesenchymal origin carry biallelic *Nf1* inactivation, including bone cells, cartilage, muscle, endothelium, fat and connective tissue cells. Although the *Nf1* *Prx1*^{-/-} mouse was generated initially as a model of long bone dysplasia, this model has subsequently provided additional insights that relate to the broad spectrum of NF1 musculoskeletal findings. Some of the important observations from this and other mouse models pertaining to developmental processes controlled by *Nf1* are described below; see also Table 2.1 for review of mouse models and bone fracture related observations.

Growth Plate Disturbances

Nf1 deficiency in cartilage causes decreased chondrocyte proliferation and premature growth plate chondrocyte hypertrophy [10]. This is caused by decreased Indian Hedgehog (Ihh) expression in the growth plate and concomitant increase of p21/Waf1 expression that leads to reduced cartilage proliferation and dwarfism of the appendicular skeleton. Reduced Ihh expression is a direct cause of premature hypertrophy and short growth [10].

Nf1 deficient cartilage strongly expresses the Sox9 transcription factor, which is localized to the nucleus and is responsible for retention of cartilaginous properties. This persistent nuclear localization of Sox9 is observed in the *Nf1* *Prx1*^{-/-} mice in the cartilage anlagen, but also in areas where it should not be expressed (e.g. during hip joint cavitation in joint cavity). Due to persistence of nuclear Sox9 expression, the hip joint cavity retains a cartilaginous phenotype and undergoes fusion with

Table 2.1 Callus cellular and structural characteristics in *Nf1* mouse models. (Updated and adapted from Stevenson et al. [39])

	<i>Nf1</i> ^{+/-} Ref [49]	<i>Nf1</i> ^{ob} ^{-/-} Refs [11, 25]	<i>Nf1</i> ^{Prx} ^{-/-} Ref [47]	<i>Nf1</i> ^{ob} ^{-/-} Ref [25]	<i>Nf1Col2</i> ^{-/-} Ref [11]	<i>Nf1</i> (flox ^{-/-}) AdCre Ref [36]
<i>Nf1</i> genetic setting	+/- (global)	-/- in mature osteoblasts only	-/- in limb mesenchymal osteoprogenitors and progeny (chondrocytes, osteoblasts, endothe- lial cells, adipocytes, muscles)	+/- (global) -/- in mature osteoblasts only	-/- in mesenchymal osteoprogenitors and progeny (chondro- cytes, osteoblasts)	+/- (global) -/- in differentiating callus cells includ- ing chondrocytes and osteoblasts
Osteoclast differentia- tion	Increased (following ovariectomy)	Increased (osteoblast-dependent)	Increased (mechanism ND)	Increased	Increased	Increased
Osteoblast differentia- tion	Decreased	Normal	Decreased	Decreased	Decreased	Decreased
Mineraliza- tion	ND	Delayed	Delayed	Delayed	Delayed	Delayed
Structural properties		Increased callus volume	Cartilaginous remnants	Reduced BV/ TV	ND	Reduced closed and open fracture union rates
		Reduced BV/TV	Reduced callus size			Increased fibrotic or undifferentiated tissue at fracture callus
		Increased cortical porosity	Altered BV/TV			
		Cartilaginous remnants	Reduced BMD			

Table 2.1 (continued)

	<i>Nf1</i> ^{+/-} Ref [49]	<i>Nf1</i> ^{ob/-} Refs [11, 25]	<i>Nf1</i> ^{px/-} Ref [47]	<i>Nf1</i> ^{ob/-} Ref [25]	<i>Nf1Col2</i> ^{-/-} Ref [11]	<i>Nf1</i> (flox/-) AdCre Ref [36]
Callus mechanical properties	ND	Decreased stiffness and maximum force	Reduced torsional stiffness	ND	Reduced vertebral stiffness, yield force, peak force	ND
			Reduced ultimate torque at failure		Reduced femoral rigidity, and bending strength	
Callus marrow	Fibrocartilaginous tissue	Normal	Fibrocartilaginous tissue	ND	ND	Fibrocartilaginous tissue

ND not determined, *BV* bone volume, *TV* trabecular volume

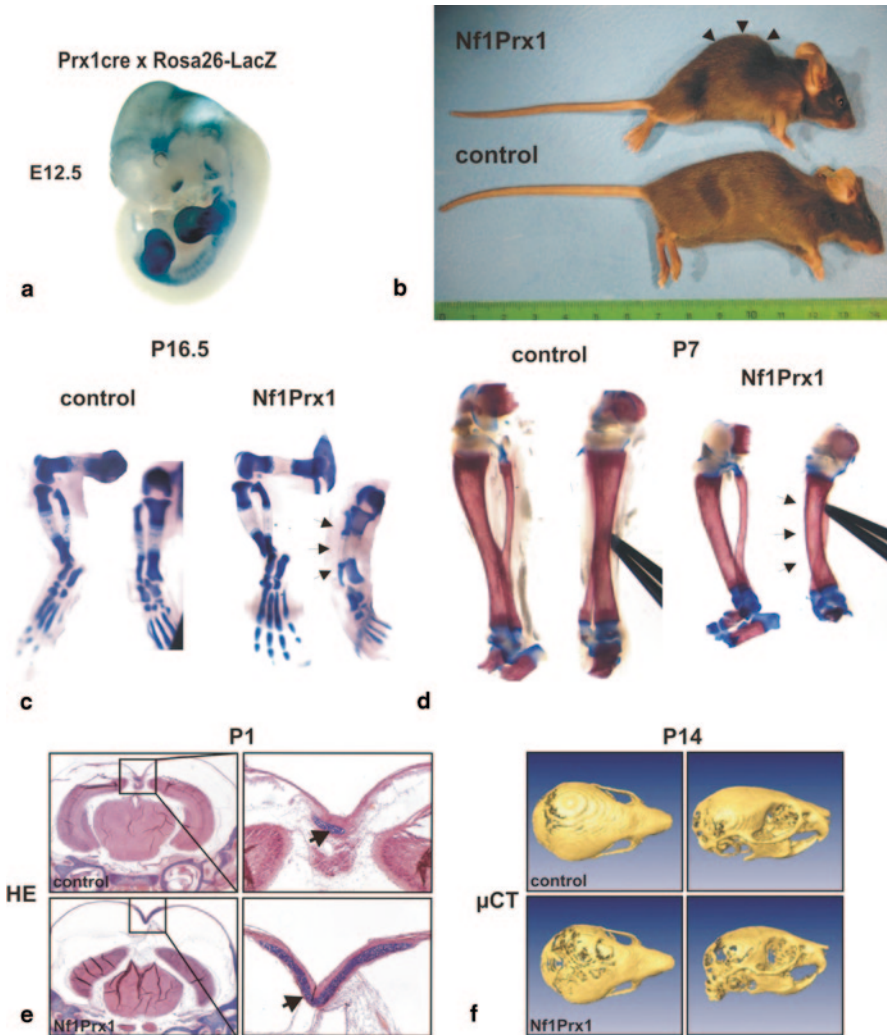


Fig. 2.4 Gross morphology and skeleton in *Nf1Prx1*^{-/-} mice. **a** Whole mount detection of the Cre recombinase activity in the cross of Rosa26-LacZ indicator mouse with Prx1-Cre mice. Note LacZ activity in the limb buds and in the cranial region. **b** A side view of the control and *Nf1Prx1*^{-/-} mutant mouse. Note that *Nf1Prx1*^{-/-} mutant mice show a profound kyphosis (arrow heads), a phenotype often presented by mice featuring muscle dystrophy. **c, d** Tibia bowing in the prenatal and postnatal development in *Nf1Prx1*^{-/-} mice. Alcian blue and Alizarin red stained skeletal preparations of E16.5 and P7 mice in side and front view. Note a bowed tibial bone (arrows) visible in the front view of the tibia both at E16.7 and P7 stage indicating a prenatal origin of the phenotype. Note also shortening of the skeletal elements in the mutant mice. **e, f** Delayed mineralization and increased cartilage formation in the *Nf1Prx1*^{-/-} cranium. **e** Hematoxylin-Eosin/Alcian Blue stained transverse sections of the P1 wt and *Nf1Prx1*^{-/-} calvaria. Right panel shows magnification of the sagittal suture with enlarged cartilage area in the *Nf1Prx1*^{-/-} mouse calvaria (arrows). **f** Delayed mineralization of the calvarial bones in the *Nf1Prx1*^{-/-} mice visualized by μ CT scan. The top view and the profile view of the skull, with poorly mineralized, transparent areas in the hind cranium of 14 days old *Nf1Prx1*^{-/-} mouse

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