NEUROFIBROMIN REGULATED SIGNALING PATHWAYS IN ENDOCHONDRAL OSSIFCIATION

by

Matthew Ross Karolak

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmacology

August, 2015

Nashville, Tennessee

Approved:

Joey Barnett, Ph.D.

Florent Elefteriou, Ph.D.

Jonathan Schoenecker, M.D., Ph.D.

Xiangli Yang, Ph.D.

Ela Knapik, M.D.

for my sister, parents, and grandparents

ACKNOWLEDGMENTS

This dissertation is the product of an intensely emotional and intellectual stress test, which would not have been completed without the support of many. Academically, I could not have asked for a better set of mentors to guide me through the intellectual rigors that this work required. It was with my undergraduate advisor, Terry Landowski, where I first discovered my interest in the skeleton as an organ system and love of the scientific method. My graduate advisor, Florent Elefteriou, helped me develop day-to-day independence in the laboratory and directed me to always ask clinically relevant research questions. I am also grateful to him for helping me develop an emotional maturity regarding my work even when my experiments seemed to be going nowhere. I must give thanks to my dissertation committee members: Joey Barnett, Jon Schoenecker, Ela Knapik, and Xiangli Yang, for their service and always offering engaging scientific discussions, technical guidance, rigorous questioning, and career advice throughout my graduate tenure. My work has been financially made possible by the National Institutes of Health (NIH) Institute for General Medical Sciences training grant 5T32GM007628 to Joey Barnett, the Children's Tumor Foundation Young Investigator Award YIA-2013-01-018 to me, and Department of Defense and NIH National Institute of Arthritis and Musculoskeletal and Skin Diseases grants W81XWH-09-01-0207 and 5R01AR055966, respectively, to Florent Elefteriou.

I am incredibly grateful to friends, past and present. I am especially grateful for the friendship and companionship of Tim and Heidi Lin, Naomi Miguel, Callie Miller, Jeremy Chan, Laura Hover, Lara Jazmin, and Amanda Groh. I thank the many friends I have at Trinity Church of Nashville and for the spiritual grounding they have helped me maintain and mature during my stay in Nashville. I am thankful to you all for reminding me of the truth found in the Gospel: that my identity is not found in my professional and personal failures or successes, but in the fact that I am an adopted child of God and by that grace alone, I am where and who I am today.

Finally, and most of all, I am grateful for my family. To my sister Amee, thank you for bringing joy, youth, and excitement to my life. To my parents Doug and Jane, who collectively fostered my love of learning from an early age and instilled in me a work ethic and academic drive that is unquestionably, entirely responsible for me completing these studies, I am incredibly grateful. I also thank my grandparents Dick and Judy who, like my parents, have always been my biggest fans and supporters of my education. And for these reasons, I dedicate this work to them.

To you all, I am forever in your debt.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

ABSTRACT

Neurofibromatosis type 1 (NF1) is the most common autosomal dominant genetic disorder occurring in 1 of every 3500 live births. NF1 is caused by loss-of-function mutations in *NF1*, the gene encoding the Ras-GAP neurofibromin. Forty percent of NF1 patients will develop orthopedic complications which often includes unilateral bowing of the extremities, fracture, and subsequent fracture healing deficits (pseudarthrosis). Because the molecular and cellular aspects mechanisms of fracture healing largely recapitulate the processes of bone development, the goal of this dissertation is to characterize the function of neurofibromin in growth plate chondrocytes and the signaling pathways it regulates during endochondral ossification. Using conditional mouse knockout models of NF1, we found that neurofibromin regulates growth plate chondrocyte proliferation, hypertrophic maturation, and matrix catabolism at the osteochondral border. Furthermore, we found that neurofibromin in prehypertrophic chondrocytes likely attenuates FGFR1 and FGFR3 signaling to inhibit chondrocyte proliferation, and neurofibromin in hypertrophic chondrocytes attenuates FGFR1 signaling to inhibit matrix catabolism as the osteochondral border. Finally, in a series of pharmacological proof-of-principle experiments, we identified C-type natriuretic peptide and the pan-FGFR inhibitor BGJ-398 as potential therapeutic agents for the treatment of NF1 pseudarthroses via their action on $Nf1^{-/-}$ chondrocytes. Further investigation of these agents in NF1 fracture healing models is warranted.

CHAPTER I

INTRODUCTION AND BACKGROUND

Overview

Neurofibromatosis type 1 (NF1)-associated skeletal dysplasia and fracture healing have severe negative impacts on affected individuals' quality of life (most of which are infants and children) and has an unknown etiology with no targeted, pharmacological therapy. Affected individuals typically exhibit unilateral tibial bowing, low bone mineral density, asymmetry of facial bones, and development of pseudarthrosis following fracture (1, 2). Evidence from biopsies and NF1 preclinical animal models, however, suggests that *NF1* loss-of-function mutations in mesenchymal bone cells, and impaired mesenchymal stem cell (MSC) differentiation into osteoblasts and impaired maturation of chondrocytes as the underlying etiology for NF1 pseudarthrosis (2-8). The hypothesis I will address in this dissertation is that the gene product of *NF1*, neurofibromin, is not only necessary for proper growth plate formation but also negatively regulates FGFR1 signaling during endochondral bone formation. We believe the developmental findings in the growth plate described in this dissertation may be translatable to fracture healing in NF1 patients.

As such, in the remainder of this chapter I will review critical publications regarding bone and growth plate development, normal fracture healing, and Neurofibromatosis type 1 which will provide the clinical context for how the developmental studies presented in the subsequent chapters are foundational to not only our understanding of basic biology of the growth plate but also to fracture healing in NF1 patients. I will also provide background as to the role of fibroblast growth factor receptor (FGFR) and MAPK signaling in normal growth plate development. Chapter I will go on to discuss the mouse models used to study the function of neurofibromin in bone development and fracture healing. The chapter will close with a description of current as well as proposed strategies in treating NF1 pseudarthroses. The proposed treatment strategies are mostly based on recent experimental findings in new NF1 mouse models and thus set the stage for the studies presented in Chapters II and III.

In Chapter II, I will address the first part of my hypothesis that neurofibromin is necessary for proper growth plate formation and present data showing that neurofibromin regulates growth plate chondrocyte proliferation, differentiation and osteoclastogenic differentiation both *in vitro* and *in vivo*. This chapter also explores the use C-type natriuretic peptide (CNP) to inhibit MAPK signaling in *Nf1*-null growth plate chondrocytes.

Chapter III is a logical extension of the studies performed in Chapter II and focuses on addressing the second part of my hypothesis, that neurofibromin negatively regulates FGFR1 signaling during endochondral bone formation. I will present data that shows that

neurofibromin indeed regulates FGFR1 signaling in growth plate chondrocytes. However, this finding is mostly specific the hypertrophic subpopulation of growth plate chondrocytes, and neurofibromin most likely also regulates both FGFR1 and FGFR3 in prehypertrophic chondrocytes. Chapter III ends with a study exploring the use of the pan-FGFR inhibitor BGJ-398 to antagonize all FGFR signaling in the growth plate in the context of *Nf1* ablation.

I will conclude with Chapter IV which begins with a broad discussion of the experimental findings of Chapters II and III and frames them in a basic bone biology context. I will then transition to a discussion of how my findings may be applied in the setting of NF1 pseudarthrosis, particularly in regards to the use of CNP or BGJ-398 to promote bone union in NF1 pseudarthrosis patients. I will also discuss the need for new mouse models of NF1, the goal of which is to test the efficacy of new drugs for NF1 pseudarthroses as well as to test hypotheses related to the underlying etiology of the NF1 pseudarthrosis and other NF1 bone manifestations.

Skeletal Development

Generation of a functional skeleton is a highly complex process that is essential for life in vertebrate animals. The skeleton protects organs from the environment, provides bodily structural support, houses the compartments necessary for hematopoiesis and respiration, and regulates many endocrine homeostatic processes. The human skeleton is composed of over 200 distinct bones each of which relies on the tight spatiotemporal signaling

processes between numerous cells types at many different stages of embryonic and postnatal development (9, 10). Approximately 1 in every 4000 births is affected by some form of skeletal abnormality highlighting the complexity of the bone development and stressing the fact that many different genes are involved in skeletal development (10, 11).

Vertebrate skeletal development is controlled by two distinct categories of developmental processes: skeletal patterning and skeletal cell differentiation from mesenchymal stem cell (MSC) progenitors. Skeletal patterning results in the various skeletal shapes of diverse skeletal elements and is influenced by the activity of skeletal morphogens such as sonic hedgehog (SHH) and patterning genes such as those belonging to the HOX gene family (9, 12). Errors in skeletal patterning arising from mutations in regional patterning genes result in skeletal dysostoses where specific skeletal elements rather than the global skeleton are affected (11, 13). Skeletal patterning relies on the precise, directional proliferation of mesenchymal progenitors that condense and ultimately differentiate into one of the two types of functional skeleton forming cells: osteoblasts and chondrocytes. Defects in the generation or function of osteoblasts and chondrocytes results in global skeletal defects called osteochondrodysplasias, osteodysplasias, or chondrodysplasias depending on which cell types are affected (11). Osteoblasts are the cells that lay down the type 1 collagen rich matrix that eventually mineralizes with hydroxyapatite. Chondrocytes are cells that produce various types of cartilage with in the body. In bone, chondrocytes produce hyaline cartilage to form the growth plates of bones residing within the axial, craniofacial, and appendicular skeletons as well as the articular surfaces of bones where bones meet at joints (9, 10).

A common MSC progenitor gives rise to both osteoblasts and chondrocytes. These progenitors trace their embryonic origins to the neural crest, lateral plate mesoderm, paraxial mesoderm, and notochord. Some neural crest and a small subset of lateral plate mesoderm progenitor cells ultimately form the most of the craniofacial skeleton. The majority of the lateral plate mesodermal cells that will become skeletal elements ultimately go on to form the appendicular skeleton and some axial skeletal elements (such as the sternum). The paraxial mesodermal cells that will become skeletal elements ultimately form the vertebral column and ribs. The only remnant of notochord derived tissues in the postnatal vertebrate resides within intervertebral disc as the nucleus pulposus (9, 10).

The mechanisms by which these distinct lineages of MSC progenitor cells give rise to mature skeletal elements can be classified as either intramembranous or endochondral ossification. In both mechanisms, the mesenchymal cells destined to become skeletal elements first condense into what is called the "mesenchymal condensation." The cells within the condensation proliferate under the influence of skeletal morphogens and patterning transcription factors. In intramembranous ossification, the mesenchymal progenitor cells differentiate directly into osteoblasts. Thus, bones formed by intramembranous formation are those that do not form with cartilaginous growth plates, and, in general, these bones comprise most of the craniofacial skeleton as well as parts of the axial skeleton (9, 10).

Endochondral Bone Formation and the Growth Plate

The second mechanism by which bones form in vertebrate animals is endochondral ossification because these bones form from "within cartilage" (**Figure 1**). That is, the pool of condensed MSC progenitor cells (**Figure 1A**) first differentiate into chondrocytes and form a cartilaginous anlage (**Figure 1B**). Chondrocytes within the avascular cartilage anlage proliferate. Chondrocytes in the center of the proliferative anlage stop proliferating and mature to hypertrophy, that is, they enlarge in size, and express markers characteristic of hypertrophic chondrocytes such as *Col10a1*, *Rankl*, *Mmp9*, *Mmp13*, and *Vegf* (**Figure 1C**). This zone of chondrocytes is known as the hypertrophic zone. Expression of VEGF promotes vascular invasion into the center of the hypertrophic zone of chondrocytes (**Figure 1D**). Simultaneously, perichondrial cells surrounding hypertrophic zone of chondrocytes differentiate directly into osteoblasts and form a bony structure called the bone collar which will become the bone cortex/diaphysis (**Figure 1D**). Vascular invasion into the hypertrophic zone in turn facilitates the arrival osteoblast precursors derived from the bone collar surrounding the cartilage, as well as circulating hematopoietic stem cells and thus bringing the cells which will eventually give rise to osteoclasts (osteoclasts are thus of the hematopoietic lineage) (**Figure 1E**). Osteoclasts are responsible for resorbing the existing mineralized matrix within the bone and can be thought of as the macrophages of bone. The cavity occupied by osteoblasts and osteoclasts is known as the primary ossification center and separates the cartilage anlage into proximal and distal cartilaginous ends of the bone which are henceforth called growth plates. Chondrocytes within each growth plate continue to proliferate, thus elongating the bone (**Figure 1F**). Chondrocytes at the ends of the bone also mature to

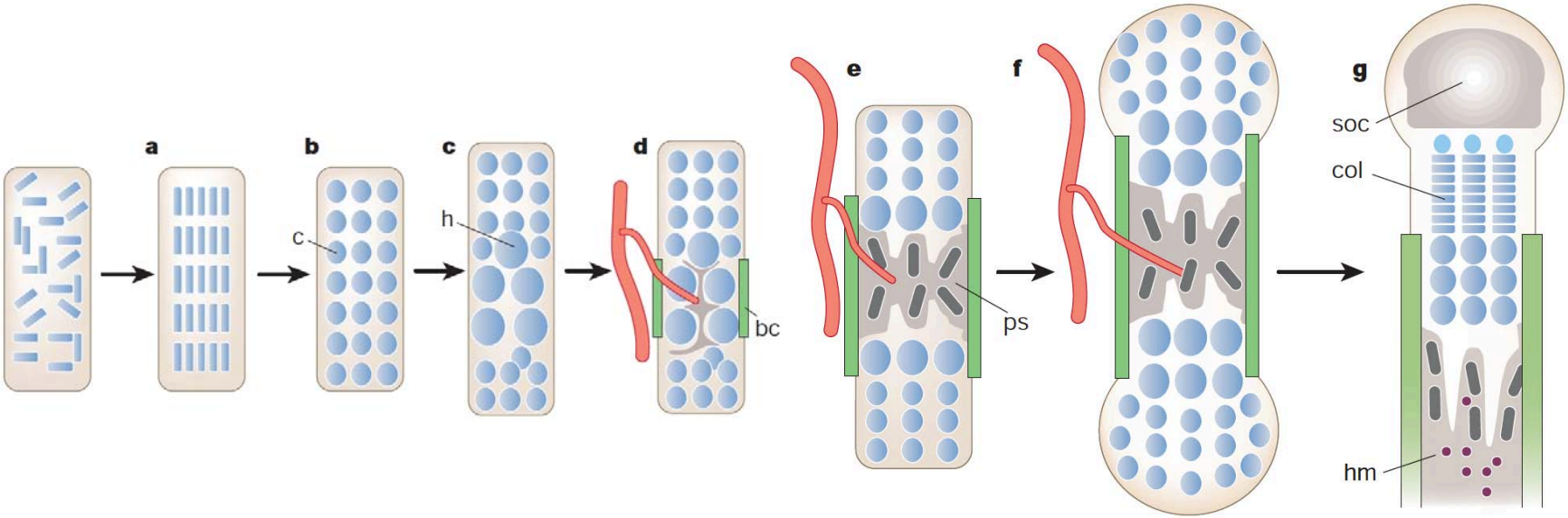

Figure 1. Endochondral Ossification

Endochondral ossification begins when mesenchymal progenitor cells (**A**) condense and (**B**) differentiate into chondrocytes (c) to form a cartilaginous anlage. (**C**) Proliferative chondrocytes mature to hypertrophy (h) and (**D**) promote differentiation of perichondrial cells into bone collar osteoblasts (bc) and vascular invasion into the cartilage mold. (**E**) Osteoblasts and hematopoietic precursors accompany vascular invasion to form the primary ossification center (ps) as (**F**) chondrocytes continue to proliferate and elongate the bone. (**G**) Mature growth plates form with proliferative chondrocyte columns (col) as vasculature invades the ends of the bone to form the secondary ossification centers (soc). A mature hematopoietic cavity (hm) also develops within the primary ossification center. Used with permission (14).

hypertrophy and promote vascular invasion to form secondary ossification centers (**Figure 1G**) (discussed more below). As this occurs, proliferating chondrocytes transition morphologically to form orderly stacks/columns of cells, collectively called the proliferation zone of the growth plate. Less proliferative chondrocytes at the very end of the growth plates (abutting the secondary ossification center) form the reserve/resting zone of the growth plate and contain a pool of chondrocytic stem-like cells which eventually enter the cell cycle, proliferate, and mature to hypertrophy along the axis of the growth plate (9, 10, 14, 15).

Upon vascular invasion and osteoblast arrival, the majority of, if not all, hypertrophic chondrocytes that abut the primary ossification center (at the osteochondral border) undergo apoptotic cell death, and the lacunae that once housed their engorged cell bodies are invaded by osteoblasts and endothelial cells to lay down new bone matrix and vascularize the bone. The mineralized type 10 collagen produced hypertrophic chondrocytes provides the first matrix scaffold of the developing bone. This calcified cartilage is used as a template for osteoblasts to lay down their type 1 collagen matrix that also mineralizes with hydroxyapatite. The area of rapid bone formation adjacent to the hypertrophic zone within the primary ossification center is referred to as the primary spongiosa where new "bone" proper is laid down by osteoblasts. The collagen fibrils initially laid down by osteoblasts in the primary spongiosa are rather disorganized with the fibrils not oriented in the same direction (woven bone). The mineralized type 10 and type 1 collagen matrices within the primary spongiosa are remodeled by the cyclical action of osteoclast mediated resorption and subsequent osteoblast matrix production

which ultimately yields a mature and organized mineralized matrix made exclusively of type 1 collagen whose fibers are oriented in the same direction (lamellar bone). The area of mature trabecular and lamellar bone is referred to as the secondary spongiosa. Continued growth plate chondrocyte proliferation is responsible for longitudinal skeletal growth whereas bone collar/periosteal differentiation into and proliferation of osteoblasts is responsible for appositional bone growth and formation of the bone diaphysis or cortex (9, 10, 14, 15).

The final major step in endochondral ossification occurs postnatally with the vascular invasion into core of the proximal and distal growth plates to form what is known as the secondary ossification center. Similar to the formation of the primary ossification center, vascular invasion into the growth plates facilitates osteoblast, vascular, and osteoclast precursor arrival. The formation of the secondary ossification center next to the primary ossification center gives the cartilaginous growth plates its namesake plate-like structure which separates the vascularized, bony primary and secondary ossification centers. Upon skeletal maturity, growth plate chondrocyte proliferation slows to a halt and the primary and secondary ossification centers bridge the growth plate (fuse), eliminating remaining growth plate cartilage, and hence terminating all future longitudinal bone growth (9, 10, 14, 15).

Molecular Regulation of the Growth Plate

Molecular regulation of the growth plate and its dynamics is highly complex relying on tightly controlled spatiotemporal signaling events. As such, significant research efforts

have been devoted to the growth plate. Dysregulation of growth plate dynamics can cause skeletal abnormalities such as gigantism on one end of the spectrum or lethal forms of dwarfism on the other. There are many endocrine, transcriptional, and epigenetic regulators of the growth plate dynamics, however these will not be discussed here (see (10, 15, 16) for reviews). Instead, the focus of this section will be on growth factor signaling important in the growth plate with a focus on those pathways that are particularly relevant to my studies in Chapters II and III. It is important to note that the effects of these signaling molecules are temporally dependent, that is the same signaling molecules can have vastly different effects depending on whether the cells they are acting on are undifferentiated mesenchymal anlage cells versus differentiated chondrocytes of the established growth plate. The focus of my studies is on regulation of differentiated chondrocytes within the existing growth plate and their hypertrophic maturation, not on chondrocyte differentiation itself (chondrogenesis) from within the mesenchymal condensation.

IHH/PTHrP Signaling in the Growth Plate

Perhaps the most well understood and studied signaling pathway involved in growth plate dynamics is the Indian hedgehog (IHH) and parathyroid hormone related protein (PTHrP) signaling pathway and feedback loop (**Figure 2**). IHH has been described as a master regulator of the growth plate because it coordinates chondrocyte proliferation/maturation as well as osteoblast differentiation (bone collar) in the perichondrium in a precise, spatiotemporal manner. The IHH/PTHrP feedback loop begins with the production of PTHrP by perichondrial cells at the ends of the primordial bone. PTHrP stimulates

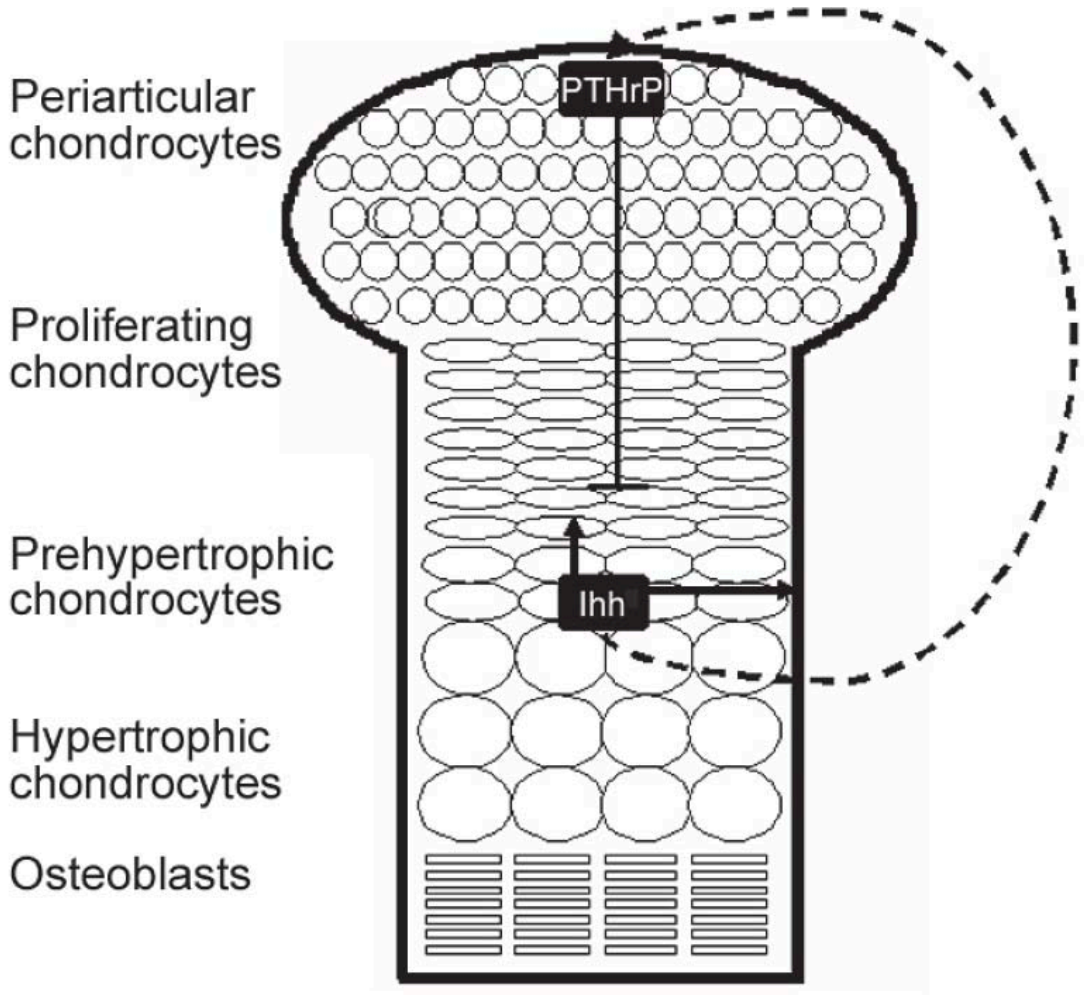

Figure 2. IHH/PTHrP Regulation of Growth Plate Dynamics
IHH/PTHrP regualtion of the growth plate begins with PTHrP secretion from the ends of growth plates which prevents proliferating chondrocytes from matureing to hypertrophy. IHH secreted by prehypertrophic chondrocytes directly stimulates chondrocyte proliferation within the proliferation zone, simulates further PTHrP secretion, and differentiation of perichondrial cells into osteoblasts. Used with permission (17).

resting zone chondrocytes to enter the cell cycle and promotes the proliferation of chondrocytes in the proliferation zone. As chondrocytes proliferate and move further away from the source of PTHrP at the ends of the bone, the chondrocytes exit the cell cycle and become prehypertrophic. Prehypertrophic chondrocytes secrete IHH which in turn promotes further synthesis of PTHrP at the distal end of the bone as well as directly promoting chondrocyte proliferation and inducing the differentiation of osteoblasts in the bone collar. Disruption of PTHrP or IHH expression, or of their receptors, results in significant defects in growth plate development (14, 18-20).

FGFs and BMPs in the Growth Plate

Also critically important in growth plate development are the bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) and their cognate receptors. The FGF and BMP pathways are generally considered to be antagonistic in function, with the BMPs generally promoting chondrocyte proliferation, while the FGFs generally inhibit chondrocyte proliferation and impair their maturation to hypertrophy. Because there are a great number of individual FGFs, BMPs, and related family members, all of which bind to their receptors in an overlapping fashion, most of our understanding of their effects have come from gain- and loss-of-function studies of the much less numerous BMP and FGF receptors (14). For a review of BMP signaling in the growth plate, see Pogue and Lyons, 2006 (21).

FGFRs in the Growth Plate and their Dysregulation

The growth plate inhibitory effects of over 22 different FGFs are mediated by 4 distinct FGF receptors, FGFRs 1-4. Each of the FGFRs binds multiple FGFs with varying affinities. The primary focus of research of FGFRs in the skeleton directed towards their distinct roles in both endochondral and intramembranous ossification. For excellent reviews regarding FGFR signaling in both intramembranous and endochondral ossification see reviews by Ornitz (22, 23). In intramembranous ossification, FGFRs 1-3 have been studied extensively due to their role in fusion of the cranial sutures, where gain-of-function mutations cause premature fusion of cranial sutures (craniosynostosis).

FGFR3

In endochondral ossification, however, research efforts have primarily been directed towards FGFR3 because *FGFR3* mutations cause achondroplastic dwarfism and hypochondroplastic shortness of stature (24-26). It was later found that these *FGFR3* mutations were gain-of-function, activating mutations and the degree of constitutive receptor activation caused by these individual mutations correlated with the severity of dwarfism, with hypochondroplasia being the most mild form, achondroplasia being the most common form of human dwarfism, and thanatophoric dysplasia being lethal (22, 27, 28). All of these forms of dwarfism affect bones formed through endochondral ossification and highlight the importance of FGFR3 signaling in growth plate chondrocytes. The effects of constitutive FGFR3 activation in chondrocytes produces short long bones, abnormal facial structure (because some bones in the skull form by endochondral ossification), and vertebral anomalies (29). Thanatophoric dysplasia is

lethal because ribs abnormalities prevent proper lung development and do not facilitate sufficient inspiration (30, 31). Similar to humans, mice harboring FGFR3 gain-of-function mutations are dwarf, and mice with mutations causing severe constitutive activation of FGFR3 die shortly after birth, as with thanatophoric dysplasia (30, 32, 33). Conversely, global and bone specific *Fgfr3* knockout results in skeletal overgrowth (**Figure 3**) (34-36).

At the molecular level, *Fgfr3* is expressed in the resting, proliferation, and prehypertrophic zones of the growth plate (37). FGFR3 signaling has been reported to exert its effects in chondrocytes via the MAPK and STAT1 pathways (38-42). FGFR3 signaling inhibits chondrocyte proliferation directly via regulation of cell cycle proteins such as p21, as well as indirectly by decreasing IHH and BMP expression (32, 40, 43). Several studies have been performed to tease apart the respective roles of STAT versus MAPK signaling in growth plate chondrocytes. These studies indicate that the direct and indirect proliferative pathways are inhibited by STAT1 signaling while the MAPK signaling pathway impairs chondrocyte maturation (44). Others, however, have shown that MAPK signaling is capable of regulating *Ihh* expression suggesting that MAPK may also be important in regulating chondrocytes proliferation, and the respective roles of MAPK and STAT1 may not be clear cut as once thought (45). Regardless of mechanism, however, both constitutively active MEK in a STAT1-null background and deletion of *Spred2*, a negative regulator of Raf in the MAPK signaling axis are sufficient to cause dwarfism in mice thus showing that MAPK is critically important for proper growth plate elongation (44, 46).

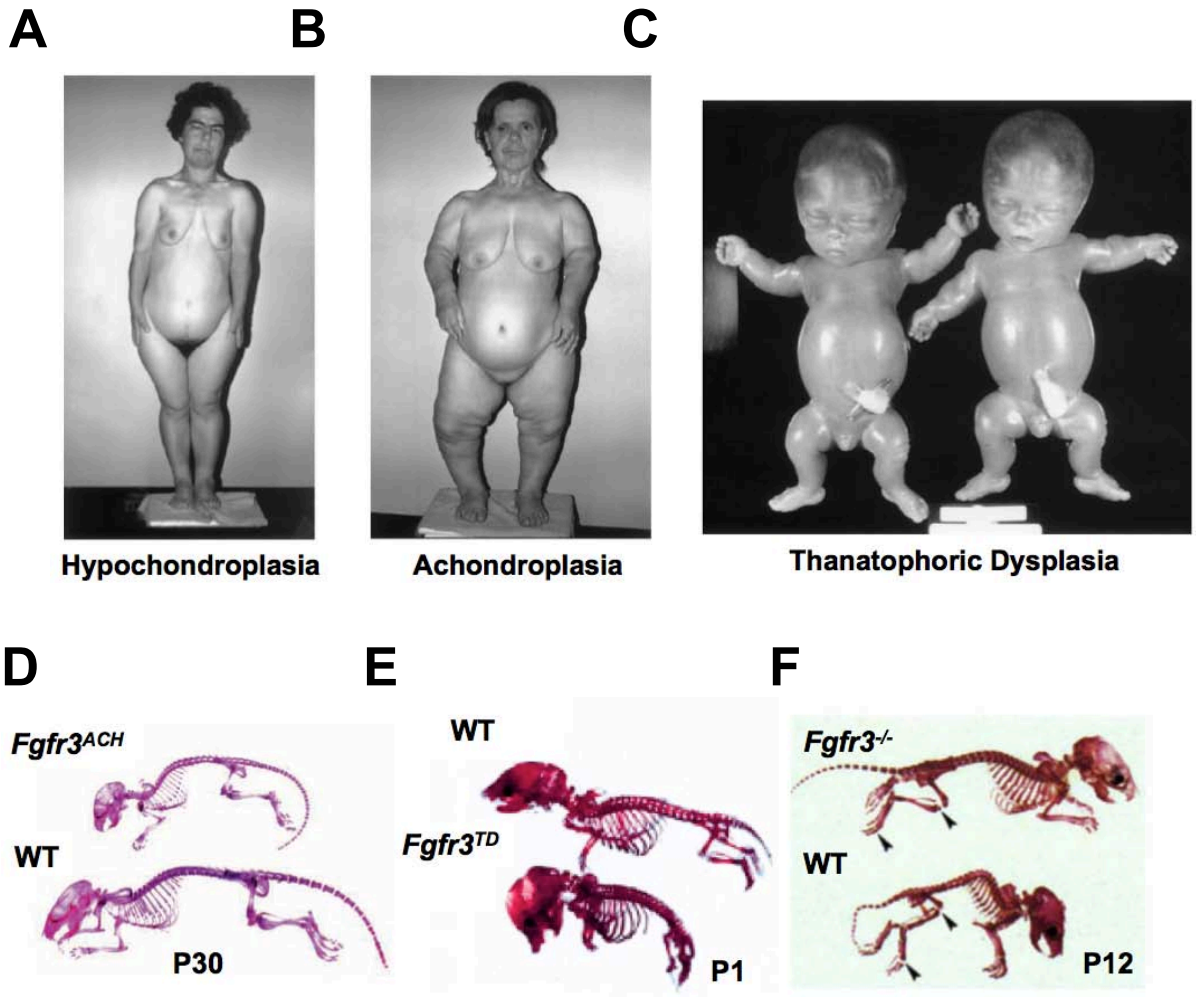

Figure 3. Manifestations of FGFR3 Gain- and Loss-of-Function Mutations in Humans and in Mice

Mutations causing varying degrees of constitutively active FGFR3 result in respective degrees of severity of dwarfism/short stature such as (**A**) hypochondroplasia, a mild form dwarfism/short stature. Note the normal physical appearance with relatively short limbs. More severe gain-of-function mutations causing more active FGFR3 results in (**B**) achondroplasia and the most severe gain-of-function mutations in FGFR3 cause the lethal (**C**) thanatophoric dysplasia. In mice, gain-of-function mutations in FGFR3 with varying degrees of severity result in (**D**) dwarfism (achondroplasia-like, shown at postnatal day 30) and (**E**) lethal dwarfism (thanatophoric dysplasia-like, shown at postnatal day 1), similar to humans. (**F**) Conversely, loss-of-function mutations in FGFR3 result in skeletal overgrowth (shown at postnatal day 12). Used with permission (30, 32, 35, 47).

FGFR1

In contrast to *Fgfr3*, *Fgfr1* expression is restricted to the prehypertrophic and hypertrophic zones of the growth plate (48, 49). Little research towards understanding the function of FGFR1 in the growth plate has been performed because, until recently, no long bone disorders related to FGFR1 signaling had been identified, but rather only one craniosynostosis causing syndrome (Pfeiffer syndrome) associated with FGFR1 gain-of-function mutation had been identified. In 2005, White and colleagues identified gain-of-function *FGFR1* mutations in patients with osteoglophonic dysplasia (50). In addition to presenting with craniosynostosis, osteoglophonic dysplasia patients are dwarf and have many abnormalities in bones formed by endochondral ossification including non-ossifying fibroma, bowing of longs bones, spontaneous fracture, and fracture healing deficits (pseudarthrosis) (**Figure 4**) (51). A study of the function constitutively active FGFR1 in cranial sutures noted that mice harboring a globally expressed, constitutively active *Fgfr1* allele in addition to having craniosynostosis, were severely dwarf (52). No follow up studies in the longs bones were performed. In 2006 however, Jacob and colleagues published the first and only study addressing the function of FGFR1 in the growth plate and long bones using a conditional loss-of-function approach. This study addressed the function of FGFR1 in osteoblasts as well as in chondrocytes. They found that the hypertrophic zone of the growth plate was expanded, which they concluded was likely due to decreased osteoclastogenesis and expression of gene responsible for matrix catabolism at the osteochondral border (49). The final downstream signaling effects of FGFR1 in the growth plate remain to be elucidated, however, it is known that FGFR1 exerts its effects via the PI3K and MAPK cascades (53-55).

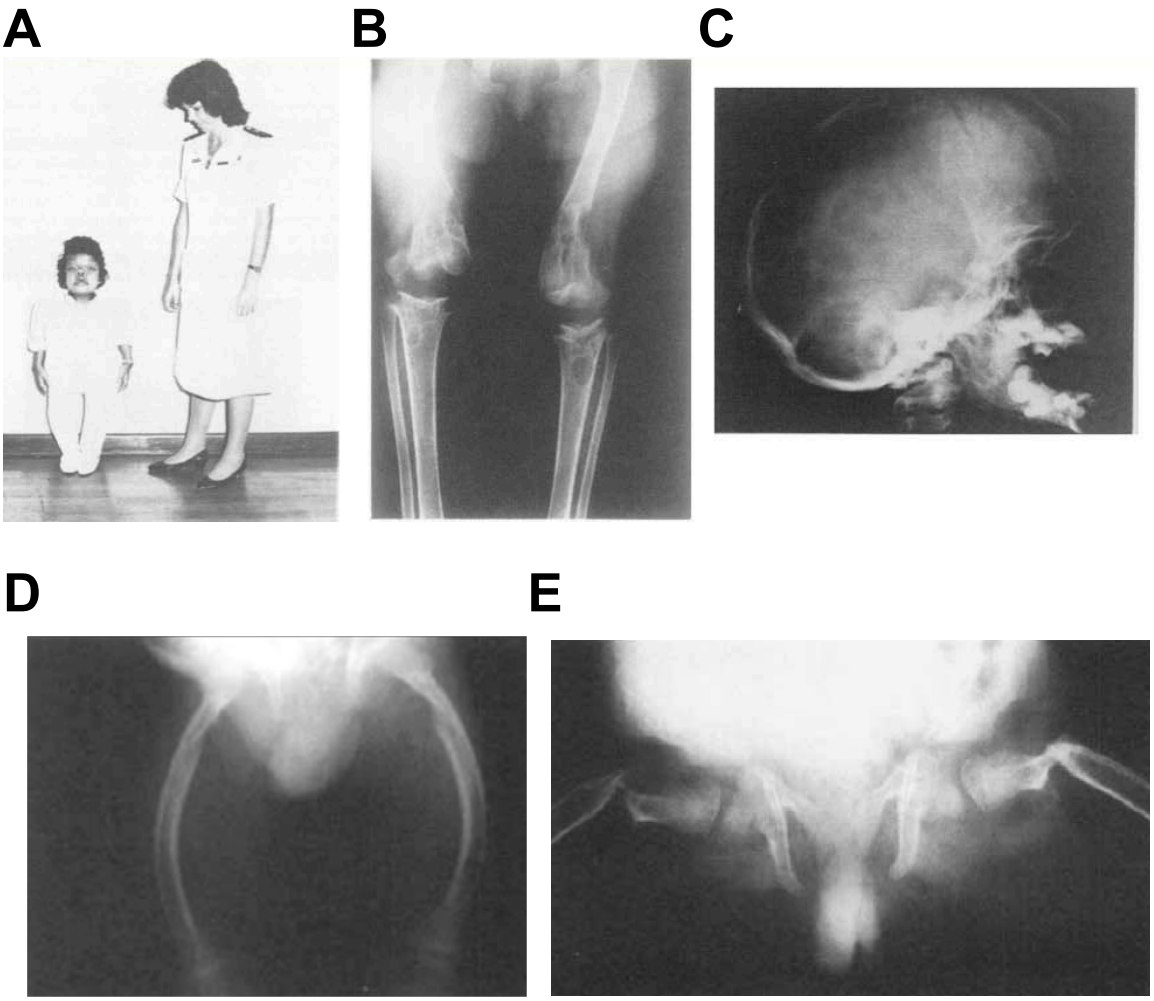

Figure 4. Osteoglophic Dysplasia and its Radiographic Findings in Humans. Osteoglophonic patients are (**A**) dwarf and have abnormal bone structure in bones formed both by endochondral and intramembranous ossification. (**B**) Long bones have abnormal shape as well as non-ossifying fribroma. (**C**) Bones formed by intramembranous ossification also exhibit abnormalities including craniosynostosis. (**D**) Osteoglophonic patients often have bowed long bones which (**E**) often spontaneously fracture and develop into pseudarthroses (bilateral femoral fractures in this case). Used with permission (51, 56, 57).

FGFR2

Fgfr2 is expressed in the perichondrium and resting zone of the established growth plate but is most widely expressed in the prechondrogenic mesenchymal condensation (48, 58). Studies addressing the function of FGFR2 in the growth plate have shown that gain-of-functions mutation in FGFR2 produce animals that are of short stature and have craniosynostosis; long bone and growth plate histological analyses were not the focus of these studies (59-61). However, loss-of-function studies of FGFR2 in the mesenchymal condensation with the *Dermo1*-Cre also yielded dwarf animals (62). In this study, the authors noted that chondrocyte proliferation was normal within the established embryonic growth plate but did not address proliferation within the mesenchymal condensation. They also documented the impairment of osteoblast proliferation and enhanced osteoclastogenesis at the osteochondral border suggesting that these abnormalities are responsible for the dwarfism phenotype. More studies are necessary to clearly elucidate the function of FGFR2 in the growth plate and mesenchymal condensation since both gain- and loss-of-function mutations in FGFR2 cause dwarfism.

FGFR4

Fgfr4 is expressed in the resting and proliferation zones of the growth plate, however, the function of FGFR4 in the growth plate remains unknown since global *Fgfr4* knockout mice have no overt skeletal phenotype (63, 64). Global *Fgfr3* and *Fgfr4* knockout mice, however, have short stature which is opposite of global knockout of *Fgfr3* alone (64). More studies, possibly aided with the use of conditional (floxed) alleles to rule of systemic effects of *Fgfr* knockout are warranted.

Postnatal Fracture Healing Recapitulates Embryonic Bone Development

It is now well understood that many tissue regenerative processes result from the reactivation of embryonic, developmental processes. Fracture healing is no different and has been described as a "postnatal developmental process" (65). As such, our understanding of endochondral ossification in the developmental setting is often able to inform our understanding of the events occurring in and regulating the more complex process of postnatal bone repair.

Fracture healing begins immediately following fracture when mesenchymal progenitors are recruited to the site of injury. It is widely believed that these progenitor cells are recruited from and are daughters of cells residing within the periosteum. A mix of both endochondral and intramembranous ossification occur which is believed to depend on proximity to the vascular supply at the site of injury. Immediately adjacent to the bone fragment at site of injury, and thus close to vascular supply, mesenchymal progenitors differentiation directly into osteoblasts and recruited mesenchymal progenitors located more distally to bone fragment, and thus with limited vascular supply and oxygen tension, differentiate into chondrocytes. The chondrocytes initially proliferate, bridge the broken bone, and connect the two bone fragments including the new bone formed via intramembranous ossification. As the vasculature from within the newly formed intramembranous bone invades into the soft, cartilaginous callus, the soft callus, proliferative chondrocytes mature to hypertrophy. Osteoblasts also invade as callus hypertrophic chondrocytes undergo apoptotic cell death. This process is an orderly transition from a cartilage dominated soft callus to a mineralized, bone dominated hard

callus which is subsequently remodeled by osteoclasts until it is fully resorbed and only bridged cortical bone remains (65-68). The fracture healing cascade is shown in **Figure 5**.

As in endochondral bone formation, FGFRs 1 and 3 have been suggested to play a role in fracture repair, although many reports primarily remain limited to the description of individual FGF expression and function or receptor expression during bone healing (69-72). From these reports, it seems that some FGFs stimulate cartilage formation during fracture healing by enhancing periosteal MSC proliferation (71, 72). FGFR1 and FGFR3 expression in soft callus chondrocytes is high during fracture healing (69, 73, 74) and one study suggested that activation of FGFR3 has deleterious effects on soft callus formation and maturation (73). No data about the role of FGFR1 during bone repair has been published.

Neurofibromin and Neurofibromatosis Type 1

An indirect regulator of ERK signaling is the Ras-GTPase activating protein (Ras-GAP) neurofibromin. Loss-of-function mutations in the *NF1* gene locus encoding the protein neurofibromin result in the rasopathy known as neurofibromatosis type 1 (NF1) (75-78). NF1 is the most common autosomal dominant genetic disorder occurring in 1 of every 3500 live births (79). NF1 morbidity ranges the spectrum from minor to severe, from occasional café au lait spots and Lisch nodules to widespread cutaneous and plexiform neurofibromas, optic nerve gliomas, malignant peripheral nerve sheath tumors,

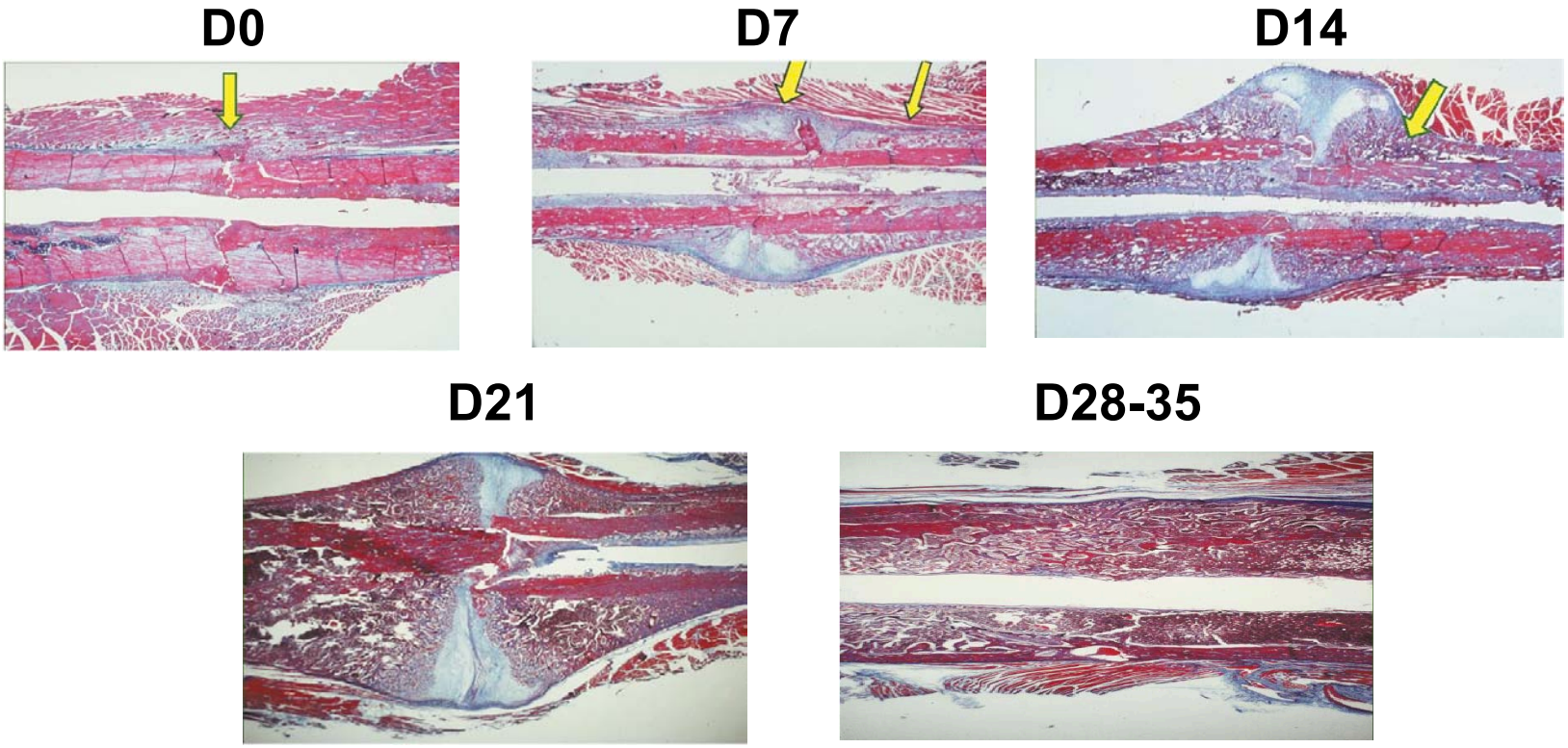

Figure 5. The Fracture Healing Cascade
Fracture healing begins immediately following injury at **D0** with the recruitment of inflammatory cells mesenchymal precursors to the site of injury. Arrow indicates site of fracture. At day 7 post-fracture **D7** early endochondral chondrogenic differentiation is visible (thick arrow) in the primordial fracture callus while intramembranous osteoblast differentiation is seen adjacent to the fracture site (thin arrow). At **D14** a robust cartilaginous response is seen in the callus center (soft callus) with a calcified cartilage front meeting osteoblasts formed by intramembranous differentiation (arrow). By **D21** post-fracture osteoblasts form the majority of the callus (hard callus) due to their replacement hypertrophic, calcified chondrocytes at the osteochondral front. The hard callus is continuously remodeled by osteoclasts and osteoblast until no remnants of the fracture site or callus can be seen between **D28-35**. Used with permission (66).

pheochromocytomas, cognitive deficits and skeletal dysplasias (80). Despite being nearly 100% penetrant (81), NF1 is a highly pleotropic disease, and the heterogeneity of the disease adds complexity to studying the mechanisms of NF1 pathogenesis (80). One prevailing theory of NF1 pathogenesis is that while patients are typically globally heterozygous null for *NF1*, only those cells and their progeny that experience complete inactivation of *NF1* are pathological, thus giving rise to a highly variable and often focal disease (5, 82, 83). Germline, homozygous *NF1* loss of function is thought to be embryonically lethal because no patients have been seen with this mutation pattern and because $Nf1^{-/-}$ mice die *in utero* (84).

The *NF1* gene locus was first identified in the late 1980s through linkage analysis in families with NF1 (75-78). A series of papers in the following few years further identified chromosomal translocations and gene mutations at the *NF1* locus as causal for NF1 (85-90). Simultaneous studies showed that the gene product of the *NF1* locus encoded a Ras GTPase activating protein (Ras GAP) that had an unusually large open reading frame of over 7.7kb encoding a ~250kD protein (91-97). These landmark papers dating back to the late 1980s and early 1990s opened the field for studies for the etiology of NF1. It has since been shown that neurofibromin's expression is widespread and is expressed in the cardiovascular system, adrenal glands, central and peripheral nervous systems, as well as in both muscle and bone (95, 98, 99). Research related to neurofibromin's function in disease to has primarily focused on its function as a Ras-GAP and effectors in the MAPK and PI3K pathways. Others have, however, hypothesized and studied non-Ras-GAP related functions of neurofibromin including its

role as a regulator of andenylyl cyclase and as a microtubule associated protein in neurons (**Figure 6**) (100-106).

Bone Manifestations

Of particular importance to my studies are the skeletal manifestations of NF1. These abnormal pediatric skeletal maladies are both global and focal. The global maladies include short stature and low bone mineral density while the focal maladies can include idiopathic or dystrophic scoliosis, kyphosis, chest wall abnormalities, asymmetry of facial bones (sphenoid bone), non-ossifying fibromas, unilateral, anterolateral tibial bowing, and pseudarthrosis (**Figure 7**) (1, 107-111). Approximately 40% of NF1 patients display focal skeletal pathologies (112, 113). Children with NF1 typically have elevated fracture risk and exhibit impaired fracture healing stemming from suspected pre-existing defects in bone formation (112, 113). Fracture and development of pseudarthrosis has significant patient morbidity as development of pseudarthrosis frequently requires multiple surgical interventions that often fail to promote bone union, and can require limb amputation (114-116). Biopsies from children with tibial NF1 pseudarthrosis are characterized by a hyperplastic hamartoma composed of poorly differentiated vimentin-positive mesenchymal cells and cartilaginous remnants, suggesting that the differentiation, maturation, and/or clearance of *Nf1*-deficient osteochondroprogenitors and progeny is severely impaired, leading to the accumulation of this abnormal tissue in bone and poor healing (5, 8). Pseudarthrotic sites are also characterized by the presence of an excessive amount of unmineralized type 1 collagen (osteoid) and an increased number osteoclasts (118).

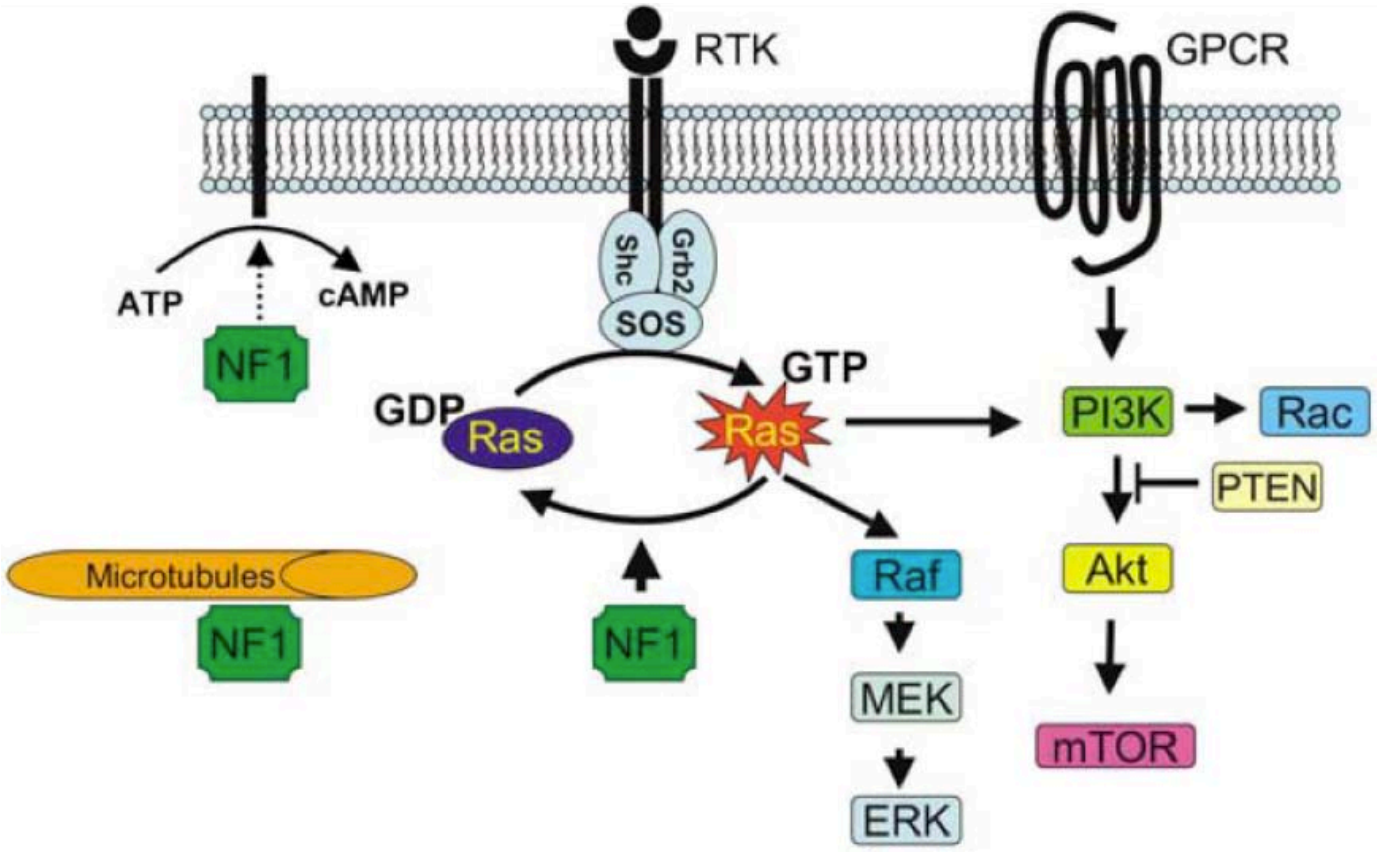

Figure 6. The Functions of Neurofibromin
Neurofibromin's canonical function is to accelerate the hydrolysis of Ras-GTP to Ras-GDP and thus inactivating Ras. Active Ras is known to activate both the MAPK and PI3K signaling pathways. Some known non-canonical roles of neurofibromin include simulating adenylyl cyclase activity in its conversion of ATP to cAMP as well as associating with microtubules in neurons. Modified and used with permission (117).

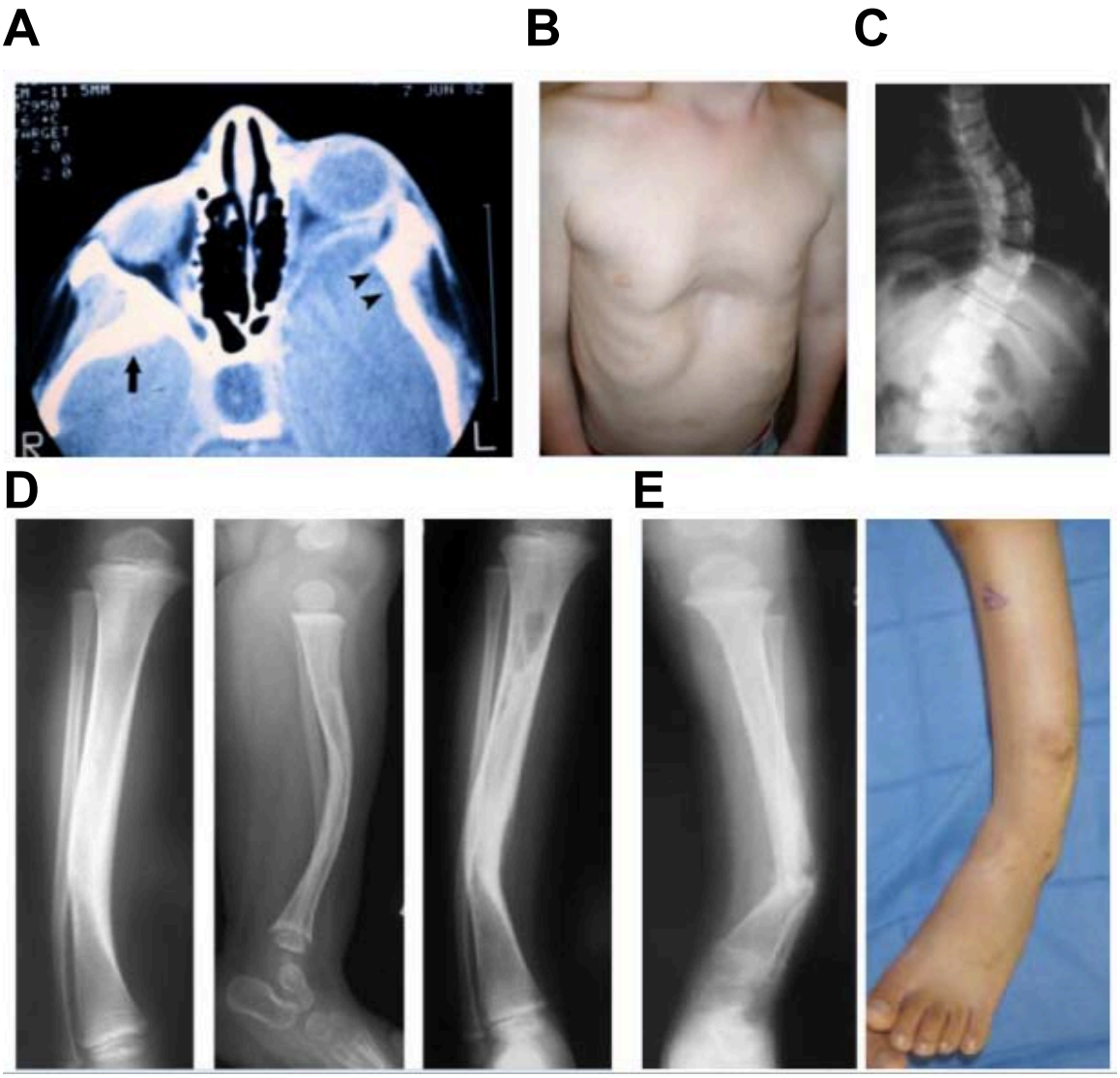

Figure 7. Focal NF1 Bone Manifestations
Focal NF1 bone manifestations include: (**A**) sphenoid wing dysplasia with the left side of the bone (arrowheads) showing abnormal structure compared to the contralateral side (arrow), (**B**) pectus excavatum, (**C**) scoliosis (both non-dystrophic, shown, and dystrophic) and kyphosis (not shown), (**D**) anterolateral bowing of the tibia (note presence of non-ossifying fibroma in the proximal tibia in the third panel), and (**E**) pseudarthrosis (radiograph, left, and physical appearance, right). Used with permission (1).

Mouse Models of the Role of Neurofibromin in Bone

In the pursuit of understanding the role of neurofibromin in bone, several approaches have been taken ranging from *in vitro* studies of human derived cells harvested from pseudarthrotic tissues to the development of mouse models to study neurofibromin *in vivo* as well as *in vitro*. Studies using human cells have been hindered by difficulty of obtaining pseudarthrotic tissues as well as isolating/culturing *Nf1*-null cells from pseudarthrotic tissue. Thus, most of our understanding of NF1's function in bone has been derived from mouse studies where genetic manipulation and availability of tissues is ethically feasible and much easier. Because most of these studies require homozygous deletion of *Nf1*, their findings must be taken with caution (since patients are heterozygous), however this observation itself has informed our understanding of NF1 bone etiology. While reading the following sections, it is important to note that *Nf1* is expression in developing and healing bone is restricted to osteoblasts of all differentiation stages, osteoclasts, and to prehypertrophic and hypertrophic growth plate chondrocytes (99, 119) and that the use of tissue and temporally specific cre recombinases to ablate *Nf1* is a powerful approach to elucidating the function of neurofibromin in each bone cell type and at a particular stages of differentiation/maturation (**Figure 8**). Furthermore, Elefteriou and Yang recently reviewed bone specific cre recombinase activity and specificity and the strengths/weaknesses associated with conditional knockout approaches (120).

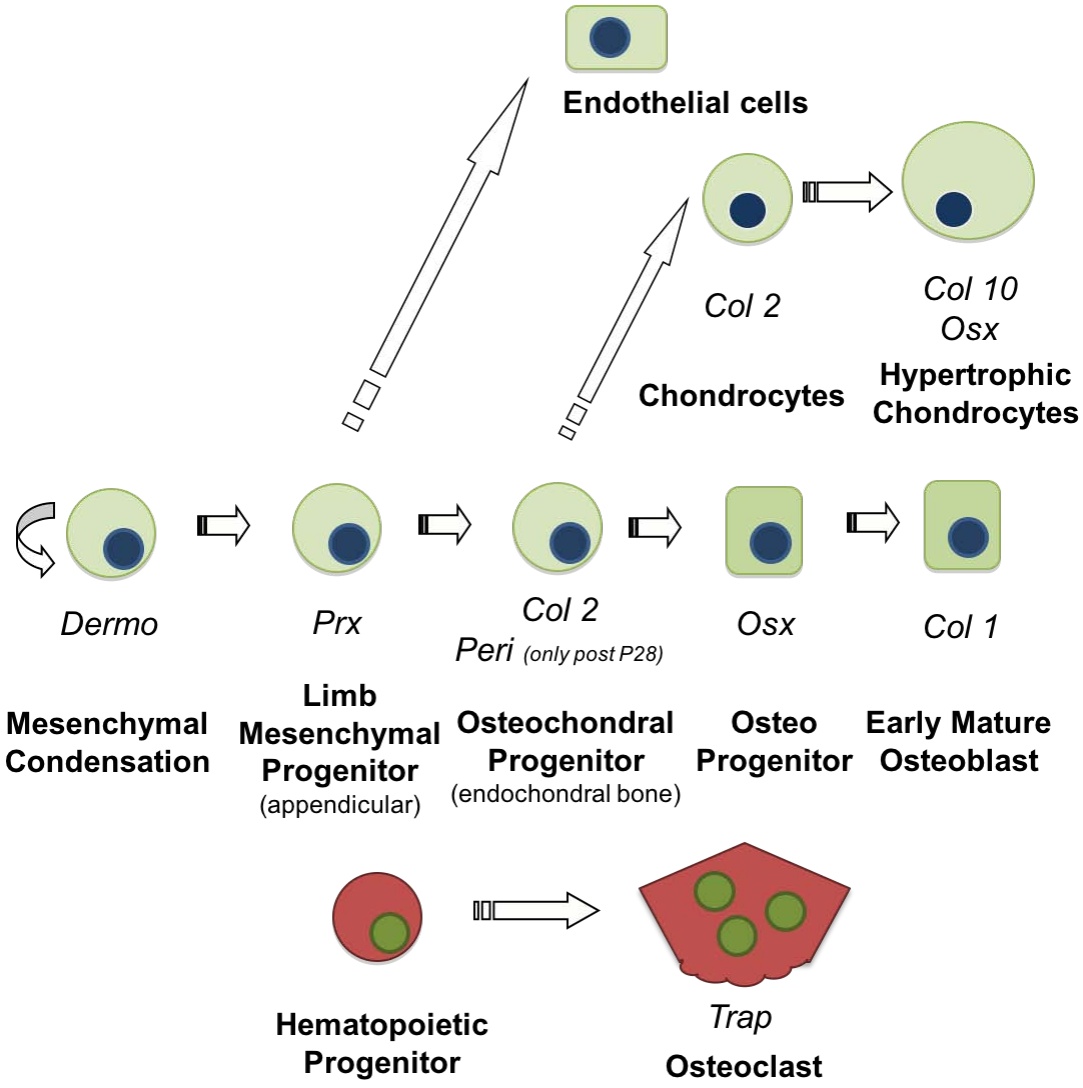

Figure 8. Cre Specificity in Skeletal Cells and Their Precursors
Promoters specific for various skeletal cell types have been used to conditionally drive cre recombinase expression to delete the floxed *Nf1* allele in mice. Shown here are the specific lineages and skeletal cell types that have been targeted for *Nf1* deletion in bone.

Nf1$^{+/-}$ *Mice*

The function of neurofibromin was first studied *in vivo* in mice in 1994 when two groups published the embryonic lethality at E14 with global *Nf1* deletion due to cardiac malformation (84, 121). Interesting, *Nf1* heterozygous mice have an elevated predisposition to various types of neoplasia as well other nonmalignant conditions observed in NF1 but display mostly normal bone development *in vivo* (121-124). *Nf1*$^{+/-}$ osteoblasts and osteoclasts have shown some minor molecular abnormalities that seem relevant to the NF1 pseudarthrosis, however, *Nf1*$^{+/-}$ mice have only a slightly impaired fracture healing phenotype (123-127). Delayed fracture healing in *Nf1*$^{+/-}$ mice was dependent on the fracture location within the bone; mid-shaft tibial fractures healed normally in both *Nf1*$^{+/-}$ and WT while distally fractured tibiae, as often occurs in patients, exhibit impaired healing versus WT (which were also delayed compared with their mid-shaft fracture, WT counterparts) (127).

Because *Nf1*$^{+/-}$ mice mostly did not exhibit phenotypes similar to those seen in NF1 patients and because *Nf1*$^{-/-}$ mice are not viable, conditional *Nf1* alleles were generated to enable the study of neurofibromin loss-of-function in specific tissues. The conditional *Nf1* allele has the sequence encoding neurofibromin's GAP domain flanked with *loxP* sequences targeting it for cre recombinase mediated deletion (128). It is unclear whether the non-GAP domain portions of the protein are translated in cre recombinase expressing tissues and progeny. Studies of tissue targeted *Nf1* deletion have revealed that, in some tissues, NF1 pathology requires an *Nf1* heterozygous genetic background (*Nf1*$^{+/-}$) to develop NF1 pathologies (129-132). In bone, however, most studies have shown that *Nf1*

loss-of-function in osteochondral progenitor cells leads to pathologies analogous to what is seen in *Nf1* pseudarthrotic tissues suggesting that a second-hit inactivation alone is sufficient for NF1 skeletal pathology (4, 133, 134). The role of the $NF1^{+/-}$ microenvironment and its contribution to focal NF1 skeletal dysplasia is, however, an active area of research.

$Nf1_{Col1}^{-/-}$ Mice

The first study using conditional knockout strategies to address the function of neurofibromin in bone was published in 2006 by Elefteriou and colleagues (135). These studies used the 2.3kb *Col1*-Cre recombinase under the control of an osteoblast specific enhancer of the *Col1a1* promoter making *Nf1* deletion specific to mature osteoblasts (136). Interestingly, these studies found that $Nf1_{Col1}^{-/-}$ mice had a high bone mass phenotype, as opposed to a low bone mass phenotype observed in NF1 patients. However, it was found that $Nf1^{-/-}$ mature osteoblasts produced copious amounts of unmineralized type 1 collagen (osteoid) which mirrored osteoidosis seen in NF1 pseudarthrotic biopsies (118, 135). $Nf1_{Col1}^{-/-}$ mice also had increased osteoclastogenesis that was dependent on osteoblast derived *Rankl* (135).

Following the initial *in vivo* and *in vitro* characterization of $Nf1_{Col1}^{-/-}$ bones and osteoblasts, Wang and colleagues further characterized the function of neurofibromin in fracture callus mature osteoblasts (137). These studies found that $Nf1_{Col1}^{-/-}$ calluses were significantly larger than WT calluses and lacked osteoclast mediated remodeling due to the presence of excessive osteoid (though osteoclastogenesis is enhanced in $Nf1_{Col1}^{-/-}$

mice, osteoclasts cannot resorb osteoid). Lack of callus remodeling led to a delay of fracture union/healing in $Nf1_{Col1}^{-/-}$ mice. Together, the two studies in $Nf1_{Col1}^{-/-}$ mice revealed important functions of neurofibromin in mature osteoblasts, however, the phenotypes of these mice indicated that the human NF1 pseudarthrotic phenotype is most likely not predominantly attributable to mature *Col1a1*-positive osteoblasts.

$Nf1^{f/-}$;$Nf1_{Col1}^{-/-}$ and $Nf1^{f/-}$;$Nf1_{Peri}^{-/-}$ Mice

Recently however, researchers in Dr. Feng-Chun Yang's laboratory generated $Nf1_{Col1}^{-/-}$ and $Nf1_{Peri}^{-/-}$ mice on an $Nf1^{+/-}$ background to investigate the effects of the *Nf1* heterozygous background on skeletal development and fracture healing. The *Periostin* driven cre recombinase is a relatively uncharacterized cre that is not commonly used but recombines to some extent in MSC progenitors residing in the periosteum only after 4 weeks of age (129); this cre recombinase was used in an attempt to address the function of *Nf1* in an MSC rich bone compartment and elucidate the function of neurofibromin in bone MSCs rather than in mature osteoblasts. No studies on $Nf1_{Peri}^{-/-}$ mice with an $Nf1^{+/+}$ background have been published. These studies showed that both the $Nf1^{f/-}$;$Nf1_{Col1}^{-/-}$ and $Nf1^{f/-}$;$Nf1_{Peri}^{-/-}$ mouse models of NF1 exhibited a low bone mass phenotype, fracture healing deficits and skeletal abnormalities including scoliosis and vertebral body shortness (129, 138, 139), similar to what has been observed in NF1 patients. Interestingly, however, these studies also showed that $Nf1_{Col1}^{-/-}$ mice with an $Nf1^{+/+}$ (WT) background had no changes in bone mass or fracture healing contradicting studies by Elefteriou, *et al*. (135, 137). No explanations were offered for these important contradictions. These studies, however, suggest a role for the haploinsufficient bone

microenvironment of NF1 patients and its non-cell-autonomous effects on bone development and *Nf1*-null bone cells. Further confirmation of the importance of the *Nf1* heterozygous genetic background is necessary, however, as our laboratory has been unable to demonstrate the importance of the $Nf1^{+/-}$ background *in vitro*, in $Nf1_{Col1}^{-/-}$ mice, or in studies using other cre drivers of recombination (unpublished data). Finally, our laboratory and others have demonstrated that an *Nf1* haploinsufficient genetic background is not necessary to develop NF1 characteristic orthopedic defects in mice (see below) and therefore, that neurofibromin can function in a cell-autonomous fashion to this end.

$Nf1_{Prx}^{-/-}$ Mice

The *Prx*-Cre transgene was used to address the function of neurofibromin in osteochondroprogenitor cells because the bone phenotypes of $Nf1_{Col1}^{-/-}$ mice did not accurately model human NF1 orthopedic manifestations. The *Prx*-Cre transgene is active in limb bud mesenchymal cells that give rise to chondrocytes, osteoblasts, endothelial cells, and muscle in the appendicular skeleton. Bone development in $Nf1_{Prx}^{-/-}$ mice was severely impaired. $Nf1_{Prx}^{-/-}$ mice were shorter than WT littermates, had growth plate abnormalities including shortened proliferation and hypertrophic zones, as well as enhanced osteoclastogenesis at the osteochondral border. Furthermore, these mice developed tibial bowing and cortical porosity (133).

Bone healing studies in $Nf1_{Prx}^{-/-}$ mice were also performed using two models of bone repair: a cortical defect of the tibia model and closed femoral fracture (3, 140). In both

models, bone repair was severely impaired in these mice and showed signs of the fibrous, hyperplastic hamartoma seen in human NF1 pseudarthroses (3, 140). The $Nf1_{Prx}^{-/-}$ mouse seems to be a better model of NF1 skeletal dysplasia over the $Nf1_{Col1}^{-/-}$ model, however several shortcomings of this model include the fact that the *Prx* transgene is active in cells that give rise to skeletal muscle making it difficult to exclude the role of *Nf1*-null muscles in the $Nf1_{Prx}^{-/-}$ phenotype, especially given that *Nf1* deficient muscle has its own distinct phenotype and skeletal loading and disuse have important effects on the skeleton (141, 142). Also, fracture studies in animals that have significantly different statures are difficult to interpret given the intrinsic variances of bone loading. Finally, the appendicular specificity of *Prx*-Cre to the appendicular skeleton only did not allow studies related to role of neurofibromin in the axial skeleton, where abnormalities such as sphenoid wing dysplasia and scoliosis have been observed.

$Nf1_{Col2}^{-/-}$ Mice

To further study the role of neurofibromin in osteochondral progenitor cells, Wang and colleagues turned to the *Col2a1* driven cre recombinase (4). These studies benefit from the fact that the *Col2*-Cre is active in the axial skeleton in addition to the appendicular skeleton, but not active in muscle progenitor cells, and thus, only recombines floxed sequences in osteochondral progenitors that give rise to the chondrocytes and osteoblasts of bones formed by endochondral ossification (4, 143).

$Nf1_{Col2}^{-/-}$ mice exhibit many phenotypes observed in NF1 skeletal dysplasia, especially those observed in the axial skeleton not previously observed in $Nf1_{Prx}^{-/-}$ mice. $Nf1_{Col2}^{-/-}$

mice exhibit progressive, dystrophic scoliosis and sternal abnormalities possibly analogous to the pectus excavatum seen in NF1 patients. $Nf1_{Col2}^{-/-}$ mice also display abnormal nucleus pulposus structure; this has not been studied in patients but is potentially related to the progressive scoliosis seen in NF1 populations. $Nf1_{Col2}^{-/-}$ mice also exhibited craniofacial abnormalities most likely caused by defective elongation of cranial bones that form by endochondral ossification; this is possibly relevant to the macrocephaly and sphenoid wing dysplasia skeletal manifestations sometimes observed in NF1 patients.

The findings in the $Nf1_{Col2}^{-/-}$ appendicular skeleton were similar to those found in $Nf1_{Prx}^{-/-}$ appendicular bones. Wang and colleagues found tibial bowing, osteoidosis, and cortical porosity in $Nf1_{Col2}^{-/-}$ bones. Furthermore, they were the first to demonstrate that NF1 patients can also have increased cortical porosity as opposed to unaffected individuals (4). *In vitro* studies of $Nf1_{Col2}^{-/-}$ bone marrow derived osteoblasts also showed that *Nf1* loss-of-function in osteoprogenitors impaired their differentiation into mature osteoblasts and lead to aberrant expression of genes promoting to formation of pyrophosphate, a known inhibitor of matrix mineralization (134). Of note, it was also found that $Nf1_{Col2}^{-/-}$ mice were severely dwarf and died shortly after weaning, thus limiting their usefulness in preclinical fracture healing studies (4).

The $Nf1_{Col2}^{-/-}$ model of NF1 skeletal dysplasia, though not ideal for preclinical fracture studies, is still extremely useful for developmental studies of neurofibromin's function during endochondral ossification due to the specificity and efficiency of the type 2

collagen cre driver. Characterization of the growth plate in $Nf1_{Col2}^{-/-}$ mice is the subject of Chapters II and III of this dissertation. Application of these findings in a more suitable, non *Col2*-Cre preclinical model of NF1 fracture healing/pseudarthrosis is currently being performed and will be discussed in Chapter IV.

$Nf1_{Osx}^{-/-}$ Mice

A new preclinical model of NF1 pseudarthrosis using the inducible *Osx*-Cre transgenic mouse was recently published by our group (134, 144). The inducible *Osx*-Cre mouse uses a Tet-off system to induce cre recombinase transcription. In other words, upon withdrawal of tetracycline or its derivatives, genetic recombination of *loxP*-flanked sequences is initiated. This *Osx*-Cre is active in osteoprogenitors in bones formed both by intramembranous and endochondral ossification, as well as in the periosteum and hypertrophic chondrocytes (145). Because it is inducible and specific to osteoprogenitors, it is also useful for the study of neurofibromin function in osteoprogenitors during fracture healing.

N'Dong and colleagues were the first to use this cre deleter mouse to ablate *Nf1 in vivo* in adult mice. Adult induced $Nf1_{Osx}^{-/-}$ mice were of normal size, but their bones exhibited poor biomechanical properties and had elevated cortical porosity. Furthermore, $Nf1_{Osx}^{-/-}$ bone marrow derived osteoblasts failed to properly differentiate and mineralize *in vitro*, highlighting the necessity of neurofibromin in osteoprogenitor to promote differentiation into mature, functional osteoblasts (134, 146).

N'Dong and colleagues went on to use $Nf1_{Osx}^{-/-}$ mice in a preclinical fracture study and locally administered the MEK inhibitor trametinib in combination with BMP-2 to promote bone union (146). They found that combination treatment promoted healing in $Nf1_{Osx}^{-/-}$ calluses better than individual treatment with trametinib or BMP-2 alone. This combination treatment was also found to be effective in promoting the differentiation of $Nf1^{-/-}$ bone marrow derived osteoblasts. These *in vivo* studies, while promising, did not analyze callus histology or address the efficacy of trametinib or BMP-2 on chondrocytic, soft callus formation or its structure. Also, because the *Osx*-transgene is active in hypertrophic chondrocytes (145, 147), and if used to inactivate *Nf1* early in development yields short mice (unpublished data), it remains to be addressed whether the *Osx*-Cre transgene recombines in osteochondroprogenitors or only osteoprogenitors contributing to the fracture callus. Periosteal expression of the *Osx*-Cre transgene and the short stature of perinatally induced $Nf1_{Osx}^{-/-}$ mice suggests the former and emphasizes the preclinical applicability of this cre recombinase for NF1 fracture healing studies. It also remains to be investigated, whether $Nf1_{Osx}^{-/-}$ mice with recombination induced perinatally develop profound axial and craniofacial skeletal phenotypes as observed in $Nf1_{Col2}^{-/-}$ mice.

$Nf1_{Trap}^{-/-}$ Mice

Because $Nf1^{+/-}$ osteoclast precursors were previously shown to exhibit hypersensitivity to pro-osteoclastogenic signals (M-CSF and RANKL) (124, 126), the *Trap*-Cre transgene was used to ablate *Nf1* in osteoclasts (148). $Nf1_{Trap}^{-/-}$ had only a mild bone phenotype, with some areas of enhanced resorptive activity but did not exhibit a low bone mass phenotype. *In vitro* analysis of $Nf1_{Trap}^{-/-}$ osteoclasts, however, showed enhanced

resorption on bone slices (148). The authors do, however, suggest that though $Nf1_{Trap}^{-/-}$ mice only had a mild phenotype, further studies of $Nf1_{Trap}^{-/-}$ mice with a global $Nf1^{+/-}$ background are necessary to conclusively evaluate the relative contribution of *Nf1*-null osteoclast to NF1 bone pathology. However, further investigation of the function of neurofibromin in osteoclasts should carefully reconcile the relative contribution of its findings with the clearly demonstrated, osteoblast-dependent, pro-osteoclastogenic phenotypes already reported in the $Nf1_{Col1}^{-/-}$ and $Nf1_{Prx}^{-/-}$ models which have significant relevance to NF1 bone manifestations found in patients (133, 135).

NF1 Pseudarthrosis Treatment and Prevention

Understanding the etiology of NF1 pseudarthrosis in pursuit of identifying new pharmacological targets in its treatment is the ultimate goal of this dissertation. As such, it is necessary to review the current standard-of-care for NF1 pseudarthrosis and highlight its shortcomings in order to adequately frame the discussion of proposed therapeutic strategies. In general, the goal of treating NF1 pseudarthrosis is to not only promote fracture healing but also to prevent its recurrence by improving bone health and function. Recent research, from our laboratory and others, has also set the stage for the repurposing of current drugs on the market or in trials for use in NF1 pseudarthroses. I will close this chapter with a discussion of these theoretical treatments, their supporting preclinical data from NF1 mouse models and also potential pitfalls associated with their use.

Current Strategies

Current strategies to treat NF1 pseudarthrosis are multi-factorial and in general aim to (i) prevent facture fracture from occurring, (ii) promote healing via surgical intervention, and (iii) promote healing via pharmaceutical intervention at the time of fracture and during surgery.

Prevention

The current strategies in treating NF1 pseudarthrosis begin even before skeletal fracture occurs. Prevention of fracture by bracing of bowed limbs may be best strategy in avoiding the significant morbidity associated with the NF1 pseudarthrosis. However, patient compliance remains an issue with long-term bracing and no prospective studies for its effectiveness have been conducted (149-151).

Surgical Intervention

If fracture does occur, surgical intervention is required to promote bone union and prevent refracture. However, there is little consensus as to the best surgical approach, and studies of appropriate statistical power remain difficult to conduct due to the relative rarity of NF1 pseudarthrosis in the general population. In general, there are two distinct components to successful treatment of NF1 pseudarthrosis, both of which are non-targeted and non-pharmacological in nature. These two components include: (i) surgical removal of fibrotic hamartomatous tissues surrounding the fracture site (including visibly thickened/diseased periosteum) and excision of diseased stumps of the fracture bone and (ii) rigid fixation of the fracture site (2, 114, 152-157). Though these surgical strategies

are generally agreed upon, the particulars of these treatments remain unstandardized and debated within the orthopedic community (2, 157). For example, the amount of normal appearing bone adjacent to the non-union site to be to surgically debrided remains unclear as does the type of rigid fixation type (Ilizarov external versus intramedullary, or use of both types) and whether rigid fixation should involve any of the numerous types of autogenous bone grafts (cancellous versus cortical versus vascularized free fibular/rib transfers and with or without the use of periosteal grafts) into the fracture site remains debated. Finally, the timing of when surgeries are performed is controversial, as some surgeons have claimed that surgical intervention before the age of 5 is associated with higher rates of healing failure and refracture (see (157) for review).

Pharmaceuticals: BMPs and Bisphosphonates

There is current anecdotal evidence for the off-label use of local BMP-2/BMP-7 soaked sponges and/or systemic bisphosphonates as pharmacological adjuvants in NF1 pseudarthrosis treatment supported by one preclinical mouse study (153, 158, 159). Nitrogenous bisphosphonates hypothetically should target not only osteoclasts within pseudarthrotic tissues, but could also possibly act on other, non-osteoclast, $NF1^{-/-}$ cells in the fracture site because of their activity in preventing post-translational modifications required for Ras activity (2, 160). The rationale behind the use of BMPs in pseudarthrotic tissue lies in BMPs osteoinductive properties and the fibrotic, hamartomatous tissue present in pseudarthroses (suggestive of a lack of osteochondral differentiation). It is important to note, however, that studies using BMP-7 for NF1 pseudarthrosis treatment have not observed statistically significant improvements in bone

union over the lack of their use in the long term mostly because refracture remained a persistent problem (159, 161). Similar results have been obtained in studies addressing combined used of BMP-2 with bisphosphonates where even combined treatment did not improve bone union or union maintenance over their lack of use (162-165). However, interpretation of these studies is difficult because non-pharmacological, surgical interventions were not standardized, and varied between many of the patients in these studies.

There are other potential pitfalls/caveats to the use of bisphosphonates aside from their potential lack of efficacy. As for bisphosphonates, it is unclear whether their use in skeletally immature children has negative effects on skeletal growth as has been demonstrated in rats (166, 167) (though not in mice (168)) and especially in a population already prone to short stature. As for use of BMPs, high-dose BMP therapy in a population prone to neoplasia may be contraindicated for bone-union as there are reports of at least two patients developing neoplasia following treatment with BMP-2 for NF1 pseudarthrosis though these reports remain limited to anecdotal case reports (169, 170).

Even with modern surgical and therapeutic interventions to promote healing in NF1 pseudarthroses, fracture healing failure and persistent pseudarthrosis still occur and are often only ameliorated by repeated surgical interventions or amputation (2). Thus, recalcitrant NF1 fracture healing necessitates the development of new therapies and techniques to prevent and treat existing NF1 pseudarthrosis.

Proposed Therapeutic Strategies

In the pursuit of new, targeted pharmacological therapies to promote bone union in NF1 pseudarthrosis, our laboratory and others have focused on not only understanding the molecular etiology of recalcitrant bone healing in NF1, but also on novel ways to deliver agents to bone that could have deleterious effects in pediatric populations if administered systemically.

Asfotase-α

Because human NF1 pseudarthrotic tissues are hypomineralized (osteoidosis) (118), and because several different mouse models are also osteoidotic ($Nf1_{Col1}^{-/-}$, including fracture calluses, $Nf1_{Col2}^{-/-}$, and $Nf1_{Prx}^{-/-}$) (4, 133, 135, 137), our laboratory hypothesized that pyrophosphate (PPi), a potent inhibitor of mineralization, and its accumulation may be the cause of osteoidosis seen in NF1 mouse models and NF1 pseudarthrotic tissues. We showed that genes regulating PPi production and extracellular localization are indeed dysregulated in $Nf1^{-/-}$ osteochondroprogenitors in mice and in tissues from human NF1 pseudarthroses (134). Furthermore, treatment of $Nf1_{Col2}^{-/-}$ and $Nf1_{Osx}^{-/-}$ mice with a recombinant, soluble, and bone localizing form of alkaline phosphatase (asfotase-α (171)) dramatically improved biomechanical properties in these mouse models (134). We therefore believe that improving mineralization may be a useful therapeutic strategy in preventing fracture in NF1 patients by improving mineralization and possibly preventing tibial bowing (134). It remains to be confirmed whether tibial bowing or cortical porosity in NF1 patients is caused by excessive production and accumulation of pyrophosphate. Because matrix composition, rigidity and stiffness have all been shown to influence

osteoprogenitor and mesenchymal progenitor cell differentiation into mature osteoblasts (172-174), the effects of asfotase-α on osteoprogenitor differentiation are also of interest since administration of exogenous alkaline phosphatase "artificially" promotes the mineralization of the collagen matrix laid down by osteoprogenitors that possess inherent difficulties in differentiation. Whether the mineralized matrix can promote cellular differentiation of *Nf1*-null osteoprogenitors is an intriguing question to be experimentally addressed. Finally, the use of asfotase-α in fracture healing setting also warrants further investigation as preventative use of asfotase-α may not be possible in all circumstances and especially because some clinicians may be weary using the drug prior to fracture due to the possibility of developing ectopic calcification (even though this side effect of asfotase-α has not been observed in its clinical trials for fatal, familial hypophosphatasia (175)).

Tgf-β Signaling Inhibitors

Many of the defects our group and others have seen in osteochondroprogenitors suggest that aberrant TGF-β signaling could be responsible for the bone phenotypes observed in NF1 patients and mouse models. TGF-β signaling is highly complex in bone residing cells, often having differing effects depending on cell type and cell differentiation status (176). The current understanding of TGF-β signaling in bone mesenchymal cells (BMCs) suggests that TGF-β promotes BMC proliferation and migration while inhibiting their differentiation into osteoblasts and other functional bone cells (177). Furthermore, TGF-β promotes osteoclastogenesis via diverse mechanisms (178-181). Because NF1 pseudarthrotic tissues are believed to exhibit impaired osteoblast differentiation, have

excessive osteoclast presence and have shared phenotypic manifestations with other orthopedic disorders with known TGF-β dysregulation, Rhodes and colleagues hypothesized that TGF-β signaling was also dysregulated in NF1 pseudarthroses and affected bone tissues (182). Using the $Nf1_{Col1}^{-/-}$ mouse model, they found that TGF-β was overexpressed *in vitro* and *in vivo* and its respective signaling pathways were overactive. They also showed that the fracture healing deficits of $Nf1_{Col1}^{-/-}$ mice could be improved with the TGF-β receptor 1 small molecule inhibitor SD-208 (182).

The use of drugs and biologics (such as the anti-TGF-β antibody 1D11) to block TGF-β signaling is controversial however because of TGF-β's anti-tumor and anti-metastatic properties and especially given that individuals with NF1 are already prone to various forms of neoplastic transformation (183). Local delivery and retention of TGF-β inhibitors would therefore be extremely critical to moving forward with this approach. Equally important however, it remains to be determined whether TGF-β signaling is also dysregulated in osteochondroprogenitors as it is this population of cells (and not mature osteoblasts targeted in the $Nf1_{Col1}^{-/-}$ model) that seems most relevant to human NF1 orthopedic pathology.

Statins

As discussed above relating to the use of bisphosphonates in NF1 pseudarthrosis, targeting post-translational modification pathways that are necessary for Ras activity is a potential strategy for treating NF1 pseudarthroses. This strategy directly targets the root pathway dysregulated in NF1 as opposed to downstream effects of aberrant Ras signaling.

Statins are a class of drug commonly used to inhibit the synthesis of cholesterol via inhibition of HMG-CoA reductase. This same pathway is necessary to produce farnesyl lipid groups that are post-translationally added to Ras to target it to the inner leaf of the cell surface membrane and allow interaction with the intracellular domains of cell-surface receptors. Use of statins in *Nf1*$^{+/-}$ mice has shown some promise in correcting cognitive defects observed in this model (184). With regards to bone, statins have been shown to have pro-anabolic properties in WT animals and have been theorized to have anti-osteoclastogenic properties via a mechanism similar to that of nitrogen containing bisphosphonates (185-188). As such, statins have been tested in two mouse models of NF1 to inhibit Ras activity in the context of bone healing (137, 140).

In the first study, Kolanczyk, *et al*. used systemic, high dose lovastatin to promote bone healing in a cortical defect model of bone repair in *Nf1*$_{Prx}^{-/-}$ mice (140). Because lovastatin was primarily developed to inhibit cholesterol synthesis, a process primarily occurring in the liver, lovastatin's high first-pass metabolic profile necessitated high systemic doses in order to have a beneficial effect on bone healing. High dose lovastatin treatment and its potential negative effects in children precipitated the studies by Wang, *et al*., in which a local, sustained delivery method was used to administer low doses of lovastatin to the fracture site in *Nf1*$_{Col1}^{-/-}$ mice. Though this mouse model does not provide a good clinical representation of the NF1 pseudarthrosis, fracture healing delays in the model were improved with local, low-dose lovastatin versus vehicle treatment (137). Importantly, these studies and others were able to show that ERK1/2 phosphorylation was decreased in bone marrow and calvarial derived osteoblasts *in vitro*

as well as in calvaria *in vivo* following systemic lovastatin treatment (4, 137, 140). Future studies utilizing local, low-dose lovastatin in a more relevant preclinical mouse model of fracture healing in NF1 are warranted as are studies with other drugs targeting required post-translational modifications of Ras such as farnesyltransferase or geranyl geranyltransferase inhibitors.

MEK1/2 Inhibitors

Another proposed strategy for treating NF1 pseudarthrosis has been to target the Ras-Raf-MEK-ERK signaling axis. The majority of the studies on the function of neurofibromin in bone and cartilage have focused on this axis as opposed to other Ras effectors such as PI3K. Targeting this axis has been the subject of intense investigation in cancer biology and cancer drug discovery. Until very recently, no ERK1/2 inhibitors had been developed and only drugs targeting Raf and MEK1/2 were available to target the MAPK axis. To date, no Ras targeting therapeutics have been successfully developed. Our laboratory and others hypothesized that the use of MEK inhibitors in the setting of NF1 pseudarthrosis may be beneficial given the dysregulation of this pathway demonstrated in osteoblasts and osteochondroprogenitors (4, 133, 135). One benefit of targeting MEK rather than Raf is that the MEK inhibitors our laboratory and others have studied are currently in clinical trials for their efficacy against the malignant manifestations of NF1 in pediatric populations. Also, targeting Raf with small molecules has been plagued by these drugs' paradoxical activation of ERK signaling aside from their inhibition of Raf. Finally, most research with Raf inhibitors has focused on inhibition of mutated, constitutively active Raf variants with limited activity on WT Raf (reviewed in (189)).

Fracture healing studies in two different mouse models of fracture healing in NF1 ($Nf1_{Osx}^{-/-}$ and locally injected cre expression vector harboring adenoviral particles into $Nf1^{f/f}$ fractures) showed that combination therapy of MEK inhibitors and recombinant BMP-2 promoted healing over vehicle or single agent therapy (146, 190). N'Dong and colleagues showed the rescue of bone marrow derived osteoblast differentiation and molecular parameters *in vitro* and significant improvements in callus biomechanical properties over $Nf1_{Osx}^{-/-}$ vehicle or single agent treated calluses (146). Importantly, given the negative side effects of systemically administered MEK inhibitors (if neoplasia are not present) and possible neoplastic promoting properties of systemically administered BMP-2, N'Dong, *et al*., administered both BMP-2 and MEK inhibitor in a locally contained, sustained release formulation (146). Both of these studies highlight the possibility of using MEK inhibitors to treat NF1 pseudarthrosis in combination with BMP-2. Further studies to investigate the mechanism behind the necessity of administering BMP-2 and MEK inhibitors together remain to be performed. Furthermore, the action of MEK inhibitors in combination with BMP-2 on $Nf1^{-/-}$ chondrocytes has not been explored and no histology on the $Nf1_{Osx}^{-/-}$ calluses was performed to assess differences in cartilage formation, hypertrophy, or transition to the hard callus. These studies would be informative for the possible beneficial effects of BMP-2 plus MEK inhibitor treatment on soft callus dynamics in NF1 pseudarthrosis.

CNP

Because MEK inhibition has the possibility of significant side effects (especially if systemically administered), our laboratory has recently become interested in the

possibility of targeting the Ras-Raf-MEK-ERK axis with C-type natriuretic peptide (CNP). CNP overexpression causes bone overgrowth in humans, and in mice, its deletion causes dwarfism similar to achondroplasia and early death (191-194). Likewise, deletion of the CNP receptor, natriuretic peptide receptor B (NPR-B), causes dwarfism in humans and in mice (195, 196). Mechanistically, it has been shown that CNP inhibition of Raf activity is mediated via the second messenger cyclic GMP (197). Others have previously shown that CNP activates NPR-B which in turn stimulates guanylyl cyclase to convert guanosine monophosphate to cyclic guanosine monophosphate (cGMP) in chondrocytes and via this mechanism, CNP regulated chondrocyte proliferation (198, 199). It wasn't until 2005 when Krejci, *et al.*, showed that cGMP activates protein kinase G (PKG) whose phosphorylation of Raf inactivates it, thus preventing terminal activation of ERK (197). Importantly, no studies have been published showing that Raf inactivation by CNP produces paradoxical ERK activation as with small molecule Raf inhibitors, suggesting that this strategy to target the Ras-Raf-MEK-ERK axis may be effective in treating NF1 pseudarthrosis. Pharmacokinetically stable CNP analogs are currently in clinical trials for treatment of achondroplasia in children because it has shown activity in mice with achondroplasia causing FGFR3 mutations (200). Because CNP's activity is restricted to cells expressing NPR-B (within the brain, cardiovascular system, and bone (200, 201)) and because there are no other known receptors of CNP, therapeutic CNP administration is it likely to have a favorable side effect profile compared to systemically administered MEK inhibitors. Since CNP was previously studied in the context of chondrocytes, my studies, described in Chapter II, also look at CNP in chondrocytes but in the context of *Nf1* loss-of-function. As will be discussed in Chapter IV, my current

work is focused on determining the function and therapeutic potential of CNP in *Nf1*-null osteoblasts.

FGFR Inhibitors

Though many downstream Ras effectors are known and their function in chondrocytes is becoming clearer, much remains to be elucidated and the upstream activators of neurofibromin-regulated Ras remain largely unknown. A hypothetical avenue of treating NF1 pseudarthrosis that has not been explored is to inhibit a cell surface receptor or a group of receptors that are primarily responsible for activating the Ras-Raf-MEK-ERK axis which neurofibromin regulates. To this end, I focused my research efforts, detailed in Chapter III, on identifying such a receptor(s) in chondrocytes. I identified FGFR1 as a potential receptor that provides a significant input into the neurofibromin regulated Ras-Raf-MEK-ERK axis in growth plate chondrocytes after an extensive literature review and after comparing human and mouse phenotypes with genetic gain- and loss-of-function mutations in various receptors. The studies in Chapter III detail the nature of the relationship between FGFR1 and neurofibromin in hypertrophic growth plate chondrocytes, speculate a possible role of neurofibromin in regulating the Ras-Raf-MEK-ERK signaling cascades emanating from both FGFR1 and 3 in the prehypertrophic growth plate chondrocytes, and provide proof-of-concept, *in vivo* evidence supporting the need for further studies to assess the potential use of the pan-FGFR inhibitor, BGJ-398, in treating NF1 pseudarthroses as a means to promote chondrocyte proliferation, hypertrophic maturation, and transition to the hard callus.

The mechanisms of action of the current and proposed pharmacological therapies and their caveats are summarized in **Table 1**.

Table 1. Current and Proposed NF1 Pseudarthrosis Therapies

Drug/Drug Class	Target	Rationale	Caveat
Bisphosphonates	(i) Osteoclasts (ii) Ras (post-translational modifications, only nitrogenous bisphosphonates)	(i) Overabundance of osteoclasts in pseudarthrotic tissues (ii) Overactive Ras is the immediate target of *NF1* loss-of-function mutation	(i) Bisphosphonate use in children with active growth plates may be problematic. (ii) Not shown to be effective for NF1 pseudarthrosis alone or in combination to BMP
BMP-2/7	Osteoblast progenitor differentiation	Osteoblast progenitors have a differentiation defect and BMP2 and 7 promote osteoblast differentiation	(i) No statistically powered, controlled clinical trials showing promotion of healing or prevention of refracture (ii) Possible contraindication in populations prone to neoplasia (iii) Requires local administration at time of surgery
Asfotase-α (bone targeted alkaline phosphatase)	PPi (Mineralization Inhibitors)	Promotes the clearance of mineralization inhibiting PPi shown to be involved in osteoidosis phenotype in NF1 mouse models and suggested in humans	(i) Ectopic calcification has not been shown but is a concern with systemic administration (ii) Unknown efficacy in fracture healing context
TGF-β Signaling Inhibitors	TGF-β Ligands and Receptors	TGF-β signaling demonstrated to be dysregulated in an NF1 mouse model targeting mature osteoblasts	(i) Unknown whether TGF-β signaling is dysregulated in osteoprogenitors (ii) Contraindicated in populations prone to neoplasia
Statins	Ras (post-translational modifications, only nitrogenous bisphosphonates)	Overactive Ras is the immediate target of *NF1* loss-of-function mutation	(i) High first pass metabolism requires high systemic dosing which may be undesirable (ii) Local administration at time of surgery
MEK Inhibitors	MEK activation	MAPK signaling known to be overactive in *Nf1*$^{-/-}$ chondrocytes, osteoblasts, and osteoprogenitors	(i) Systemic administration has multiple side effects (ii) May not be effective alone
C-Type Natriuretic Peptide Analogues (CNP)	Raf inhibition via NPR-B receptor agonism	(i) Targets MAPK signaling indirectly via a receptor with limited tissue expression (ii) Efficacy in WT and Nf1$^{-/-}$ chondrocytes *in vivo*	(i) No demonstrated efficacy in WT or *Nf1*$^{-/-}$ osteoblasts (ii) No demonstrated efficacy in in a fracture healing context
FGFR Inhibitors	FGF Receptors	(i) Phenotypic similarity between bone manifestations of FGFR activating mutations and NF1 (ii) Efficacy in WT and *Nf1*$^{-/-}$ chondrocytes *in vivo*	(i) No demonstrated efficacy in in a fracture healing context (ii) Systemic administration may have side effects

CHAPTER II

THE RAS-GTPASE ACTIVITY OF NEUROFIBROMIN RESTRAINS ERK-DEPENDENT FGFR SIGNALING DURING ENDOCHONDRAL BONE FORMATION

This is a pre-copyedited, author-produced, adapted version of an article accepted for publication in *Human Molecular Genetics* following peer review. The version of record is: Ono, K., Karolak, M.R., N'Dong, J.D.L.C., Wang, W., Yang, X. and Elefteriou, F. (2013) The Ras-GTPase activity of neurofibromin restrains ERK-dependent FGFR signaling during endochondral bone formation. *Hum. Mol. Gen.*, 22, 3048–3062.

Introduction

As discussed in Chapter I, vertebrate long bones are formed through endochondral ossification, a highly coordinated process regulated by paracrine and hormonal factors and gives long bones their shape and structure, with two distal cartilaginous growth plates at the epiphyses and a bony region (diaphysis) extending in between, until growth plate closure (14, 202, 203). The fibroblast growth factors (FGFs) and their receptors (FGFRs) play an important role during skeletal development. Activating mutations in *FGFR1* and *FGFR2* cause syndromes characterized by craniosynostosis (Pfieffer, Crouzon, and Apert syndromes) (204-206), whereas *FGFR3* activating mutations are typically associated with achondroplastic (thanatophoric dysplasia) and hypochondroplastic dwarfism (24, 25, 207).

Because *FGFR1*-activating mutation (P252R) causes craniosynostosis, FGFR1 function has been associated primarily with flat bone growth and skull formation. However, FGFR1 is expressed in the growth plate during development and displays a distinct spatial distribution with FGFR3. Activating missense mutations in FGFR1 cause Osteoglophonic dysplasia (OD), a "crossover" disorder that has skeletal phenotypes associated with *FGFR1, FGFR2* and *FGFR3* mutations, including dwarfism (50). FGFR1 may thus contribute to endochondral bone formation; however, this has been very little explored.

Loss of *NF1* function is associated with constitutive activation of Ras and downstream signaling, including ERK1/2. We have shown previously that neurofibromin is expressed in growth plate chondrocytes and that conditional mutant mice lacking *Nf1* in chondrocytes ($Nf1_{Col2}^{-/-}$ mice) have a reduced stature postnatally (4). The observations that FGFRs signal through ERK1/2, that neurofibromin negatively regulates ERK1/2, and that mice with constitutive activation of FGFR1 or FGFR3 share a number of endochondral bone formation phenotypes with $Nf1_{Col2}^{-/-}$ mice (27, 30, 40, 43, 52, 208, 209), led us to hypothesize that neurofibromin may be an important regulator of FGFR signaling, controlling the dynamics of growth plate elongation, maturation and catabolism during development. We show here that neurofibromin is required for multiple steps of endochondral bone formation during limb development, and provide evidence that the GTPase activity of neurofibromin restrains FGFR-Ras-ERK1/2 signaling in postmitotic differentiated chondrocytes to allow proper chondrocyte proliferation, maturation and growth plate catabolism.

Materials and Methods

Animals and Drugs

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University Medical Center. WT and $Nf1_{Col2}^{-/-}$ were generated by crossing $Nf1^{flox/flox}$ mice and $Nf1^{flox/+}$ mice; *a1(II) collagen-Cre* mice (128, 143). $Nf1^{flox/flox}$ mice and $Nf1^{flox/flox}$ mice; *a1(II) collagen-Cre* mice were used as WT and cKO, respectively. NC-2 (100 or 300 mg/kg) or PBS (phosphate buffer saline vehicle) was injected daily and subcutaneously from birth for 18 days. NC-2 is composed of the human CNP22 sequence, preceded by six amino acids from the propeptide, fused to the C-terminus of the Fc domain of human IgG1 by using an intervening glycine-rich linker $(GGGGS)_2$. The compound binds specifically to the NPR-B receptor and stimulates intracellular production of cGMP with a 60nM EC_{50} (Alexion, private communication).

Cell Culture

Primary chondrocytes were extracted from the rib cages of P0 (newborn) mice. The cartilaginous part of the rib was dissected and the soft tissue was removed, then digested by collagenase D (3 mg/ml, Roche, Nutley, New Jersey, USA) and trypsin/ethylenediamine-tetraacetic acid (EDTA) ($0.625x10^{-3}$%, GIBCO, Grand Island, NY, USA) in serum free DMEM medium (Invitrogen, Grand Island, NY, USA) for 1h. Digestion medium was then discarded and samples were further digested overnight at 37 °C (210). The extracted cells were filtered through 40mm nylon mesh (BD Bioscience, Bedford, MA, USA), collected by centrifugation and used as primary chondrocytes.

Primary chondrocyte cell cultures were maintained in DMEM+/+, DMEM supplemented with 10% FBS, 100 I.U./mL penicillin, and 100μg/mL streptomycin (Cellgro, Manassas, VA, USA) in a humidified, 5% CO_2 incubator at 37°C. After reaching confluence, primary chondrocytes were lifted using 0.25 % trypsin (Invitrogen, Grand Island, NY, USA), and replated into experimental wells. To induce differentiation, cells were treated 50 mg/mL ascorbic acid (Sigma, St. Louis, MO, USA) and 5 mM β-glycerophosphate (Sigma, St. Louis, MO, USA). For micromass culture, 15 ml of concentrated ($2x10^7$ cells/ml) cells were plated on 6-well (211). After 4 hours of incubation, 2mL of DMEM+/+ was slowly added. FGF2 (10 ng/ml, R&D systems, Minneapolis, MN, USA) and EGF (100 ng/ml, R&D systems, Minneapolis, MN, USA) were used to stimulate primary chondrocytes. Osteoclastogenesis assays were performed with spleen-derived osteoclast precursors co-cultured with rib-derived primary chondrocytes. Osteoclast precursor cells were separated from the single-cell suspension of whole spleens by a ficoll gradient (LSM, MP Biomedicals, Solon, OH, USA) and plated ($5x10^5$ cells/ml) on confluent primary chondrocytes in DMEM+/+ (212). Osteoclastogenesis was induced following treatment with 10^{-8} M of $1,25(OH)_2$ vitamin D_3 (Sigma, St. Louis, MO, USA) (213).

RT-PCR, Genomic PCR and qPCR

Total RNA was extracted using TRIzol (Invitrogen, Grand Island, NY, USA), and cDNAs were synthesized following DNase I treatment using the high-capacity cDNA reverse-transcription kit (Applied Biosystems, USA). Quantitative PCR (qPCR) was performed by using TaqMan gene expression assays or SYBR green qPCR. The probe

and primer sets for *Rankl* (Mm00441908_m1), *Opg* (Mm00441906_m1), *Ihh* (Mm00439613_m1), *Runx2* (Mm00501578_m1), *Tnsap* (Mm00475834_m1), *Fgfr1* (Mm00438930_m1), *Fgfr3* (Mm00433294_m1) and the normalizer *Hprt* (Mm00446968_m1) were obtained from Applied Biosystems (Foster City, CA, USA). The primers were: *Mmp9* (forward; GCCCTACAGCGCCCCCTACT, reverse; AGACACGCCCCTTGCTGAACA), *Opn* (forward; CTCCTTGCGCCACAGAATG, reverse; TGGGCAACAGGGATGACA), *Col10a1* (forward; GGCAGCAGCATTACGACCCAAGAT, reverse; GAATAACAGACACCACCCCCTCAC), *Nf1* (forward; GTATTGAATTGAAGCACCTTTGTTTGG, reverse; CTGCCCAAGGCTCCCCCAG). The primers were; *Npr-b* (forward; TGTACCATGACCCCGACCTT, reverse; CCCGTTGGCTCTGATGAAGT), *Gapdh* (forward; ACCACAGTCCATGCCATCAC, reverse; TCCACCACCCTGTTGCTGTA). Specificity of amplification was verified by the presence of a single peak on the dissociation curve. Specific amplification conditions are available upon request. For genotyping, genomic DNA was isolated from tail tips by sodium hydroxide digestion, and PCR was performed using primers P1, P2 and P4, as described by Zhu *et al*, (128), resulting in a 280bp *Cre*-mediated recombination band and a 350bp non-recombined band. The *a1(II) collagen-Cre* transgene was detected using the forward primer: GAGTTGATA GCTGGCTGGTGGCAGATG and reverse: TCCTCCTGCTCCTAGGGCCTCCTGCAT to generate a 700bp band.

Histology

Paraformaldehyde-fixed samples were decalcified in 0.5 M EDTA (pH 8.0) overnight or up to one week, depending on the age of the mice, and then dehydrated in graded series of ethanol, cleared in xylenes and embedded in paraffin. 5 mm sagittal sections were cut and stained with hematoxylin and eosin. Immunohistochemistry was performed according to standard protocols using an antibody against Neurofibromin (sc-67, Santa Cruz, Santa Cruz, CA, USA), Phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204, E10 mouse mAb #9106, Cell Signaling, Boston, MA, USA), MMP9 (ab38898, Abcam, Cambridge, MA, USA) or a non-immune IgG antibody, followed by horseradish peroxidase detection of the secondary antibody. *In vivo* proliferation assays were performed following intraperitoneal (IP) injection with BrdU (0.1mg/g) 2 hours prior to sacrifice. Embryos were then harvested and processed for embedding and sectioning. BrdU was detected with a BrdU staining kit (Invitrogen, Grand Island, NY, USA) following the manufacturer's instructions. TRAP staining was performed by using the leukocyte acid phosphatase staining kit (Sigma, St. Louis, MO, USA). Histomorphometric measurements were performed using the Bioquant Analysis System (BIOQUANT image analysis corporation, Nashville, TN, USA). *Fgfr1* in situ hybridization was performed on 5 mm sagittal paraffin sections. Sections were stored at 4°C until hybridization. The *Fgfr1* probe used is a 706bp fragment of the 3' *Fgfr1* UTR (sequence available upon request). Anti-sense [^{35}S]-uridine triphosphate (Perkin Elmer, Waltham, MA, USA) probes were synthesized for hybridization.

Western Blotting

Lysate from primary chondrocytes were prepared in RIPA buffer containing protease and phosphatase inhibitors (Roche, Nutley, New Jersey, USA). Proteins were separated by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membranes were then immunoblotted with indicated primary antibodies, followed by incubation with a horseradish peroxidase-coupled anti-rabbit IgG antibody (sc-2004, Santa Cruz, Santa Cruz, CA, USA). Protein bands were visualized via chemiluminescence. Primary antibodies used for immunoblotting were ERK1/2 (#9102), phospho Erk1/2 (#9101), p70S6K (#9202), phospho p70S6K (#9205) and Caspase-9 antibodies (#9504, Cell Signaling, Boston, MA, USA).

Results

Neurofibromin Expression Is Enriched in Differentiated Chondrocytes

We have previously reported the expression of neurofibromin in the bone mesenchymal lineage, including mesenchymal osteochondroprogenitor cells, osteoblasts and chondrocytes (4). To further delineate neurofibromin expression within the growth plate and in the context of chondrocyte differentiation, the expression pattern of neurofibromin was investigated on tissue sections by immunocytochemistry and in differentiating primary chondrocytes *in vitro* by quantitative RT-PCR. Examination of neurofibromin immunoreactivity in the femoral growth plate of newborn mice (P0) indicated that neurofibromin is not expressed in resting and proliferating chondrocytes, whereas it is highly expressed in prehypertrophic and hypertrophic chondrocytes (**Figure 9A**), where

FGFR3 (34, 37, 48, 58) and FGFR1 are expressed (**Figure 9B**). In order to examine the dynamics of *Nf1* expression during chondrocyte differentiation, primary rib chondrocytes were extracted by sequential digestions, cultured in high-density micromass conditions, differentiated using ascorbic acid (211) and RNAs were collected at different time points following ascorbic acid induction. Quantitative RT-PCR analyses showed that the expression of *Col10a1,* a marker gene expressed by differentiated chondrocytes, increased with time, thus attesting for proper *in vitro* chondrocyte differentiation (**Figure 10A**). *Nf1* mRNA expression increased during chondrocyte differentiation (**Figure 10B**), with a pattern similar to the one of *Fgfr1* expression (**Figure 10C**) In contrast, *Fgfr3* expression was rather decreased during chondrocyte differentiation (**Figure 10D**). *Nf1* is thus expressed at late stages of chondrocyte differentiation, suggesting a role of neurofibromin in chondrocyte maturation and hypertrophy.

Neurofibromin Restrains ERK1/2 Signaling in Chondrocytes

Stimulation of FGFR signaling by FGF ligands or following activating mutation in FGFR3 leads to RAS-ERK pathway activation in chondrocytes (44, 191, 214, 215). Similarly, as neurofibromin is a negative regulator of RAS activity, inactivating mutations in *Nf1* lead to constitutive RAS-ERK activation in multiple cell types (216-218); however, such activation and its functional consequence have not been investigated in chondrocytes. To determine whether neurofibromin activity regulates ERK1/2 activity in chondrocytes, we used mice lacking *Nf1* specifically in this lineage, generated by crossing transgenic *Col2a1-Cre* deleter mice with $Nf1^{flox/flox}$ mice (4, 128). Long bone epiphyses extracted from newborn $Nf1_{Col2}^{-/-}$ mice showed a 80% reduction in *Nf1* mRNA

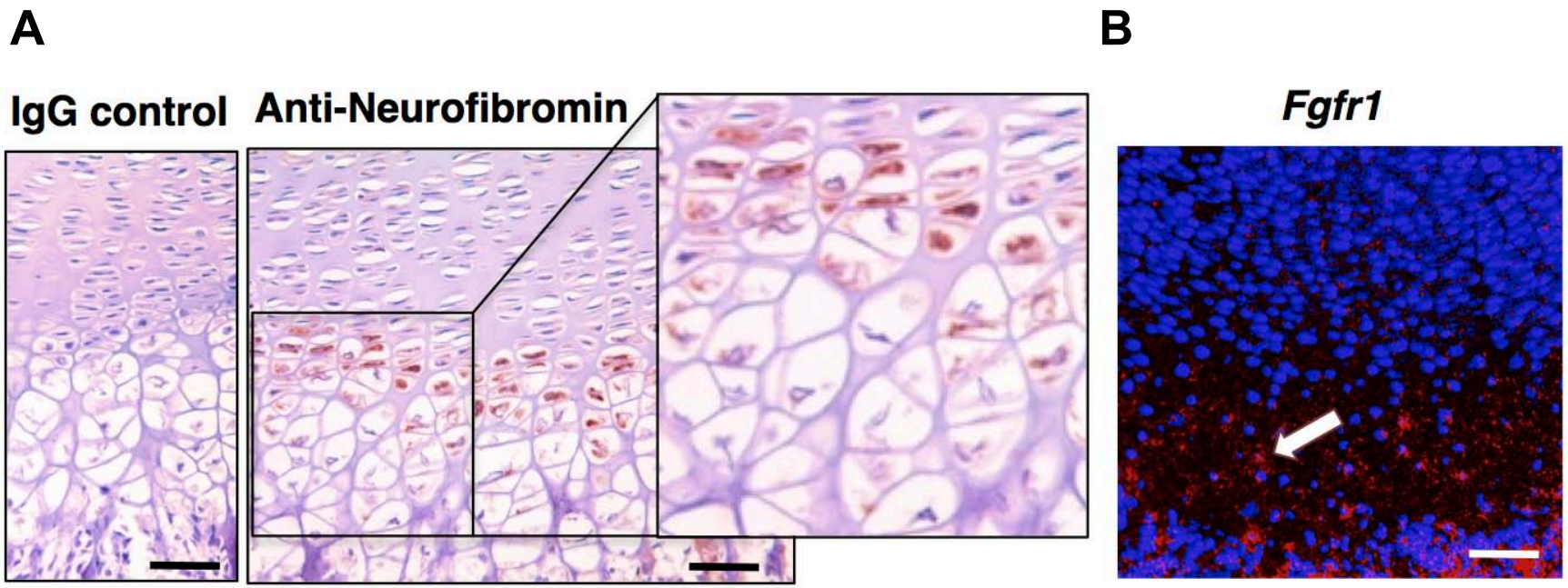

Figure 9. Neurofibromin and *Fgfr1* Are Expressed in Differentiated Growth Plate Chondrocytes
(**A**) Neurofibromin immunoreactivity in P0 mouse tibiae prehypertrophic and hypertrophic chondrocytes (left: control isotype IgG, right: anti-neurofibromin antibody). Scale bar: 100 μm. (**B**) *In situ* hybridization for *Fgfr1* (red signal) and Hoechst nuclear staining (blue signal). Scale bar: 100 μm. Arrow points to hypertrophic *Fgfr1*-positive chondrocytes.

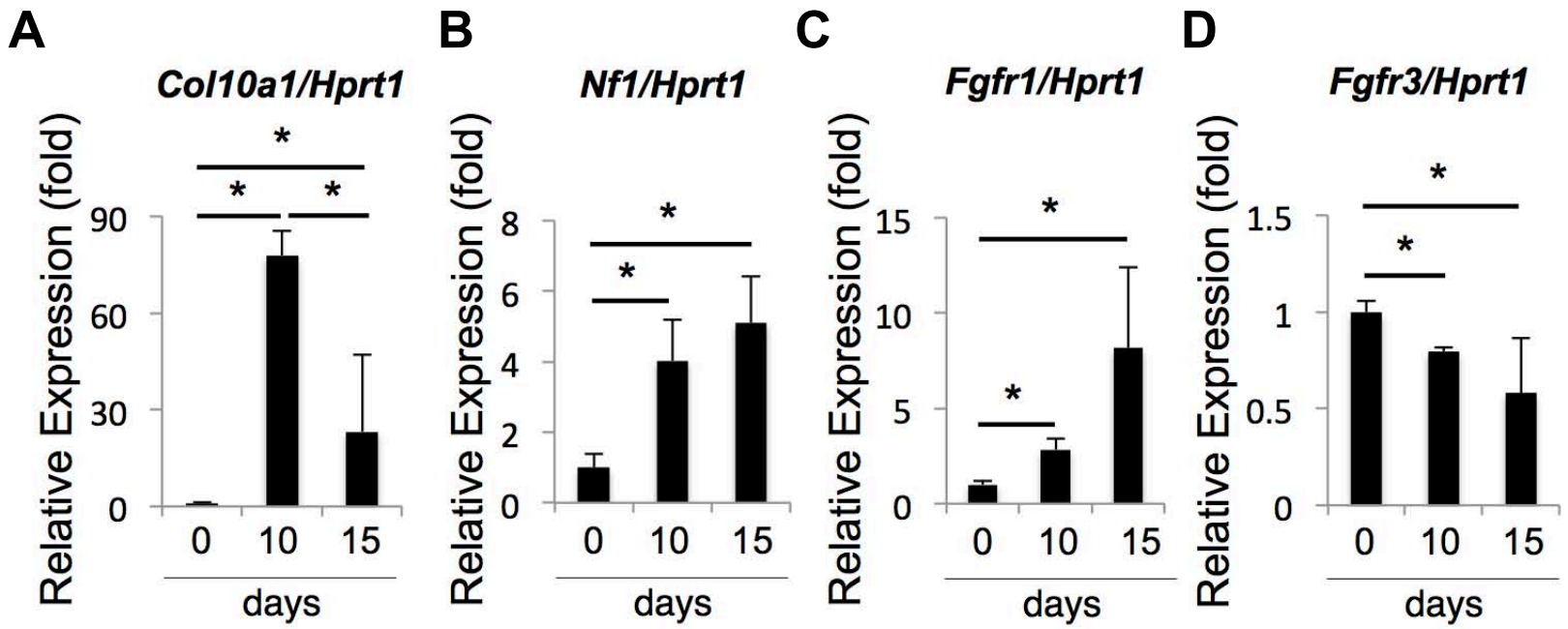

Figure 10. Gene Expression Patterns of Differntiating Chondrocytes *In Vitro* Mimic *In Vivo* Expression Patterns
(A-D) mRNA expression level of *Col10a1* (A), *Nf1* (B), *Fgfr1* (C) and *Fgfr3* (D) in primary chondrocytes high density pellets during differentiation *in vitro*, measured by qPCR (*:$P<0.01$, n=3).

expression compared to WT littermates (**Figure 11A**), despite the presence of contaminating *Col2a1*-negative tissues/cells in tissue preparations from mutant $Nf1_{Col2}^{-/-}$ (*Nf1*$^{flox/flox}$*;Col2a1-Cre*) pups. Primary rib chondrocytes extracted from four day-old WT and mutant $Nf1_{Col2}^{-/-}$ pups were then prepared, cultured for 3 days in the presence of ascorbic acid, and assessed for ERK1/2 phosphorylation by Western blot analyses. As observed in other lineages, a marked increase in ERK1/2 phosphorylation was detected in *Nf1*-deficient chondrocyte cultures compared to WT chondrocytes cultures, whereas p70S6K phosphorylation was not affected (**Figure 11B**). Increased phospho-ERK immunoreactivity was also observed in prehypertrophic and hypertrophic chondrocytes from 10 day-old $Nf1_{Col2}^{-/-}$ mice compared to WT littermates (see **Figure 25A**). These results confirm efficient recombination of the floxed *Nf1* allele by the mouse *Col2a1-Cre* transgene *in vivo* and *in vitro*, and show that it leads to reduced *Nf1* expression and ERK1/2 activation in $Nf1^{-/-}$ chondrocytes.

Nf1 in Prehypertrophic Chondrocytes Is Required for Proper Chondrocyte Proliferation

The activation of ERK1/2 in $Nf1^{-/-}$ chondrocytes and in chondrocytes expressing an activated form of FGFR3, along with the overlapping growth plate expression of neurofibromin, FGFR1 and FGFR3 during endochondral bone formation, led us to hypothesize that neurofibromin is a negative regulator of FGFR signaling in mature chondrocytes. In order to test this hypothesis, we analyzed in detail the progression of bone growth in $Nf1_{Col2}^{-/-}$ mice during embryonic and postnatal development, and then related the observed phenotypes to known developmental and molecular defects associated with FGFR mutant mice.

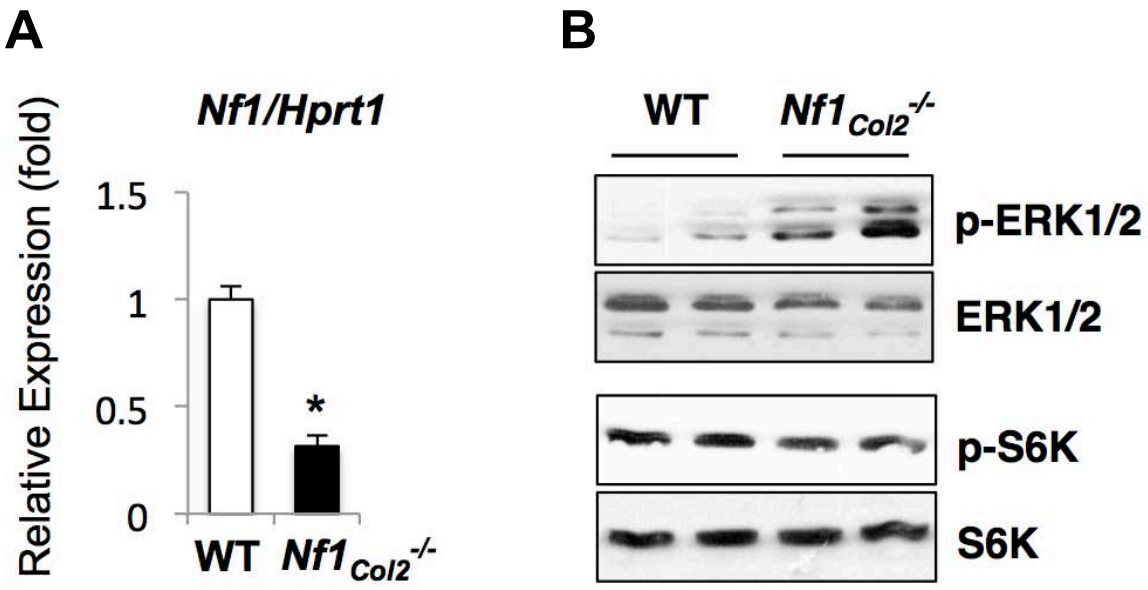

Figure 11. *Nf1* Deletion by the *Col2*-Cre Deleter Is Efficient and Leads to ERK1/2 Activation

(**A**) *Nf1* mRNA expression is reduced in the cartilage of $Nf1_{Col2}^{-/-}$ mice (*:P<0.01, qPCR, n=3). (**B**) ERK1/2 activation, assessed by Western blot analysis, is increased in primary chondrocytes extracted from $Nf1_{Col2}^{-/-}$ mice compared to WT mice, whereas phosphorylated-p70S6K is not changed (n=2-3).

We have previously shown that $Nf1_{Col2}^{-/-}$ mice, at birth, are morphologically indistinguishable from WT littermates and have comparable body weight. However, size difference appears and become significant 5 days after birth and increases progressively thereafter (4). H&E staining and subsequent growth plate histological analyses at E14 and E15 did not reveal significant difference in the formation of primary ossification centers between WT and $Nf1_{Col2}^{-/-}$ embryos (**Figure 12A**), as observed in mice lacking *Fgfr1* in chondrocytes (49) and despite growth plate expression of neurofibromin at this developmental stage (99). No obvious difference in vascular invasion between genotypes could be detected either upon CD31 staining of E16.5 tibiae (**Figure 12B**). However, starting at P0, the length of both proliferating and hypertrophic zones was significantly shorter in $Nf1_{Col2}^{-/-}$ mice compared to WT mice, although gross body size differences could not be detected. Shortening of the proliferating zone worsened with time and was most pronounced at 3 weeks of age, at which time point the proliferating zone of $Nf1_{Col2}^{-/-}$ mice was less than 60% of WT mice (**Figure 13A,B**). The shortening of the hypertrophic zone, in contrast, was most severe at P0 (**Figure 13A,C**). The formation of secondary ossification centers (SOC), initiated with vascular invasion into hypertrophic chondrocytes in the middle of the distal epiphyses, was delayed in $Nf1_{Col2}^{-/-}$ mice. At 1week of age, SOCs were not detected in the proximal tibiae of $Nf1_{Col2}^{-/-}$ mice, whereas they were already present in WT tibiae, as evidenced by the presence of hypertrophic chondrocytes and red blood cells (**Figure 13A**). Similar phenotypes and delay in the formation of SOCs were also observed in mice with FGFR3 activating mutations (33, 219). Ablation of *Nf1* in chondrocytes thus causes dysregulation of endochondral ossification and results in postnatal growth retardation.

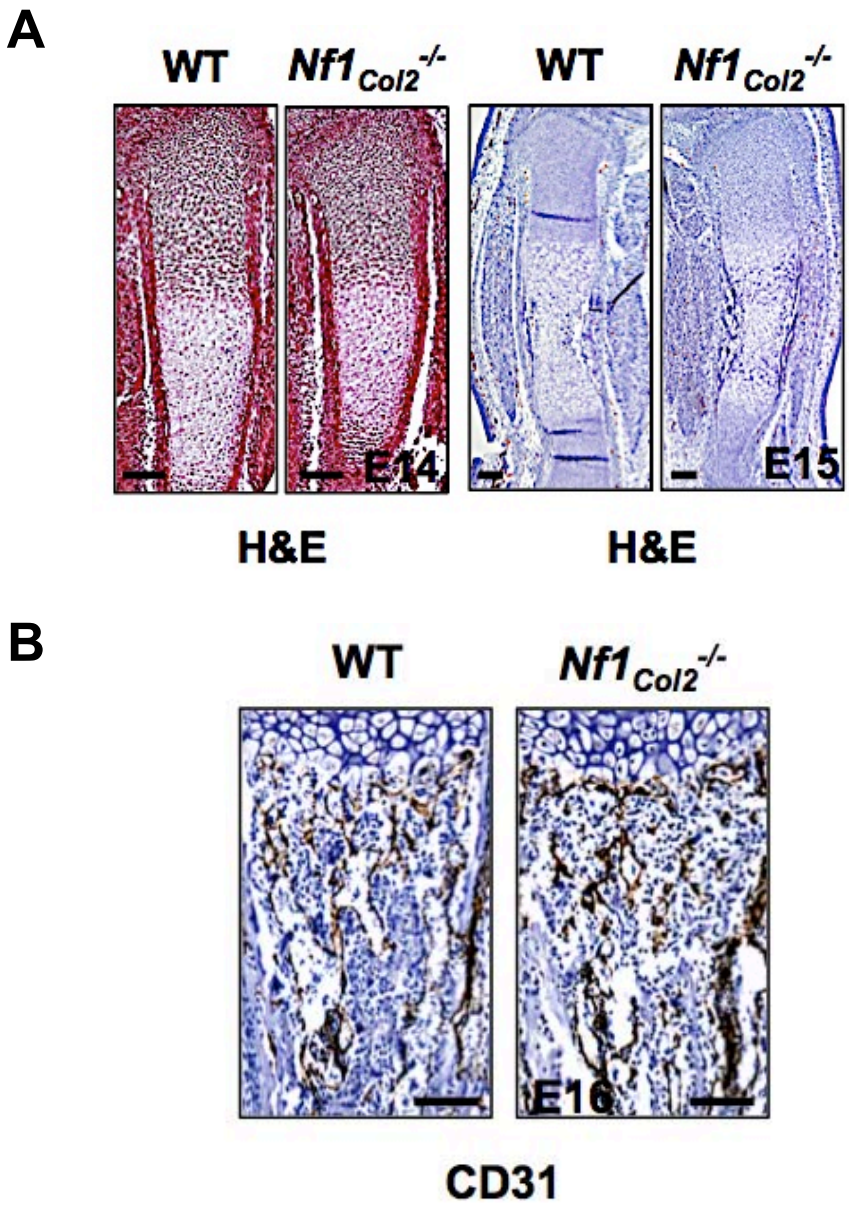

Figure 12. Embryonic Stages of Endochondral Ossfiication Are Unaffected by *Nf1* Loss-of-Function

(**A**) No significant difference in the formation of the primary ossification center are observed between WT and $Nf1_{Col2}^{-/-}$ tibial growth plates at E14 and E15. Scale bar: 100 um, H&E staining. (**B**) No siginificant difference in vascular invasion was detected in tibial primary spongiosae from $Nf1_{Col2}^{-/-}$ and WT mice at E16, as measured by CD31 immunohistochemistry (blood vessels, brown staining). Scale bar: 200 μm.

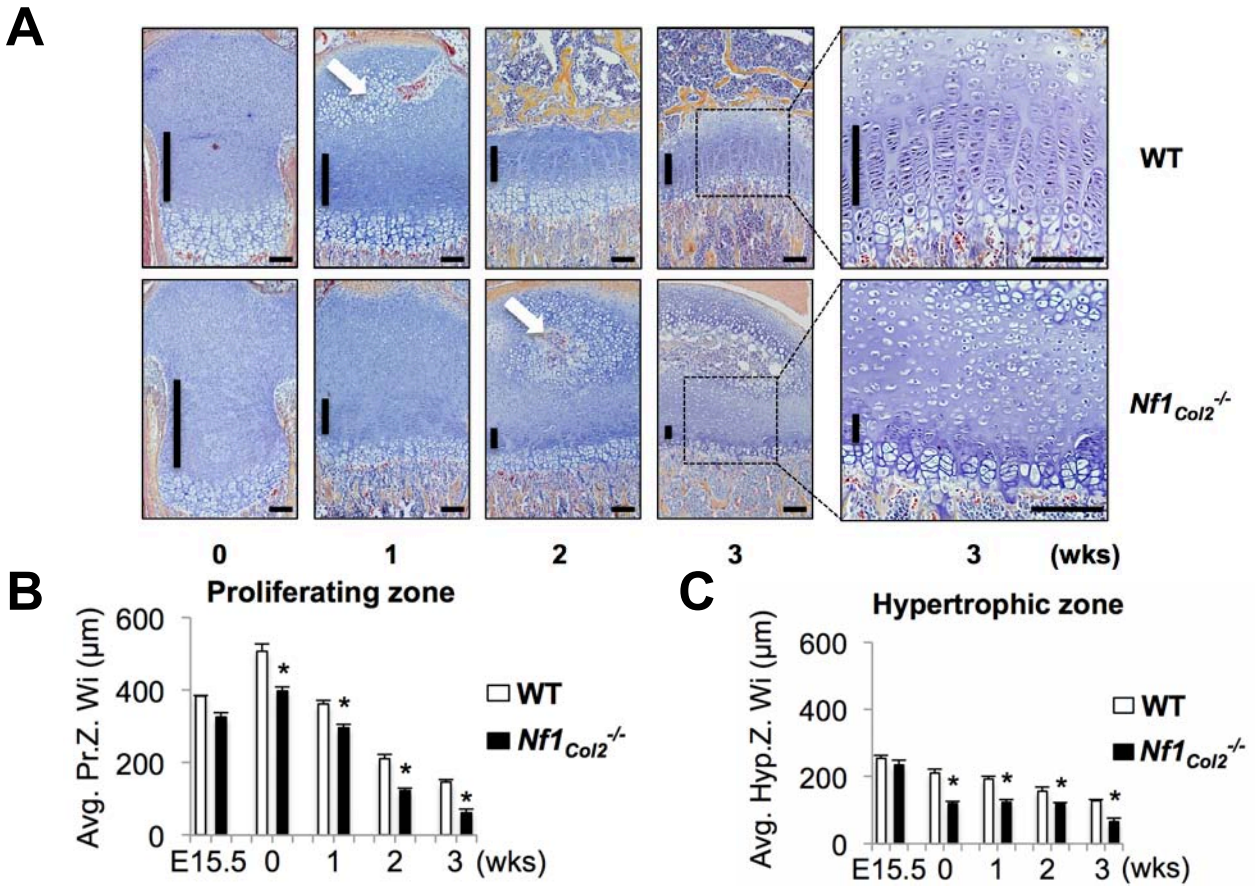

Figure 13. Postnatal Endochondral Bone Formation Is Defective in *Nf1*$_{Col2}^{-/-}$ Mice

(**A**) Sections of tibial growth plates at birth and at weekly intervals thereafter. The length of the proliferating zone is shown by the vertical black lines. Scale bar: 100 μm, H&E staining. (**B, C**) Length quantification of the proliferating (B) and hypertrophic (C) zones at E15.5, P0, 1, 2 and 3 weeks of age (*:P<0.01, n=3). The length of proliferating and hypertrophic zones was significantly shorter in *Nf1*$_{Col2}^{-/-}$ mice compared to WT mice from birth.

The progressive postnatal reduction in size of the proliferating zone in $Nf1_{Col2}^{-/-}$ mice suggested that neurofibromin regulates chondrocyte proliferation. To address this hypothesis, the effect of *Nf1* deficiency on chondrocyte proliferation was assessed by *in vivo* BrdU labeling. At P0, no significant difference was observed in the labeling index of tibial proliferative chondrocytes between $Nf1_{Col2}^{-/-}$ mice and WT littermates (**Figure 14A**). In contrast, chondrocyte proliferation was significantly decreased postnatally at P14 in the tibiae from $Nf1_{Col2}^{-/-}$ mice compared to WT littermates (**Figure 14B**). Since neurofibromin is not detected in proliferative chondrocytes (**Figure 9A**), we reasoned that a factor(s) released by postmitotic $Nf1^{-/-}$ prehypertrophic and/or hypertrophic chondrocytes may indirectly affect the proliferative activity of adjacent *Nf1*-negative chondrocytes, in a paracrine manner. In addition, since activation of FGFR3 represses chondrocyte proliferation through its inhibition of IHH signaling (23, 32, 43), we reasoned that *Nf1* deficiency in prehypertrophic chondrocytes may reduce *Ihh* expression, thus possibly leading to the observed reduced chondrocyte proliferation. To address this hypothesis, gene expression studies were performed using long bone cartilage tissues excised from $Nf1_{Col2}^{-/-}$ and WT littermates, and high cell density micromass cultures of primary chondrocytes prepared from WT and $Nf1_{Col2}^{-/-}$ ribs, differentiated or not *in vitro* with ascorbic acid (220-222). In agreement with the reduced proliferative index observed in the growth plates of $Nf1_{Col2}^{-/-}$ mice *in vivo*, *Ihh* expression was significantly decreased in growth plate cartilage from $Nf1_{Col2}^{-/-}$ mice compared to WT littermates (**Figure 15A)**. In micromass cultures, *Ihh* expression was strongly decreased as well, in both immature and differentiated cultures (**Figure 15B)**. *Pthrp* expression was very low in both chondrocyte cultures and cartilage of both WT and mutant mice (beyond limits of

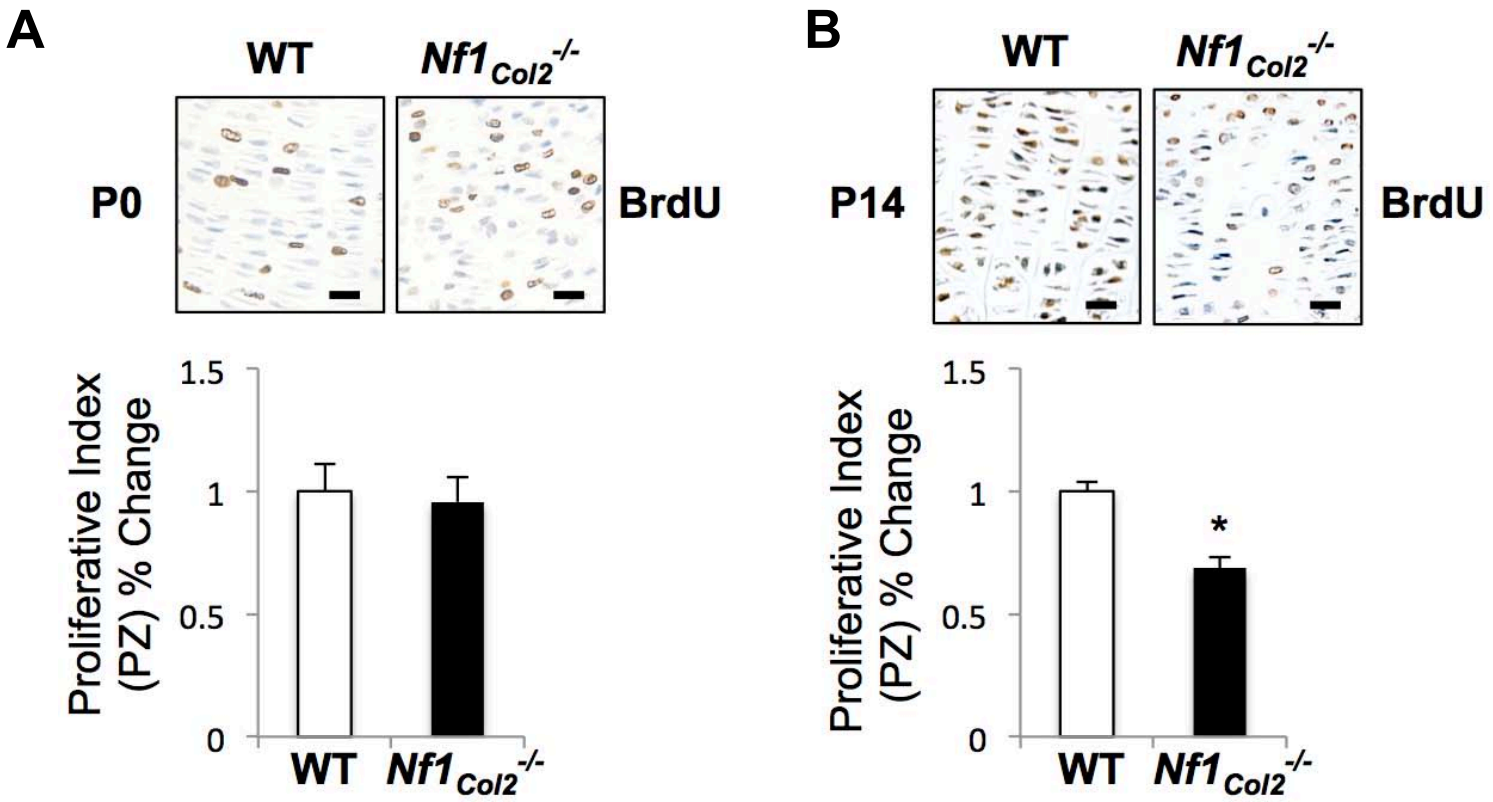

Figure 14. Decreased Postnatal Chondrocyte Proliferation in *Nf1*$_{Col2}^{-/-}$ Mice

(**A, B**) Tibial proximal growth plate BrdU immunohistochemistry and quantification of the proliferative index at P0 (A) and P14 (B) (PZ: Proliferating zone, scale bar: 50 μm, *:P<0.01, n=5), showed reduced chondrocyte proliferation at postnatal stage only (Scale bar: 50 μm, *:P<0.01, n=5).

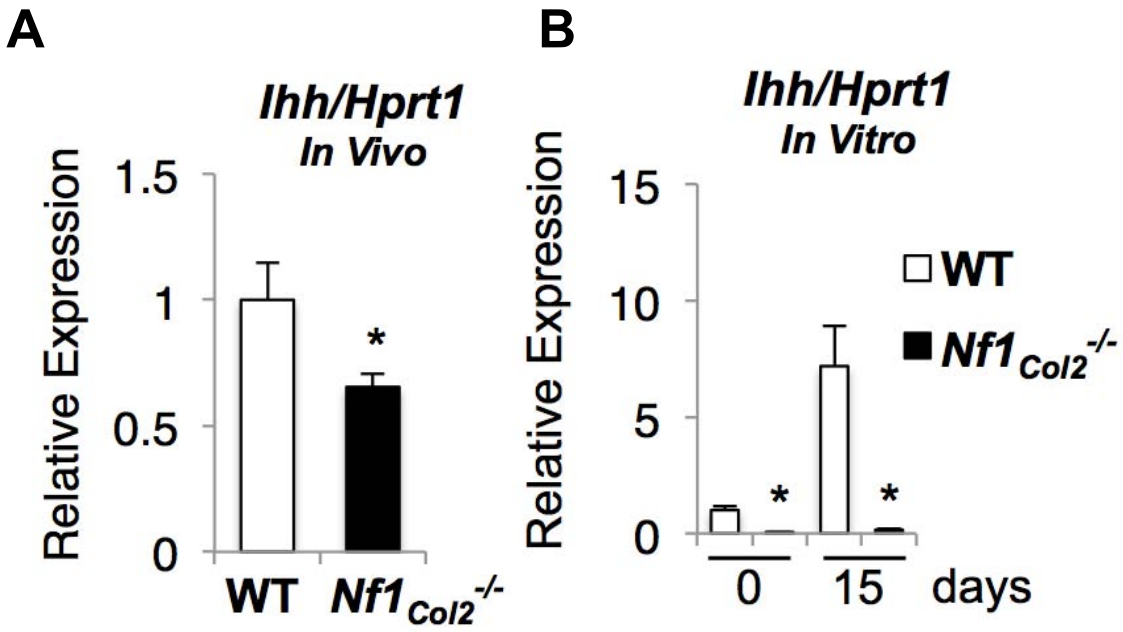

Figure 15. ***Nf1*** **Loss-of-Function Causes Reduced Chondrocytic** ***Ihh*** **Expression** ***In Vitro*** **and** ***In Vivo***

(**A, B**) *Ihh* mRNA expression measured in long bone epiphyseal cartilage from P0 WT or $Nf1_{Col2}^{-/-}$ mice (A, *:P<0.01, qPCR, n=4) and in micromass cultured primary chondrocytes at the proliferative (d0) and differentiated (D15, following ascorbic acid treatment) stages. (B, *:P<0.01, qPCR, n=3). *Ihh* expression is decreased, in the cartilage and chondrocyte cultures from $Nf1_{Col2}^{-/-}$ mice compared to WT littermates.

detection and thus not shown). These data suggest that suboptimal IHH levels in the growth plates of $Nf1_{Col2}^{-/-}$ mice may contribute to the observed reduction in chondrocyte proliferation. This phenotype is also in agreement with the increase in chondrocyte proliferation, differentiation, and IHH signaling observed in mice deficient for FGFR3 or FGF18 (223), and with the decrease in *Ihh* observed upon constitutive activation of FGFR3 (43). Despite this supporting evidence, a genetic IHH gain-of-function experiment in $Nf1_{Col2}^{-/-}$ mice will be necessary to definitively prove the contribution of reduced Ihh signaling to the chondrocyte proliferation phenotype of $Nf1_{Col2}^{-/-}$ mice.

Nf1 Is Required for Proper Chondrocyte Hypertrophy and Apoptosis

The shortening of the hypertrophic zone observed in $Nf1_{Col2}^{-/-}$ mice can conceivably be caused by a decreased pool of proliferating chondrocytes, defective or premature chondrocyte differentiation, an increase in chondrocyte apoptosis, or by premature/overt catabolism of growth plate cartilage. The results of our gene expression studies indicated that *Opn* expression was increased in differentiated (*Col10a1*-positive) mutant chondrocyte cultures and growth plates (**Figure 16A,B**), as observed in chondrocytes treated with FGF9 (224) or in mice with FGFR3 activating mutations (33, 225). *Col10a1* expression, however, was decreased in chondrocyte cultures and growth plates from $Nf1_{Col2}^{-/-}$ pups compared to WT littermates (**Figure 17A,B**), as observed in mice with constitutive FGFR3 activation (43, 225), but converse to $FGF18^{-/-}$ and $FGFR3^{-/-}$ mice (223). The decreased expression of *Ihh* and *Col10a1* observed in absence of *Nf1* is thus suggestive of impaired or delayed differentiation.

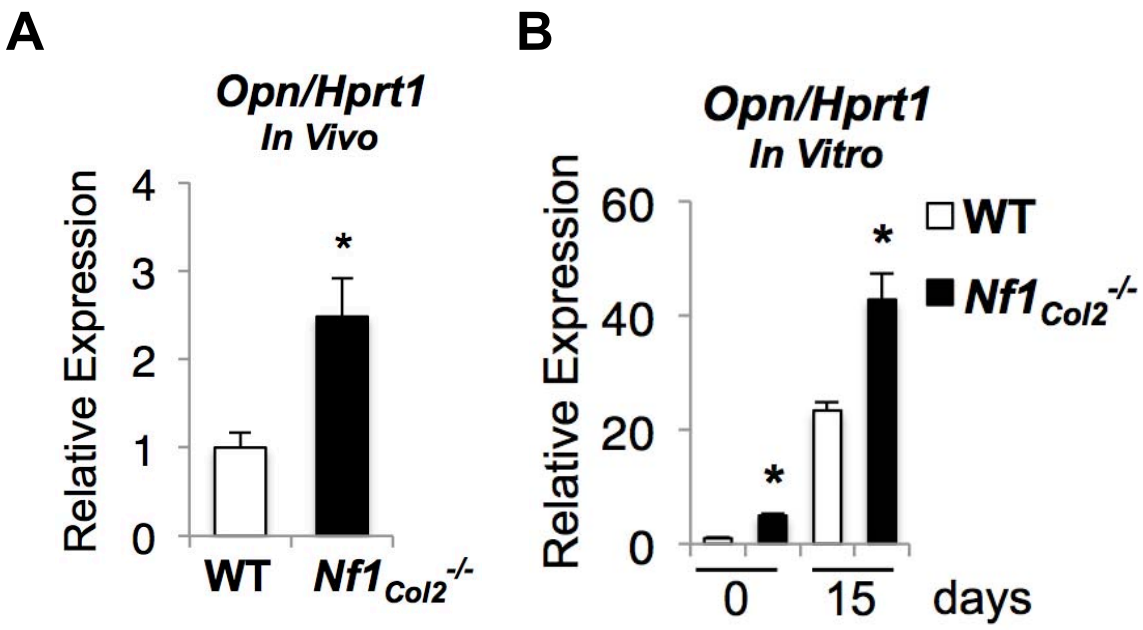

Figure 16. ***Nf1*** **Loss-of-Function Causes Increased Chondrocytic** ***Opn*** **Expression** ***In Vitro*** **and** ***In Vivo***

(A, B) *Opn* mRNA expression measured in long bone epiphyseal cartilage from P0 WT or $Nf1_{Col2}^{-/-}$ mice (A, *:$P<0.01$, qPCR, $n=4$) and in micromass cultured primary chondrocytes at the proliferative (d0) and differentiated (D15, following ascorbic acid treatment) stages. (B, *:$P<0.01$, qPCR, $n=3$). *Opn* expression is increased, in the cartilage and chondrocyte cultures from $Nf1_{Col2}^{-/-}$ mice compared to WT littermates.

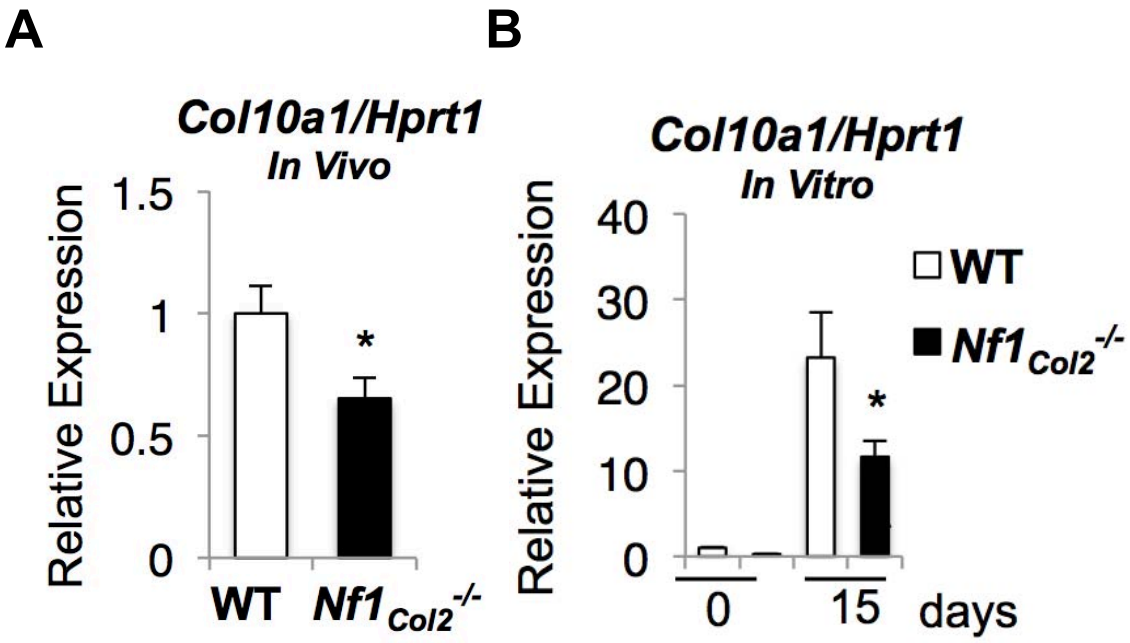

Figure 17. ***Nf1*** **Loss-of-Function Causes Reduced Chondrocytic** ***Col10a1*** **Expression** ***In Vitro*** **and** ***In Vivo***

(**A, B**) *Col10a1* mRNA expression measured in long bone epiphyseal cartilage from P0 WT or $Nf1_{Col2}^{-/-}$ mice (A, *:P<0.01, qPCR, n=4) and in micromass cultured primary chondrocytes at the proliferative (d0) and differentiated (D15, following ascorbic acid treatment) stages. (B, *:P<0.01, qPCR, n=3). *Col10a1* expression is decreased, in the cartilage and chondrocyte cultures from $Nf1_{Col2}^{-/-}$ mice compared to WT littermates.

Both FGFR3 activation (41) and phosphate (221) trigger hypertrophic chondrocyte apoptosis via the activation of ERK. Therefore, we hypothesized that lack of *Nf1* in hypertrophic chondrocytes, by inducing ERK activation, may promote apoptosis and explain, in part, the shortening of the hypertrophic zone observed in $Nf1_{Col2}^{-/-}$ pups. Since chondrocyte apoptosis is activated by a caspase-9 mediated mitochondrial pathway (221), we measured caspase-9 activation by Western blotting, using high cell density primary chondrocytes micromass cultures from WT and $Nf1_{Col2}^{-/-}$ mice. Treatment of ascorbic acid-differentiated chondrocytes with 25 mM phosphate for 2 hours did not affect cleavage of caspase-9 in WT cells but clearly induced caspase-9 cleavage into its active form in $Nf1^{-/-}$ chondrocytes (**Figure 18**). These results indicate that loss of *Nf1* function in hypertrophic chondrocytes promotes their sensitivity to apoptosis.

Nf1 Is Required for Proper Growth Plate Catabolism

MMP9 and 13 are expressed by hypertrophic chondrocytes and work synergistically to degrade type I and II collagens (226). In addition, the expression of these two genes in chondrocytes is increased by FGF2 treatment (197, 227). We thus examined *Mmp9* and *Mmp13* expression in chondrocyte cultures from WT and $Nf1_{Col2}^{-/-}$ mice to address whether shortening of the hypertrophic zone in $Nf1_{Col2}^{-/-}$ mice could stem from an MMP-dependent increase or premature growth plate catabolism caused by unrestrained FGFR signaling. In agreement with the aforementioned observations, we detected a significant increase in the expression of both genes in ascorbic acid-differentiated $Nf1^{-/-}$ chondrocytes compared to WT controls (**Figure 19A,B**). These results are also in agreement with the immunoreactivity of MMP9 in the last row of hypertrophic

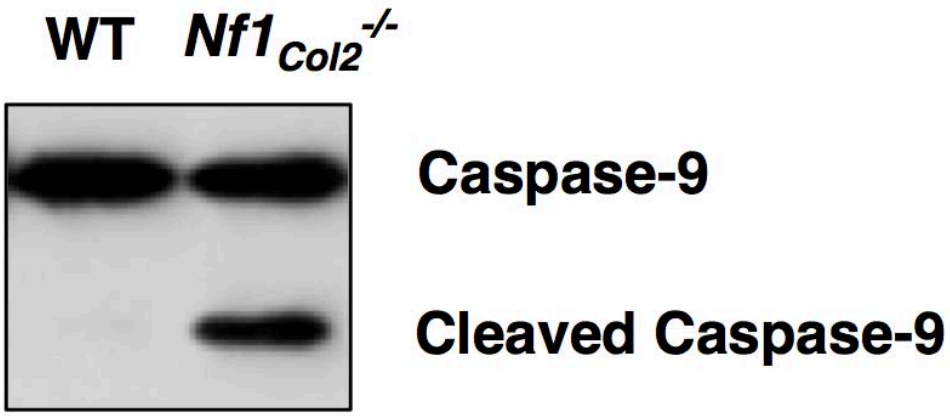

Figure 18. Increased Sensitivity to Apoptosis in *Nf1*$_{Col2}^{-/-}$ Chondrocytes
Total and cleaved caspase-9 in differentiated primary chondrocytes from WT or *Nf1*$_{Col2}^{-/-}$ mice (n=2, Western blotting). *Nf1*$^{-/-}$ chondrocytes are more sensitive to apoptosis than WT chondrocytes upon phosphate treatment.

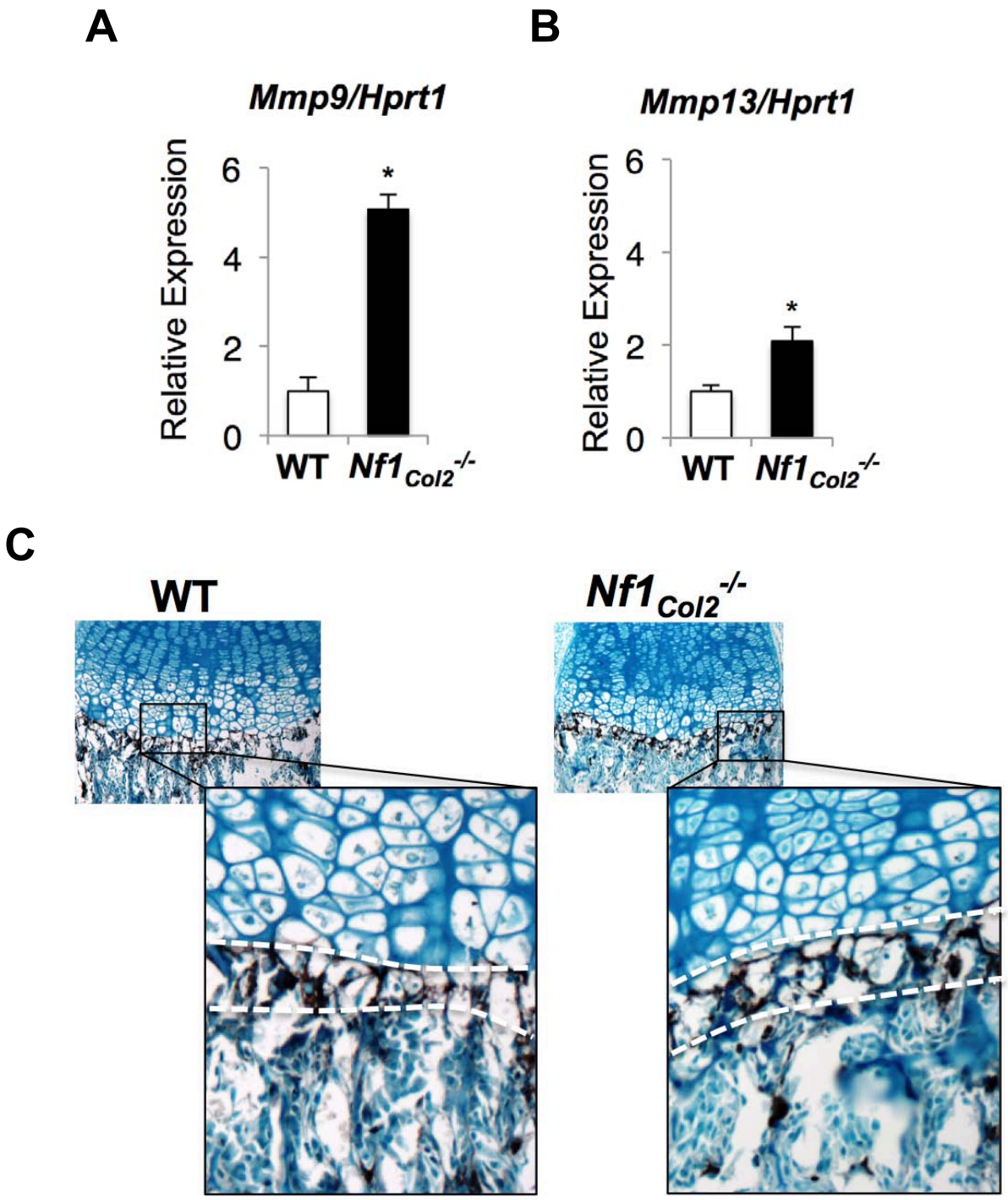

Figure 19. MMPs 9 and 13 Are Dysregulated in *Nf1*$^{-/-}$ Chondrocytes
(**A, B**) Reduced expression of *Mmp9* (A) and *Mmp13* (B) mRNA expression in *Nf1*$^{-/-}$ primary chondrocytes *in vitro* compared to WT chondrocytes (*:P<0.05, n=5). (**C**) MMP9 immunoreactivity (brown staining) n 5µm sections from WT and *Nf1*$_{Col2}^{-/-}$ mice, counterstained with Alcian blue (P0). Dotted white lines demark the last rows of hypertrophic chondrocytes.

chondrocytes (**Figure 19C** and (226, 228)), with the decrease in *Mmp9* expression observed in mice lacking *Fgfr1* in chondrocytes (49), with the increased hypertrophic chondrocyte zone in *Mmp9* and *13*-deficient mice (226), and suggest that neurofibromin, by limiting the expression of *Mmp9* and *13*, inhibits growth plate extracellular matrix degradation.

Replacement of growth plate cartilage by bone matrix is also dependent on matrix degradation by osteoclasts/chondroclasts, as demonstrated by the elongation of the hypertrophic zone and the presence of cartilaginous remnants in mice treated with pharmacological inhibitors of osteoclast activity (bisphosphonates) and in osteopetrotic mutant mice that lack osteoclasts (229). Osteoclast differentiation is dependent on osteoclastogenic cytokines, including RANKL and osteoprotegerin (OPG), produced by bone cells, including osteoblasts and osteocytes. The contribution of chondrocytes to osteoclastogenesis has been less investigated, but recent evidence indicates that matrix embedded cells, including chondrocytes and osteocytes, are essential sources of RANKL in bones (230, 231). We have shown that lack of *Nf1* in both immature and mature osteoblasts promotes *Rankl* expression and thus osteoclastogenesis (4, 135), and other have reported that ERK1/2 inactivation in chondrocytes and osteoblasts decreases *Rankl* expression and osteoclastogenesis (232). Thus, as ERK1/2 is constitutively active in $Nf1^{-/-}$ chondrocytes, we reasoned that increased *Rankl* expression and enhanced osteoclast formation and activity could cause excessive resorption and shortening of the hypertrophic zone in $Nf1_{Col2}^{-/-}$ pups. To address this hypothesis, mature osteoclasts were identified by TRAP staining in bone of newborn WT and $Nf1_{Col2}^{-/-}$ mice and their number

was quantified at the chondro-osseous border. A clear increase in the number of TRAP-positive multinuclear mature osteoclasts was observed in the tibial chondro-osseous area of $Nf1_{Col2}^{-/-}$ mice compared to WT pups (**Figure 20A**), similarly to what has been observed in mice characterized by FGFR3 activating mutation (33). In agreement with this phenotype, *Rankl* and *Opn* expression was significantly increased in differentiated $Nf1^{-/-}$ primary chondrocytes compared to WT controls, and this increase could be prevented by short term pre-treatment of the cultures with the ERK1/2 inhibitor U0126 (**Figure 20B**). In contrast, chondrocytic *Opg* expression was not affected by *Nf1* loss-of-function (**Figure 20C**). *Opn* expression was also increased in $Nf1^{-/-}$ chondrocytes (**Figure 20D**), possibly contributing to the enhanced osteoclastogenesis observed in $Nf1_{Col2}^{-/-}$ mice (233, 234).

To address whether the increased osteoclastogenesis and cartilage resorption observed in $Nf1_{Col2}^{-/-}$ mice had a chondrocytic origin, an *ex vivo* chondrocyte-monocyte *in vitro* co-culture system was used. Primary chondrocyte cultures from WT and $Nf1_{Col2}^{-/-}$ pups were prepared and WT spleen monocytes (used as osteoclast progenitors) were purified and plated on the top of the chondrocyte cultures, in the presence or absence of 1,25 OH-vitamin D_3. As shown in **Figure 21A,B**, osteoclast area, measured as readout of the osteoclastogenic potential of WT and $Nf1^{-/-}$ chondrocytes, was significantly increased in co-cultures containing $Nf1^{-/-}$ chondrocytes following 5 days of differentiation, even in the absence of vitamin D, compared to cultures containing WT chondrocytes (**Figure 21A,B**). Consistent with the increase in osteoclastogenesis observed in $Nf1_{Col2}^{-/-}$ pups, the amount of calcified bone in the primary spongiosa of newborn $Nf1_{Col2}^{-/-}$ pups was significantly

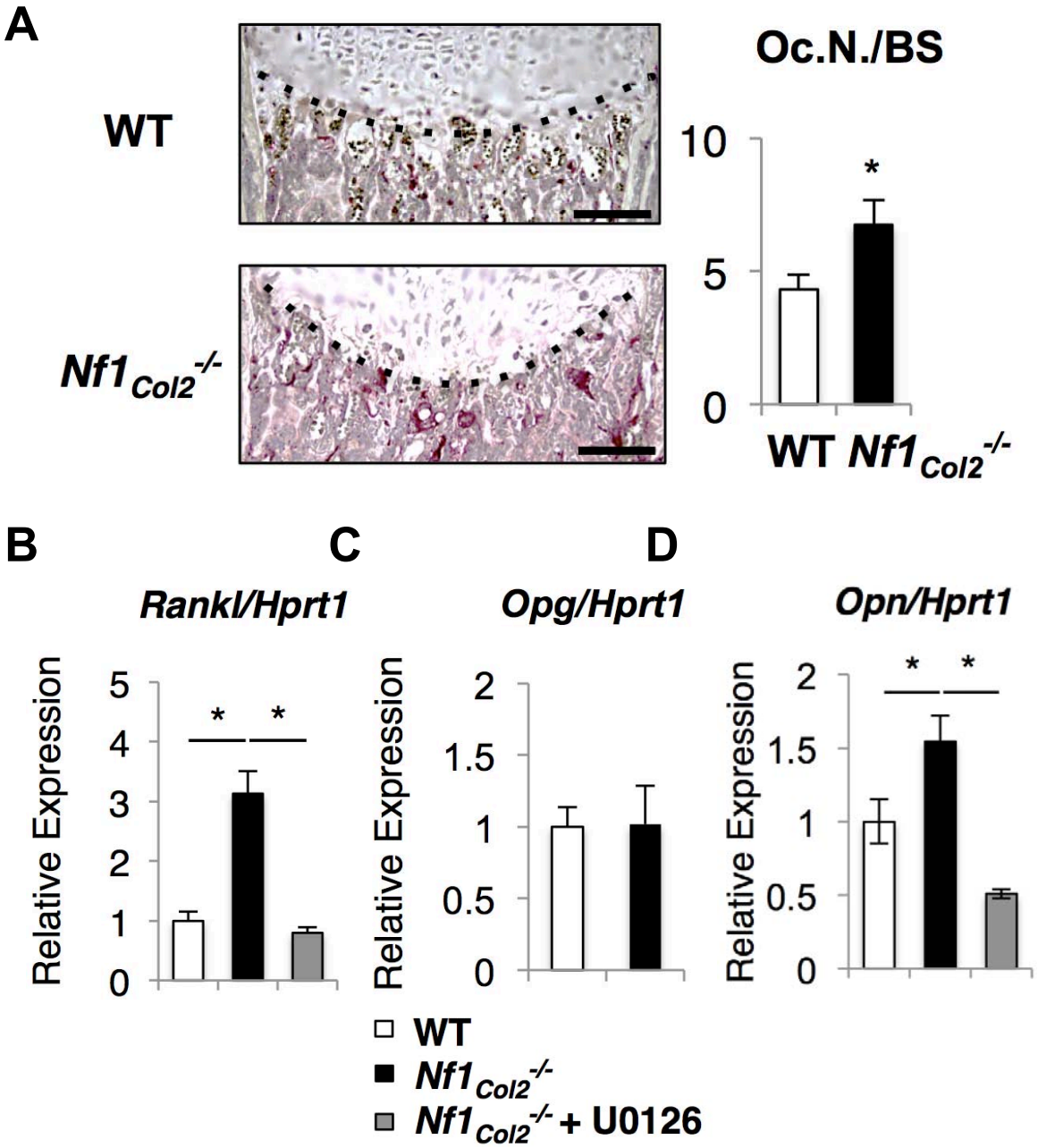

Figure 20. Enhanced Growth Plate Catabolism in $Nf1_{Col2}^{-/-}$ Mice
(**A**) TRAP staining and quantification of the number of TRAP-positive (red) osteoclasts per osteochondral surface in the proximal tibiae in WT and $Nf1_{Col2}^{-/-}$ mice at P0 (*:P<0.01, n=5). Scale bar: 100 μm. The number of osteoclasts is increased in the chondro-osseous area of $Nf1_{Col2}^{-/-}$ pups compared to WT. (**B-D**) *Rankl* (B) *Opg* (C) and *Opn* (D) mRNA expression in differentiated primary chondrocytes with or without MEK inhibitor (U0126, 10 μM, 6h) (*:P<0.01, n=3). *Rankl* and *Opn* expression are significantly increased in primary chondrocytes from $Nf1_{Col2}^{-/-}$ mice compared to WT mice, and this increase is attenuated by U0126; *Opg* expression is unaffected by *Nf1* loss-of-function.

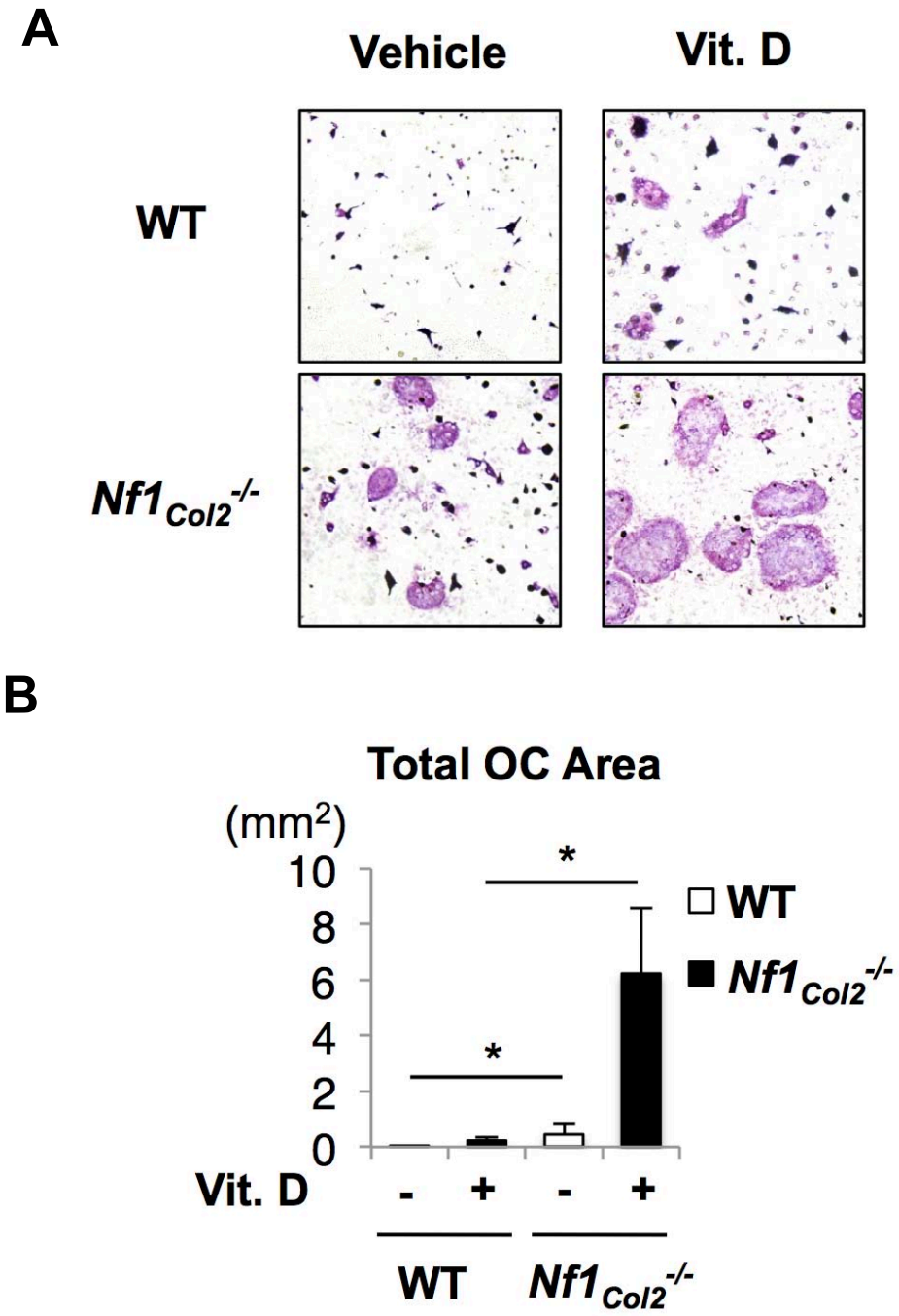

Figure 21. ***Nf1*-Null Chondrocytes Have Increased Osteoclastogenic Potential *In Vitro***

(**A**) Representative pictures of TRAP-stained chondrocyte-monocyte co-cultures using WT splenocytes and primary chondrocytes from WT and $Nf1_{Col2}^{-/-}$ mice and (**B**) quantification of osteoclast area per plate in these co-cultures (*:$P<0.01$, $n=4$). Osteoclastogenic activity is significantly increased in co-cultures containing $Nf1^{-/-}$ chondrocytes compared to WT chondrocytes.

reduced compared to WT littermates (**Figure 22**). These findings indicate that neurofibromin in differentiated chondrocytes restrains osteoclastogenesis and cartilage matrix degradation in an ERK, MMP9/13 and RANKL-dependent manner.

Neurofibromin Restrains ERK1/2 Signaling Downstream of FGFR

The phenotypic and gene expression similarities reported above, between mice characterized by FGFR activation in chondrocytes and $Nf1_{Col2}^{-/-}$ mice, support the notion that neurofibromin acts as a brake on FGFR signaling in chondrocytes. In such case, we reasoned that ERK1/2 activation upon FGFR stimulation in WT chondrocytes should be controlled and limited in time, whereas it should be prolonged in *Nf1*-deficient chondrocytes. To address this question, primary chondrocytes extracted from P4 WT and mutant $Nf1_{Col2}^{-/-}$ pups were prepared, cultured for 7 days and assessed by Western analyses for their response to FGF2 pulse-stimulation. Upon FGF2 treatment (10 ng/ml, 5 min), maximal ERK1/2 activation was observed after 5 min of treatment in WT chondrocytes, and activation was progressively dampened at later time points to return close to baseline within 30 min (**Figure 23A**). In contrast, in $Nf1^{-/-}$ chondrocytes, ERK activation was detectable in non-treated cultures and remained sustained at the 10 and 30 min time points. As a control, ERK1/2 activation in response to epithelial growth factor (EGF, 100 ng/ml) was similar between WT and $Nf1^{-/-}$ chondrocytes (**Figure 23B**). These results thus indicate that neurofibromin is a negative regulator of ERK1/2 activation downstream of FGFR signaling in differentiated chondrocytes.

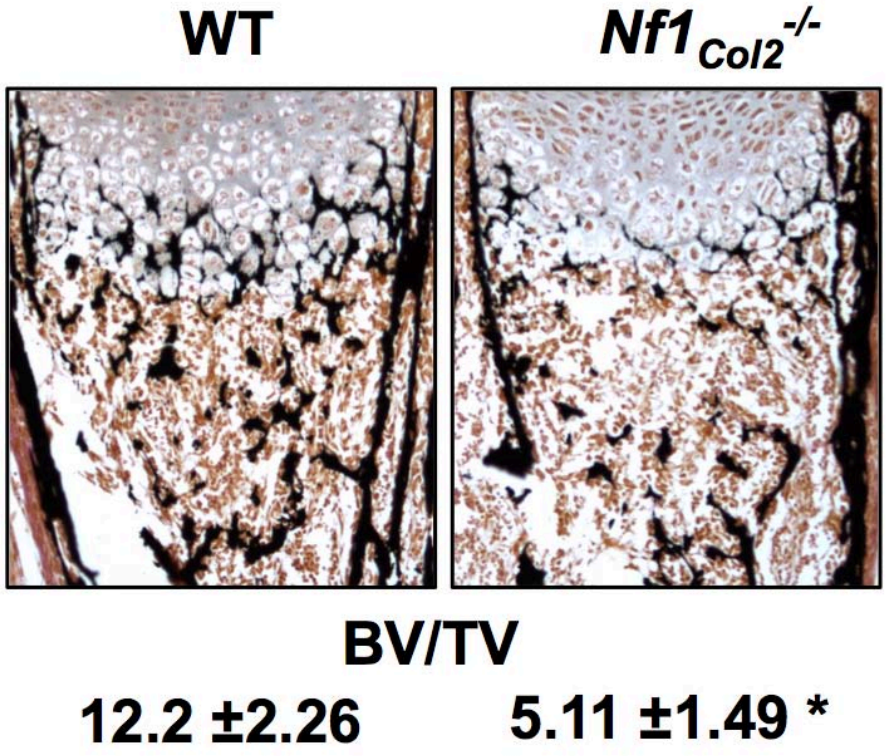

Figure 22. Newborn *Nf1*$_{Col2}^{-/-}$ Mice Have a Lower BV/TV Ratio
Von Kossa/Van Gieson staining for mineralized and unmineralized bone revealed a decreased tibial trabecular bone volume to total volume ratio (BV/TV, *:P<0.01, n=5) in P0 *Nf1*$_{Col2}^{-/-}$ pups compared to WT littermates in the primary spongiosa.

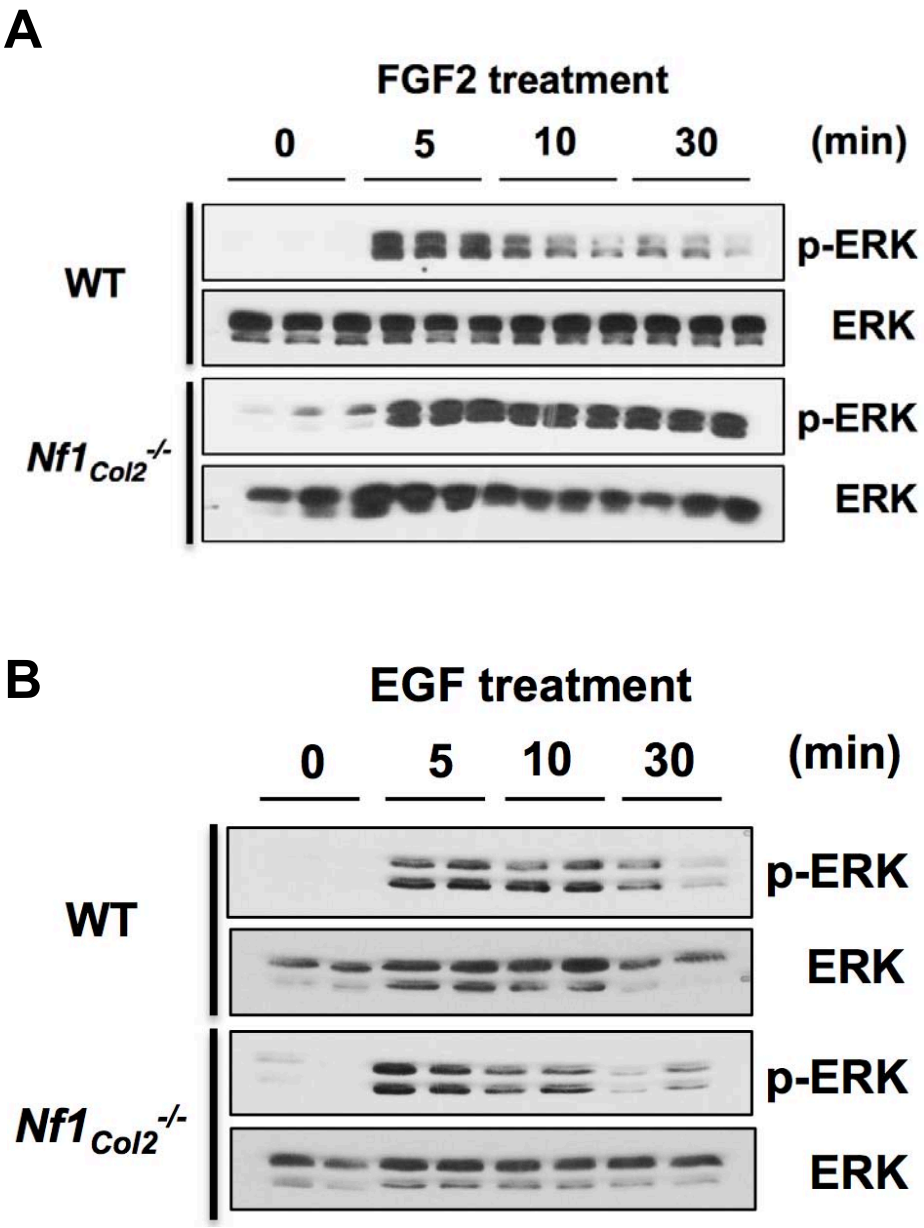

Figure 23. Neurofibromin Restrains ERK-Dependent FGFR Signaling in Chondrocytes

(**A, B**) Confluent primary chondrocytes from WT or $Nf1_{Col2}^{-/-}$ mice were serum-starved and harvested following FGF2 (10 ng/ml, 5 min) (A) or EGF (100 ng/ml, 5 min) (B) pulse treatment. Cells were collected at indicated periods of time after treatment. ERK1/2 activation was transient in WT chondrocytes but remained sustained in $Nf1^{-/-}$ chondrocytes in response to FGF2, whereas no difference between genotype was observed following EGF treatment.

The CNP Analog NC-2K Increases Bone Growth in $Nf1_{Col2}^{-/-}$ Mice

Recombinant forms of CNP have shown promising beneficial effects in preclinical models of dwarfism characterized by FGFR3 activation (191, 200). CNP is a member of the natriuretic peptide family encoded by *Nppc,* which, through activating *cyclic* guanosine monophosphate (cGMP) and PKG, blocks activation of the ERK pathway by inhibiting Raf1 (197, 235). If ERK1/2 constitutive activation in $Nf1^{-/-}$ chondrocytes contributes to the observed abnormalities in endochondral bone formation observed in $Nf1_{Col2}^{-/-}$ mice, we thus reasoned that restraining ERK activation by CNP should correct these defects. To address this hypothesis, we first verified the expression of *Npr-b*, the receptor for CNP, in differentiating primary chondrocytes from WT and $Nf1_{Col2}^{-/-}$ chondrocyte cultures. Chondrocytes from both WT and $Nf1_{Col2}^{-/-}$ mice showed clear expression of *Npr-b* throughout chondrogenic differentiation (**Figure 24A**), as previously described by *in situ* hybridization (192, 200, 236). *Npr-b* expression was detected at every differentiation stages, with a more pronounced expression level in differentiated chondrocytes (2 weeks post-induction). Importantly, *Nf1* deficiency did not significantly alter the level of expression of *Npr-b*. Next, ERK1/2 activation was assessed in WT and $Nf1^{-/-}$ primary chondrocytes treated with increasing doses of NC-2, a stable recombinant form of CNP (see Material and Methods). At the three different doses tested, NC-2 strongly suppressed the enhanced ERK1/2 phosphorylation typical of $Nf1^{-/-}$ chondrocytes *in vitro* (**Figure 24B**).

Unlike CNP, which has a half-life of around 3 min, the circulating half-life of NC-2 is ~20h when injected subcutaneously in mice, making it very useful for *in vivo* studies.

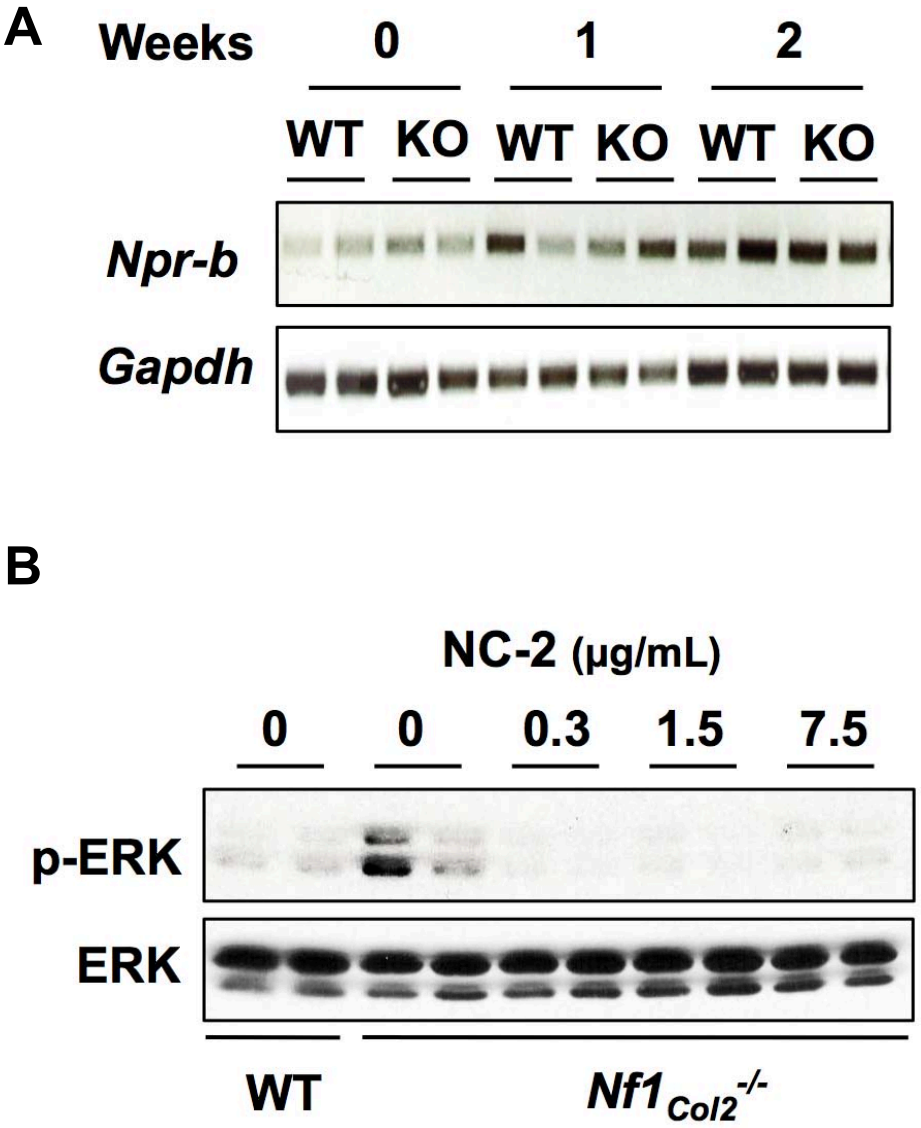

Figure 24. Treatment with the CNP Analog NC-2 Reduces ERK Activation in *Nf1*-Null Chondrocytes

(**A**) Expression of the CNP receptor *Npr-b* was measured by RT-PCR throughout chondrogenic differentiation in primary rib chondrocyte cultrues from both WT and $Nf1_{Col2}^{-/-}$ mice (WT chondrocytes, KO; $Nf1^{-/-}$ chondrocytes). *Npr-b* is expressed throughout chondrogenic differentiation in primary chondrocytes from WT and $Nf1_{Col2}^{-/-}$ mice. (**B**) Decreased phospho-ERK1/2 status in serum-starved chondrocytes from $Nf1_{Col2}^{-/-}$ mice following NC-2 treatment for 30 min, measured by Western blot analysis (n=2).

Based on our *in vitro* proof-of-concept data, $Nf1_{Col2}^{-/-}$ mice were treated subcutaneously and daily with vehicle or two doses of NC-2 (100 and 300 mg/kg) during their early postnatal growth period (from birth to 18 days of age). As observed *in vitro*, NC-2 treatment (300mg/kg) for 10 days was able to substantially attenuate p-ERK immunoreactivity in the growth plates of $Nf1_{Col2}^{-/-}$ mice, although the effect of treatment on growth plate structure was not yet very visible, except for a striking improvement in the formation of the proliferating chondrocyte columns (**Figure 25A**). In agreement with our previous analyses at an earlier age (4), $Nf1_{Col2}^{-/-}$ mice displayed, following 18 days of vehicle treatment, a 40% reduction in naso-anal length at time of sacrifice compared to WT littermates, whereas $Nf1_{Col2}^{-/-}$ mice treated with NC-2 displayed a significantly increased body size at both dose concentrations tested compared to vehicle-treated $Nf1_{Col2}^{-/-}$ mice (**Figure 25B**). At endpoint, the difference in body length between vehicle and NC-2-treated $Nf1_{Col2}^{-/-}$ mice was reduced from 40% to 15%. Growth plate histological analyses indicated that the shortening of the proliferating and hypertrophic zones observed in $Nf1_{Col2}^{-/-}$ mice were significantly corrected following daily NC-2 treatment, reaching sizes not significantly different from WT littermates (as quantified in **Figure 26A-C**). The columnar disorganization of proliferating chondrocytes notable in $Nf1_{Col2}^{-/-}$ mice was also corrected following NC-2 treatment (**Figure 25A and 26A**). These results support the notion that *Nf1* controls postnatal endochondral bone formation in an ERK-dependent fashion.

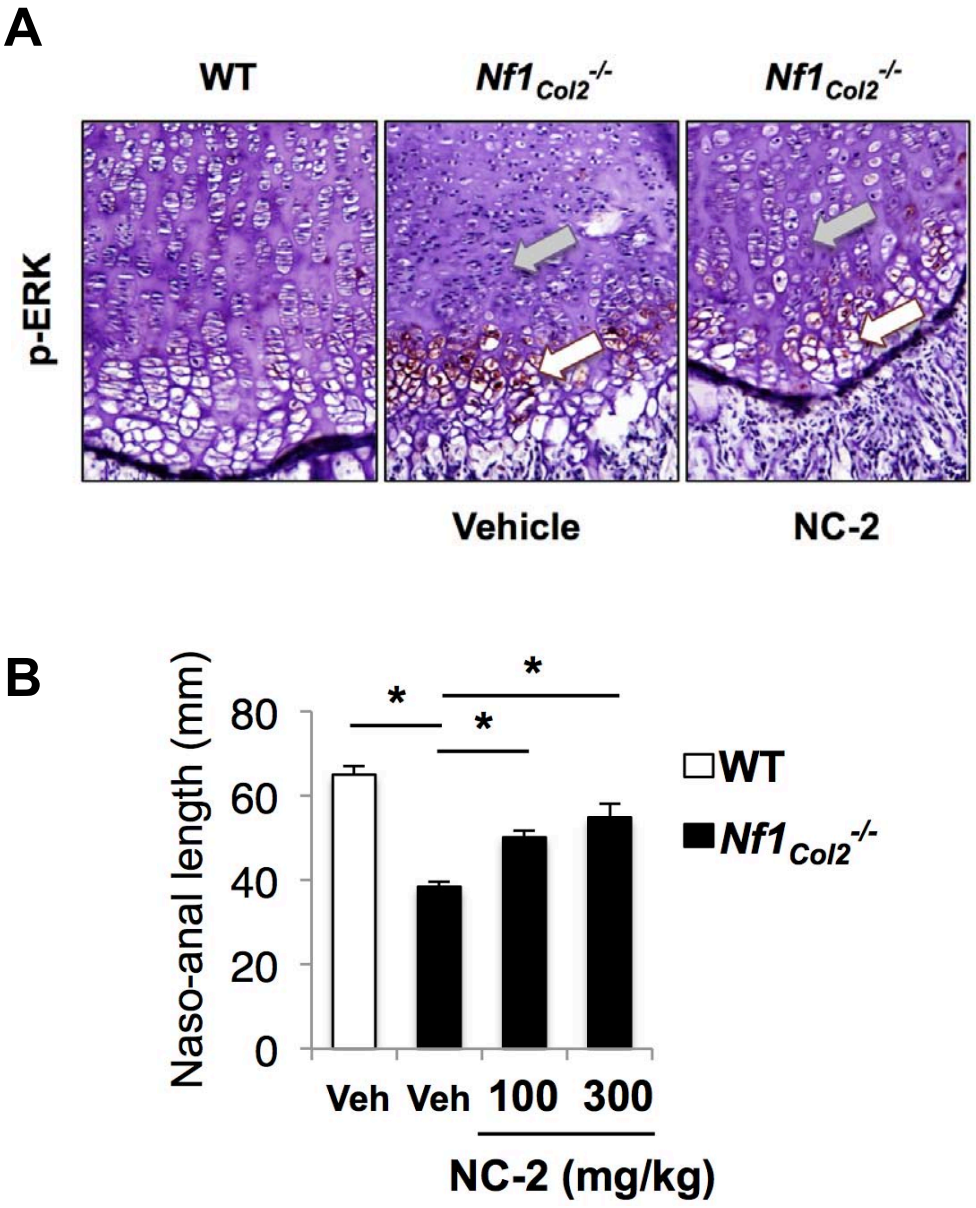

Figure 25. NC-2 Reduces ERK Activation and Improves Body Length in $Nf1_{Col2}^{-/-}$ Mice

(**A**) Reduced *in vivo* p-ERK immunoreactivity in the tibial growth plate of $Nf1_{Col2}^{-/-}$ mice (P10) following daily treatment with NC-2 (300mg/kg) compared with $Nf1_{Col2}^{-/-}$ mice treated with vehicle (n=2). Gray arrows show the changes in the formation of proliferative columns. White arrows show the immunoreactivity (brown) for p-ERK1/2. (**B**) NC-2 treatment corrects the growth retardation of $Nf1_{Col2}^{-/-}$ mice as shown by increased naso-anal length (mm) of $Nf1_{Col2}^{-/-}$ mice treated with increasing doses of NC-2 (*:P <0.01, n=7).

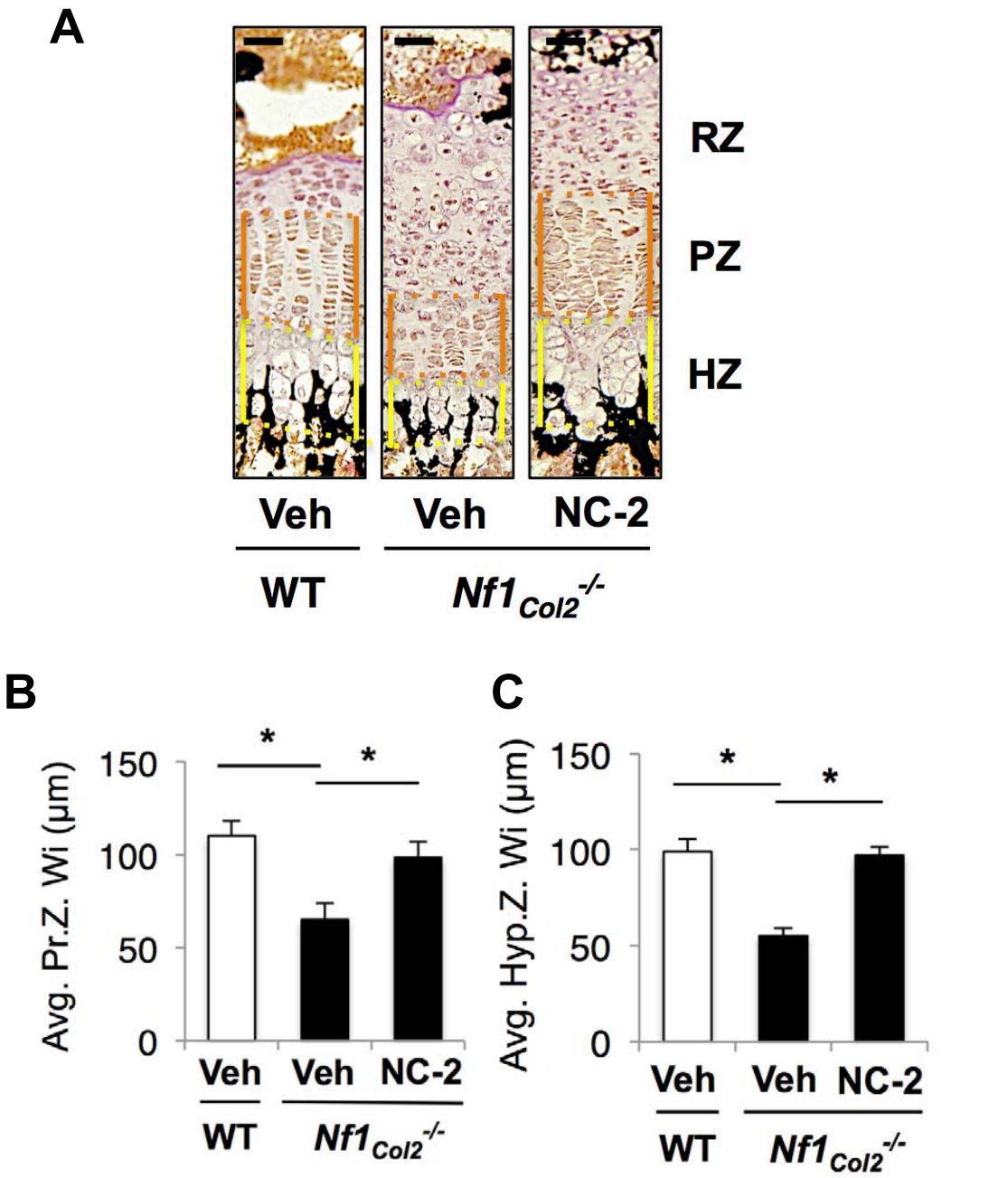

Figure 26. NC-2 Treatment Improves Growth Plate Elongation in $Nf1_{Col2}^{-/-}$ Mice
(**A**) Representative images of Von Kossa/Van Gieson stained, undecalcified femur sections from 18 day-old WT and $Nf1_{Col2}^{-/-}$ mice treated with NC-2 (300mg/kg) or vehicle. Scale bar; 50 µm. Proliferative (PZ) and hypertrophic (HZ) zones are depicted by orange and yellow bars respecively, and appear more organized and longer with NC-2 treatment in $Nf1_{Col2}^{-/-}$ mice compared with vehicle treated $Nf1_{Col2}^{-/-}$ mice. (**B, C**) NC-2 treatment improved the shortened proliferating (B) and hypertrophic (C) zone lengths typical of $Nf1_{Col2}^{-/-}$ mice (*:P <0.01, n=7) versus vehicle treated $Nf1_{Col2}^{-/-}$ mice

Discussion

FGFRs play a critical role during bone development, as best demonstrated by the various forms of skeletal dysplasias associated with mutations in *Fgfr1* and *Fgfr3* in mice and humans (see (237) for review). Over the last 10 years, genetic mouse models have been instrumental to the characterization of FGFR downstream signaling pathways, amongst which the kinases MEK1 and STAT1 play a predominant role (30, 40, 44, 208, 238-240). In this study, we provide evidence that the Ras-GTPase activity of neurofibromin is required for proper chondrocyte proliferation, hypertrophy and growth plate catabolism, and regulates ERK1/2-dependent FGFR signaling in differentiated chondrocytes.

Loss of *Nf1* function in postmitotic chondrocytes led to the inhibition of chondrocyte proliferation. Both *Fgfr3* and *Ihh* are important genes controlling chondrocyte proliferation during embryonic and postnatal growth (241-243). IHH promotes chondrocyte proliferation (244-246) and activation of FGFR3 represses chondrocyte proliferation through its inhibition of IHH signaling (23, 32) as well as IHH-independent pathway(s) (19). Because neurofibromin is not detected in proliferative chondrocytes (99) but rather expressed in prehypertrophic chondrocytes (where both FGFR1 and FGFR3 are expressed) and hypertrophic chondrocytes (where FGFR1 is expressed) (32, 49), we reasoned that the reduced chondrocyte proliferation observed in $Nf1_{Col2}^{-/-}$ mice must stem from alteration in the secretion of paracrine factor(s) by prehypertrophic or hypertrophic $Nf1^{-/-}$ chondrocytes. The strongly reduced expression of *Ihh* in $Nf1^{-/-}$ chondrocyte cultures and growth plates, as well as the common phenotypic growth plate features between

Nf1$_{Col2}^{-/-}$ mice and mice lacking *Ihh* in type II collagen-expressing chondrocytes (247) lead us to propose that neurofibromin is an important regulator of *Ihh* expression in prehypertrophic chondrocytes. Whether neurofibromin regulates *Ihh* expression downstream of FGFR3 or FGFR1, or both, in prehypertrophic chondrocytes, and the nature of the signaling pathway between Ras and *Ihh* transcriptional activation remains to be determined.

The distinct expression patterns of FGFR1 and FGFR3 in the growth plate and the overlapping localization of neurofibromin and FGFR1 in hypertrophic chondrocytes, as well as in the perichondrium (48, 49, 58, 219, 248), suggest that neurofibromin controls FGFR1 signaling in hypertrophic chondrocytes. This hypothesis is supported by the strikingly similar phenotypes shared by *Nf1*$_{Col2}^{-/-}$ mice and mice overexpressing a chimeric FGFR containing the extracellular domain of FGFR3$_{ach}$ replaced with the intracellular domain of FGFR1, under the control of the *Col2a1*-cre promoter (219). Conversely, mice with *Col2a1*-cre-driven chondrocyte-specific inactivation of *Fgfr1* exhibit phenotypes opposite to *Nf1*$_{Col2}^{-/-}$ mice, including expansion of their hypertrophic type 10 collagen-positive zone, reduced *Opn* and *Mmp9* expression and decreased number of TRAP-positive osteoclasts at the chondro-osseous junction at embryonic stages (49). Despite this collection of concordant indirect evidence, genetic crosses between *Fgfr* and *Nf1* mutant mice will be required to further dissect the intricate regulatory mechanisms linking neurofibromin and FGFR signaling in chondrocytes.

The role of neurofibromin in growth plate development appears to be particularly important during postnatal growth, as $Nf1_{Col2}^{-/-}$ mice are dwarf postnatally but born with a size similar to WT littermates. This suggests that neurofibromin plays a non-redundant and necessary role during the embryonic to postnatal transition period, but does not exclude that its activity is required during embryonic growth plate development and osteogenesis, as supported by the shortening of the hypertrophic zone, the reduced osteogenesis and increased cortical bone porosity that are measurable at birth already. The alterations in gene expression and the length reduction of the hypertrophic zone in $Nf1_{Col2}^{-/-}$ embryos are indicative of altered chondrocyte maturation in absence of *Nf1*. The similar *in situ* staining pattern for *Ihh* expression between WT and $Nf1_{Col2}^{-/-}$ pups (4) suggests that the reduced expression of *Ihh* observed in $Nf1^{-/-}$ chondrocyte cultures and in the growth plates of $Nf1_{Col2}^{-/-}$ mice is due to suppression of *Ihh* gene expression/protein synthesis rather than a reduction in the number of cells expressing *Ihh*. On the other hand, increased osteoclastogenesis in transgenic mice expressing the *Fgfr3* (G369C/+) activating mutation has been reported, and the phenotype was attributed to a direct effect of FGFR3 activation in osteoclasts (249). Our gene expression and chondrocyte/monocyte co-culture data point to chondrocyte-derived RANKL, MMP9 and 13, and possibly OPN as alternative or additional cause(s) of increased osteoclastogenesis and premature cartilage removal in this mouse model and possibly in patients with chondrodysplasia (224, 250-253). In addition, the increase in *Mmp9* and *Mmp13* expression in $Nf1^{-/-}$ chondrocytes, and shortening of the hypertrophic zone in $Nf1_{Col2}^{-/-}$ embryos, are in agreement with previous reports indicating that FGF2 stimulates *Mmp9* and *Mmp13* expression in chondrocytes (227), and with the elongated hypertrophic zone

observed in mice lacking MMP9 and 13 (226). It is also noteworthy that the MMP9 immunoreactivity pattern was distinct from the distribution of osteo/chondroclasts at the osteochondral border, indicating that increased MMP9 activity from both mature osteoclasts and hypertrophic chondrocytes may contribute to the promotion of growth plate catabolism in $Nf1_{Col2}^{-/-}$ mice. These data together thus indicate that neurofibromin activity in hypertrophic chondrocytes is necessary during embryonic development to limit cartilage matrix degradation at the ossification front. It is noteworthy that *Rankl* and *Opn* appear both to be direct targets of neurofibromin signaling in chondrocytes, as their expression is reduced upon short-term ERK inhibition. This observation suggests that because of unrestrained ERK1/2 activity, both genes are ectopically expressed by $Nf1^{-/-}$ hypertrophic chondrocytes *in vivo*, hence explaining the increase in genes commonly considered as hypertrophic chondrocyte markers, in the setting of reduced expression of *Ihh and Col10a1*.

It is at this point still unclear if the shortening of the hypertrophic zone observed in $Nf1_{Col2}^{-/-}$ mice is predominantly caused by altered differentiation of $Nf1^{-/-}$ chondrocytes, their increased sensitivity to apoptosis, or by premature or enhanced growth plate cartilage resorption. Since the mineralized cartilage matrix serves as a template for bone formation and since osteogenesis is coupled to chondrogenesis (254-256), it is also possible that the increased growth plate catabolism observed in $Nf1_{Col2}^{-/-}$ mice contributes, along with the low level of chondrocyte-derived IHH, to the reduced osteogenesis and increased cortical bone porosity measured in these mice (4). The fact that the type II collagen-cre transgene inactivates the floxed *Nf1* gene not only in chondrocytes, but also

in embryonic osteochondroprogenitors (4, 49) giving rise to bone marrow osteoblasts in this particular mouse model, however, did not allow us to tease apart the relative contribution of *Nf1* in type II collagen-expressing cells to growth plate development versus bone formation. The signaling mechanism whereby neurofibromin controls *Rankl, Mmp9* and *Mmp13* expression also remains to be identified. Regardless of the respective contribution of *Nf1* in osteoblasts and chondrocytes, these data identify *Nf1* as a necessary component for normal growth plate homeostasis during development, regulating chondrocyte proliferation, differentiation, apoptosis and growth plate catabolism (**Figure 27**).

Mutations in *NF1* in patients with neurofibromatosis type I cause various bone abnormalities, of which two of the more severe are tibial pseudarthrosis (non-union following fracture) and dystrophic scoliosis. NF1 patients are haploinsufficient for *NF1* and, as observed in $Nf1^{+/-}$ mice, do not present with a significant reduction in size, although they are on average slightly shorter (see (1) for review). The clinical presentation of the NF1 focal dysplasias and results from genetic mouse models support a model whereby somatic *NF1* loss of heterozygosity in a subset of bone marrow osteochondroprogenitors causes the focal and dystrophic skeletal maladies in NF1 (4, 5, 129, 133). The severity of the bone growth phenotype in $Nf1_{Col2}^{-/-}$ mice contrasts, however, with the nearly normal body size of NF1 patients and suggests that *NF1* loss-of-function in chondroprogenitors or chondrocytes during early development is not the cause of the moderate size reduction seen in NF1 patients. Our results suggest, however, that loss of neurofibromin function in growth plate osteochondroprogenitors might

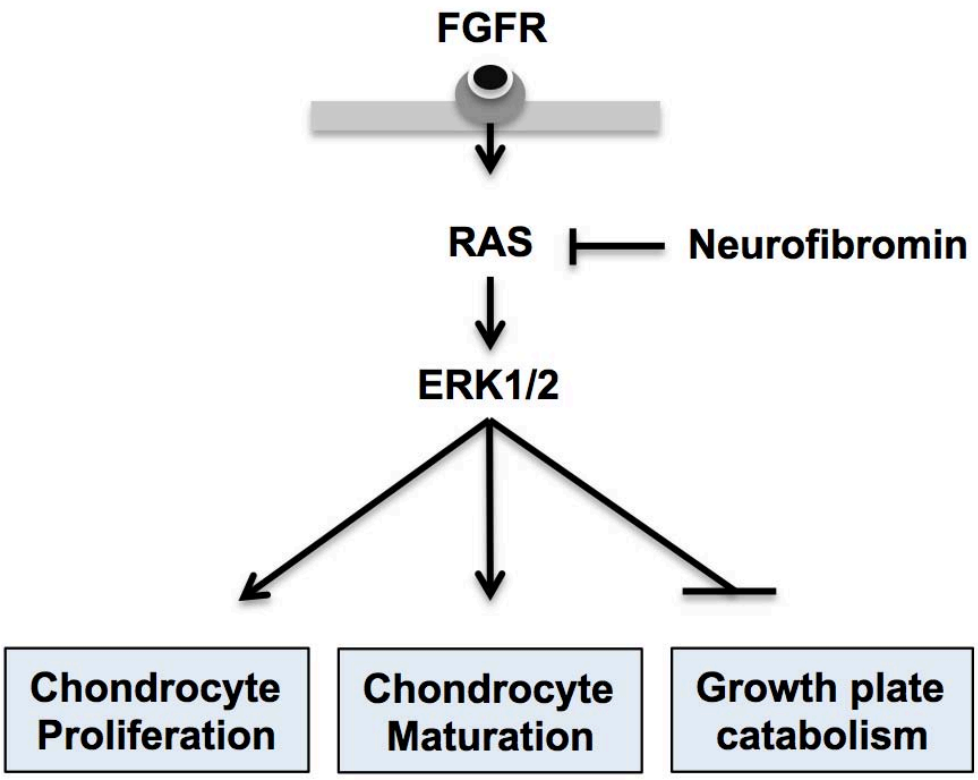

Figure 27. Model Summarizing the Role of Neurofibromin During Growth Plate Development

Neurofibromin, expressed in prehypertrophic and hypertrophic chondrocytes, restrains Ras-ERK1/2-dependent FGFR signaling, thereby promoting chondrocyte proliferation and maturation, and inhibiting growth plate catabolism to maintain proper growth plate homestasis**.**

contribute to the severe delay of fracture healing observed in some of these patients, as bone repair requires the timely transition through endochondral bone formation steps, where developmental genes are reactivated in adults. If this is verified, the ability of the NPR-B agonist NC-2 to blunt ERK chronic activation typical of *Nf1*$^{-/-}$ chondrocytes and to reverse the chondrocyte phenotypes of *Nf1*$_{Col2}^{-/-}$ mice might translate to beneficial outcomes in the treatment of NF1 pseudarthrosis. Lastly, although the majority of known cases of chondrodysplasias are caused by mutations in *FGFR1* or *FGFR3*, this study raises the possibility that some of the ACH cases with no mutation detected in these two receptors could stem from somatic inactivating mutations of *NF1*.

Summary

The severe defects in growth plate development caused by chondrocyte ERK1/2 gain or loss-of-function suggest that tight spatial and temporal regulation of MAPK signaling is necessary to achieve harmonious growth plate elongation and structure. We provide here evidence that neurofibromin, via its Ras GTPase-activating activity, controls ERK1/2-dependent FGFR signaling in chondrocytes. We show first that neurofibromin is expressed in FGFR-positive prehypertrophic and hypertrophic chondrocytes during growth plate endochondral ossification. Using mice lacking *Nf1* in type II collagen-expressing cells, (*Nf1*$_{Col2}^{-/-}$ mutant mice), we then show that lack of neurofibromin in postmitotic chondrocytes triggers a number of phenotypes reminiscent of the ones observed in mice characterized by FGFR gain-of-function mutations. Those include dwarfism, constitutive ERK1/2 activation, strongly reduced *Ihh* expression and decreased

chondrocyte proliferation and maturation, increased chondrocytic expression of *Rankl, Mmp9* and *Mmp13* and enhanced growth plate osteoclastogenesis, as well as increased sensitivity to caspase-9 mediated apoptosis. Using wild type (WT) and $Nf1^{-/-}$ chondrocyte cultures *in vitro*, we show that FGF2 pulse-stimulation triggers rapid ERK1/2 phosphorylation in both genotypes, but that returns to basal level is delayed in $Nf1^{-/-}$ chondrocytes. Importantly, *in vivo* ERK1/2 inhibition by daily injection of a recombinant form of C-type natriuretic peptide (CNP) to postnatal pups for 18 days was able to correct the short stature of $Nf1_{Col2}^{-/-}$ mice. Together, these results underscore the requirement of neurofibromin and ERK1/2 for normal endochondral bone formation and support the notion that neurofibromin, by restraining RAS-ERK1/2 signaling, is a negative regulator of FGFR signaling in differentiating chondrocytes.

CHAPTER III

FGFR1 SIGNALING IN HYPERTROPHIC CHONDROCYTES IS ATTENUATED BY THE RAS-GAP NEUROFIBROMIN DURING ENDOCHONDRAL BONE FORMATION

This is a pre-copyedited, author-produced, adapted version of an article accepted for publication in *Human Molecular Genetics* following peer review. The version of record is: Karolak, M.R., Yang, X. and Elefteriou, F. (2015) FGFR1 signaling in hypertrophic chondrocytes is attenuated by the Ras-GAP neurofibromin during endochondral bone formation. *Hum. Mol. Gen.*, 24,2252-2264.

Introduction

As discussed in Chapter I, endochondral bone formation is a complex, highly regulated and orderly multi-step process by which long bones elongate during development and heal following fracture. Failure or dysregulation of any of these steps during development typically leads to shortened stature or dwarfism, structural bone abnormalities (11), whereas failure during fracture healing in adults leads to fracture non-union (pseudarthrosis) (257).

In Chapter II, I explored the function of neurofibromin in growth plate chondrocytes both *in vitro* and *in vivo*. These studies suggested a role for neurofibromin in attenuating

FGFR signaling in growth plate chondrocytes. Fibroblast growth factor (FGF) signaling during endochondral bone formation has been the subject of intense scientific inquiry. As discussed in Chapters I and II, FGFRs 1 and 3 were shown to be expressed in distinct zones: FGFR3 in proliferating chondrocyte columns and FGFR1 in hypertrophic chondrocytes (**Figure 9B** and (34, 37, 48, 49, 58)). The non-overlapping expression patterns of FGFR1-FGFR3 suggest that these receptors have unique functions, mediated by differences in their ligand-binding specificity and/or downstream signaling. In addition, human cases indicate that FGFR1 signaling is crucially important for endochondral ossification as activating mutations in *FGFR1* cause osteoglophonic dysplasia (50, 258).

Loss-of-function mutations in *NF1* cause chronic and unregulated Ras and ERK1/2 activity and results in neurofibromatosis type-1 (NF1). Interestingly, NF1 skeletal pathologies, including non-ossifying bone lesions, pseudarthrosis, shortened stature, idiopathic or dystrophic scoliosis, asymmetry of facial bones, osteosclerosis, tibial bowing, hypertelorism, pectus excavatum and low bone mineral density (1, 107-110), are highly reminiscent of the bone manifestations observed in individuals with osteoglophonic dysplasia, although more focal and heterogeneous than the latter. Our previous studies have shown that *Nf1* deletion in *Col2a1*-positive murine chondrocytes leads to Ras-ERK1/2 chronic activation, to a reduction in the proliferation and hypertrophic zones of the growth plate and to dwarfism compared with wild-type (WT) littermates ((4) and **Figures 11, 13, 23, 25**). In addition, these mice display an increased number of osteoclasts at the osteochondral border, and *Nf1*-deficient chondrocytes

favored osteoclastogenesis *ex vivo*, suggesting that in chondrocytes, neurofibromin serves to limit osteoclastogenesis and growth plate catabolism during development.

Genetic mouse models with *Nf1* deficiency or *Fgfr1* gain- or loss-of-function mutations support a negative association between FGFR1 signaling and neurofibromin activity. First, we found that the activation of ERK1/2 by FGF2 treatment is dampened to baseline activity within 30 min in WT chondrocytes, whereas it lasts more than 1h in *Nf1*-deficient chondrocytes. These findings suggested that neurofibromin acts as a brake on FGFR signaling in chondrocytes (**Figure 23**). Second, the hypertrophic zone of $Fgfr1^{Col2cKO}$ mice, in which *Fgfr1* is ablated in *Col2a1*-positive chondrocytes, is longer than in WT littermates, in contrast to the shortened hypertrophic zones of the $Nf1^{Col2cKO}$ mice, which lack *Nf1* in the same *Col2a1*-positive cells ((49) and **Figure 13**). Third, $Fgfr1^{Pro250Arg}$ mice, harboring a constitutively active $Fgfr1^{Pro250Arg}$ allele at the endogenous *Fgfr1* locus, are dwarfed as are $Nf1^{Col2cKO}$ mice (4, 52). Lastly, at the molecular level, the growth plate expression of matrix metalloproteinase 9 (*Mmp9*) and osteopontin (*Opn*), two proteins contributing to growth plate catabolism and osteoclastogenesis at the osteochondral front, are downregulated in $Fgfr1^{Col2cKO}$ mice while they are upregulated in $Nf1^{Col2cKO}$ mice ((49) and **Figures 16, 19, 20**). Therefore, constitutive activation of FGFR1 in $Fgfr1^{Pro250Arg}$ mice leads to similar phenotypes to the ones observed in $Nf1^{Col2cKO}$ mice lacking *Nf1* in chondrocytes, whereas loss of FGFR1 in $Fgfr1^{Col2cKO}$ mice triggers phenotypes that are mostly opposite to the ones observed in $Nf1^{Col2cKO}$ mice. Collectively, these observations led us to hypothesize that neurofibromin acts as a brake on FGFR1 signaling in the developing growth plate. In this study, we generated double mutant mice

lacking both *Nf1* and *Fgfr1* in *Col2a1*-positive chondrocytes to ask whether loss of activity of neurofibromin and thus activation of Ras-ERK1/2 signaling reverses the aberrant hypertrophic zone length and decreased osteoclastogenesis caused by lack of FGFR1 signaling in $Fgfr1^{Col2cKO}$ mice (see **Figure 30**). Our results support a model whereby neurofibromin controls: (i) the cartilage catabolism-promoting function of FGFR1 signaling in hypertrophic chondrocytes and (ii) the organization and extension of proliferative chondrocyte columns, via regulation of FGFR3 (and possibly FGFR1) signaling in prehypertrophic chondrocytes.

Materials and Methods

Animals and Drugs

The Vanderbilt University Institutional Animal Care and Use Committee (IACUC) approved all animal procedures. To generate $Dbl^{Col2cKO}$ mice, we employed a two-armed breeding scheme (**Figure 28**). In arm one, we bred *Col2a1-Cre*;$Fgfr1^{flox/flox}$;$Nf1^{flox/+}$ males with $Fgfr1^{flox/flox}$;$Nf1^{flox/+}$ females to generate WT (cre-negative), $Fgfr1^{Col2cKO}$ (*Col2a1-Cre*;$Fgfr1^{flox/flox}$;$Nf1^{+/+}$;) and $Dbl^{Col2cKO}$ (*Col2a1-Cre*;$Fgfr1^{flox/flox}$;$Nf1^{flox/flox}$) mice (55, 128, 143). In arm two, we bred *Col2a1-Cre*;$Fgfr1^{flox/+}$;$Nf1^{flox/+}$ males with $Fgfr1^{flox/+}$;$Nf1^{flox/flox}$ females to generate WT (cre-negative), $Nf1^{Col2cKO}$ (*Col2a1-Cre*;$Fgfr1^{+/+}$;$Nf1^{flox/flox}$;) and $Dbl^{Col2cKO}$ (*Col2a1-Cre*; $Fgfr1^{flox/flox}$;$Nf1^{flox/flox}$) mice. Arm two used *Col2a1-Cre*;$Fgfr1^{flox/+}$;$Nf1^{flox/+}$ males rather than *Col2a1-Cre*;$Fgfr1^{flox/+}$;$Nf1^{flox/flox}$ males because *Col2a1-Cre*;$Nf1^{flox/flox}$ mice are infertile. Progenies were obtained at a Mendelian ratio (~12.5% $Fgfr1^{Col2cKO}$, ~6.25% $Nf1^{Col2cKO}$ and ~12.5% or ~6.25% $Dbl^{Col2cKO}$, depending

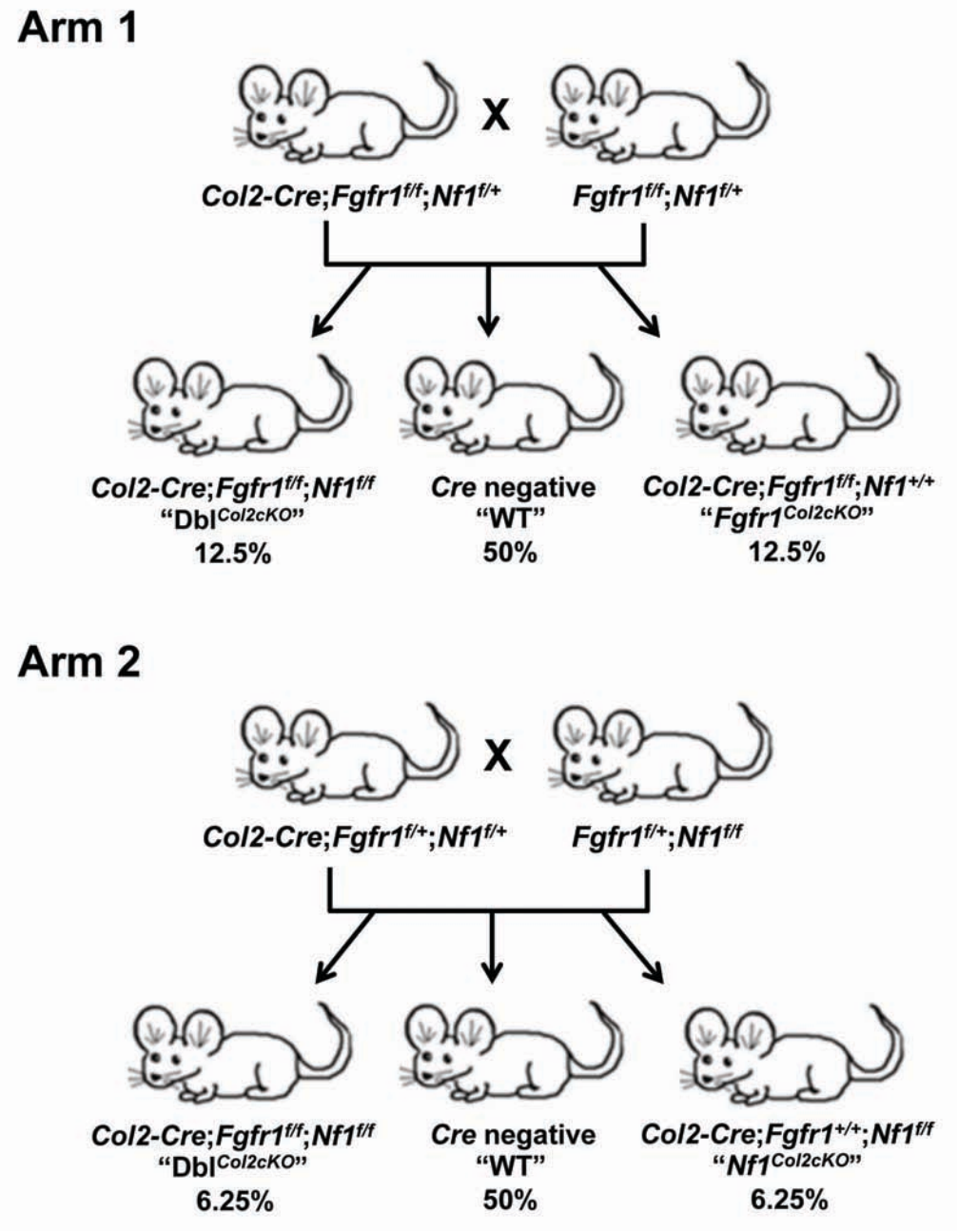

Figure 28. Breeding Scheme Generating Littermate WT, *Fgfr1*^{Col2cKO}, *Nf1*^{Col2cKO} and Dbl^{Col2cKO} Mice

Arm 1 generated WT (50%), *Fgfr1*^{Col2cKO} (12.5%*)* and Dbl^{Col2cKO} (12.5%) mice littermates. Arm 2 generated WT (50%), *Nf1*^{Col2cKO} (6.25%) and Dbl^{Col2cKO} (6.25%) mice. "Cre negative" pups were used as WT mice. Arm 2 used *Col2-Cre*;*Fgfr1*$^{f/+}$;*Nf1*$^{f/+}$ breeders because *Col2-Cre*; *Fgfr1*$^{f/+}$;*Nf1*$^{f/f}$ mice are not fertile. f: flox, +: non-floxed, WT allele.

on breeding arm), indicating the absence of embryonic lethality.

Nf1^{Col10cKO} and WT littermate mice were obtained by breeding *Col10-Cre*;*Nf1*$^{flox/flox}$ males with *Nf1*$^{flox/flox}$ females (128, 259). *Col10-Cre*;*Rosa26*:*lacZ* mice were obtained by breeding homozygous *Col10-Cre* mice with homozygous ROSA26 reporter mice (260). WT and *Nf1*^{Col2cKO} littermate mice for drug studies were obtained by breeding *Col2-Cre*;*Nf1*$^{flox/+}$ males with *Nf1*$^{flox/flox}$ females (128, 143). *Fgfr1* (B6.129S4-*Fgfr1tm5.1Sor*/J) *and Nf1* (STOCK *Nf1tm1Par*/J) floxed mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). *Col2-Cre* mice were originally obtained from Dr. Gerard Karsenty (Columbia University). *Col10-Cre*;*Rosa26*:*lacZ* mice were a gracious gift from Dr. Douglas Mortlock (Vanderbilt University, Nashville, TN, USA). *Col10-Cre* mice were originally generated by Dr. K. von der Mark (University of Erlangen-Nuremberg, Germany).

Tissues were harvested at the indicated times and fixed at 4°C for 24h in freshly prepared 4% paraformaldehyde (Sigma, St. Louis, MO, USA). Body and tibia lengths were assessed by caliper and postmortem Faxitron X-Ray (Tucson, AZ, USA), respectively. Body length was measured while animals were in the prone position and taken from the tip of the nose to the anus with a digital caliper. BGJ-398 (Selleckchem, Houston, TX, USA) or vehicle (PEG300/D5W, 2:1, v/v) was injected subcutaneously daily from birth and for 18 days. BGJ-398 is most potent at FGFRs 1-3 with *in vitro* IC50s of 0.9nM-1.4nM but has off-target, sub-optimal activity at other non-FGFRs at IC50 concentrations of 180nM or greater (261).

Histology

Fixed tissues were decalcified for up to 1 week (depending on age) in 20% EDTA at 4°C, followed by dehydration by graded ethanol series, cleared in xylenes (or Histo-Clear for β-galactosidase staining, National Diagnostics, Atlanta, GA, USA) and embedded in paraffin. Serial, 5-micron sagittal sections were cut, Histo-Clear deparaffinized and rehydrated by graded ethanol series. Sections were then stained with hematoxylin and eosin using standard protocols. For osteoclast analyses, sections were TRAP-stained using standard protocols. For β -galactosidase staining, tissues were stained prior to fixation using standard protocols. Briefly, tissues were washed with phosphate buffered 0.02% NP-40, 0.01% deoxycholate and 2mM $MgCl_2$ followed by overnight incubation in β-galactosidase staining solution (phosphate buffered 0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/mL X-Gal) at room temperature. Tissues were then fixed, decalcified, processed and sectioned as described earlier. Sections were counterstained with nuclear fast red.

Histomorphometric Analyses

Histomorphometric measurements were performed using the Bioquant Analysis System (BIOQUANT Image Analysis Corporation, Nashville, TN, USA). To determine the width of the proliferation and hypertrophic zones of the growth plate, we used the BIOQUANT software to employ a direct measurement technique. Specifically, the border of a zone and the border which constitutes the interface between adjacent zones were identified manually by blinded, properly trained laboratory members. The border between resting chondrocytes and the proliferation zone was defined by the appearance of a

proliferative column of four or more chondrocytes of flattened morphology. The interface between the proliferation zone and hypertrophic zones was defined by the appearance of cells of larger size (hypertrophy) at the distal end of proliferative columns. The termination of the hypertrophic zone was defined as the interface between hypertrophic chondrocytes and the primary spongiosa. Along each defined border, BIOQUANT software selected a set of systematically random points with a fixed interval set by the laboratory member. For the neighborhood around each of these points, BIOQUANT computed a vector orthogonal to the interface. Each vector was extended across each respective zone until it intersected the opposite border; the length of each vector was recorded. The width of the zone was then reported as the mean of all such vectors. The width measured by this technique does not attempt to compensate for oblique sectioning, however, all sections included for measurement were orthogonally sectioned and at a similar position within each bone. The osteochondral border surface used for osteoclast measurements was manually defined by blinded, properly trained laboratory members as the interface between the hypertrophic chondrocyte and primary spongiosa compartments, across the entire bone element. Once the interface between these two zones was defined, BIOQUANT computed the length of the interface using standard 2-dimensional distance computations to generate a surface measurement. TRAP-positive osteoclasts in contact with this osteochondral border were counted for analyses.

In Situ Hybridization (ISH)

Tissues were fixed, decalcified, processed and sectioned as described earlier. Sections were stored at 4 degrees centigrade until use. ISH was performed by standard protocols.

Fgfr1, *Fgfr3, Ihh* ISH was performed using probes to the respective 3' UTRs (*Fgfr1* and *Fgfr3* sequences available upon request, *Ihh* as previously published (262)). *Nf1* ISH was performed using a probe to the 5' translated region of the mRNA (99). Sense and anti-sense [35S]-uridine triphosphate (Perkin Elmer, Waltham, MA, USA) probes were synthesized for hybridization as described previously (262, 263). Sections were stained with Hoechst 33258 to identify nuclei. Images were processed using Adobe Photoshop (San Jose, CA, USA).

Immunohistochemistry

Tissues were fixed, decalcified, processed and sectioned as described earlier. Antigens were retrieved on graded ethanol rehydrated sections using DeCal Retrieval Solution per manufacturer's instructions (Biogenex, Fremont, CA, USA). Immunostaining was performed using standard protocols with anti-phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204, E10 mouse mAb #9106, Cell Signaling, Boston, MA, USA) antibodies or non-immune IgG1 antibodies, followed by ImmPACT NovaRED (Vector Laboratories, Burlingame, CA, USA) horseradish peroxidase detection of the secondary antibody. Immunostained sections were counterstained with hematoxylin.

X-Ray Imaging

Radiographs were obtained using a digital cabinet X-ray system (LX-60, Faxitron X-Ray, USA) at 35kV and exposed for 4 seconds.

Genomic PCR, RT-PCR and qPCR

For genotyping, genomic DNA was isolated from tail biopsies by sodium hydroxide digestion and PCR was performed using appropriate primers. The *Nf1* floxed allele was detected with primers P1, P3 and P4, as previously described (128). The *Fgfr1* floxed allele was detected with primers intr5.53 and intr5.53 to generate a 750bp band for the floxed allele and a 564bp band for the WT allele, as previously described (55). The *Col2-Cre* transgene was detected using the forward primer: GAGTTGATAGCTGGCTGGTGGCAGATG and reverse: TCCTCCTGCTCCTAGGGCCTCCTGCAT to generate a 700bp band (143). The *Col10-Cre* transgene was detected using the P1 and P5 primers to generate a 305bp band, as previously described (259). Total RNA was extracted from snap-frozen P5 (to avoid the presence of the secondary ossification center) murine growth plates (1 distal femoral epiphysis and 1 proximal tibial epiphysis per sample) using TRIzol (Life Technologies, Grand Island, NY, USA), and cDNAs were synthesized from 1 microgram RNAs following DNase I treatment using the high-capacity cDNA reverse-transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR (qPCR) were performed using TaqMan gene expression assays. The probe and primer sets for *Rankl* (Mm00441908_m1), *Opg* (Mm00435462_m1), *Fgfr1* (Mm00438924_m1), *Fgfr3* (Mm00433294_m1) and the normalizer *Hprt* (Mm00446968_m1) were obtained from Life Technologies. Normalized fold expression values for *Rankl*, *Opg*, *Fgfr1* and *Fgfr3* were calculated using relative starting quantity values obtained using standard curve qPCR and normalized by relative starting quantity values obtained from standard curve *Hprt* qPCR.

Statistical Analysis

All experimental values were analyzed using GraphPad PRISM (v6.0, La Jolla, CA). When experimental conditions involved two groups, an unpaired t-test was used to determine differences between groups with significance determined by obtaining a *P*-value of <0.05. When experimental conditions involved three or more groups, one-way ANOVA was used to determine differences between groups. If differences were detected by ANOVA ($P<0.05$), *post hoc* significance was calculated by Holm-Sidak's method correcting for multiple comparisons with significance determined by obtaining an adjusted p value of <0.05. For analyses tracking body length and weight, repeated measure two-way ANOVA was used; *post hoc* calculated significance, if any, is graphed only for the terminal time point (P18). Graphs of growth plate zone lengths were graphed as grouped analyses for space saving purposes but were analyzed by one-way ANOVA at each time point specified. Data are presented as mean ± SEM.

Results

Fgfr1, Fgfr3 and Nf1 Expression Is Localized to Distinct Zones in the Growth Plate

A first requirement for neurofibromin to control FGFR1 signaling is for the two proteins to be expressed in the same chondrocyte population. To address this question, we performed *in situ* hybridization (ISH) on serial paraffin sections on postnatal (P) WT long bone growth plates, using Indian hedgehog (*Ihh*) as a marker of the prehypertrophic zone. At both P0 and P7, *Fgfr1* and *Nf1* transcripts were both detected in the hypertrophic zone, with minimal detectable expression in the proliferation zone, where *Fgfr3* was localized

primarily. Both *Fgfr1* and *Nf1* transcripts had weaker expression detectable in the prehypertrophic zone (**Figure 29**). Thus *Fgfr1* expression overlapped with *Nf1* expression in the prehypertrophic, hypertrophic and perichondrium areas, while *Fgfr3* expression only overlapped with *Nf1* expression in prehypertrophic chondrocytes at both P0 and P7. Although co-expression does not prove functional interaction, these results suggest that neurofibromin may regulate FGFR1 signaling rather than FGFR3 signaling in hypertrophic growth plate chondrocytes.

Nf1 Ablation in Fgfr1-Deficient Chondrocytes Reverses the Alterations in Growth Plate Maturation/Hypertrophy Observed in Fgfr1^{Col2cKO} Mice

The overlap in *Fgfr1* and *Nf1* expression in postmitotic prehypertrophic and hypertrophic chondrocytes (**Figures 9, 29**) (34, 48, 49, 99), the largely opposite growth plate phenotypes between *Fgfr1^{Col2cKO}* and *Nf1^{Col2cKO}* mice ((4, 49) and **Chapter II**) and the fact that FGFR activation and *Nf1* mutation/ablation both trigger Ras-Raf-MEK-ERK activation in chondrocytes (**Figure 23**), led us to the hypothesis that neurofibromin inhibits, among the many receptor tyrosine kinases (RTKs) expressed in the growth plate, the signaling of FGFR1 in mature chondrocytes (**Figure 30**). This hypothesis is not functionally testable *in vitro* due to the dynamic nature of growth plate development and challenges in generating maturing chondrocytes *in vitro*, and can be best addressed genetically and *in vivo*, by either overexpressing neurofibromin in chondrocytes to rescue the phenotypes of cells with activation of FGFR1, or by reducing neurofibromin activity in cells lacking FGFR1 to artificially reactivate Ras-ERK1/2 signaling in these cells. In the latter option, we reasoned that lack of neurofibromin, leading to Ras-ERK1/2

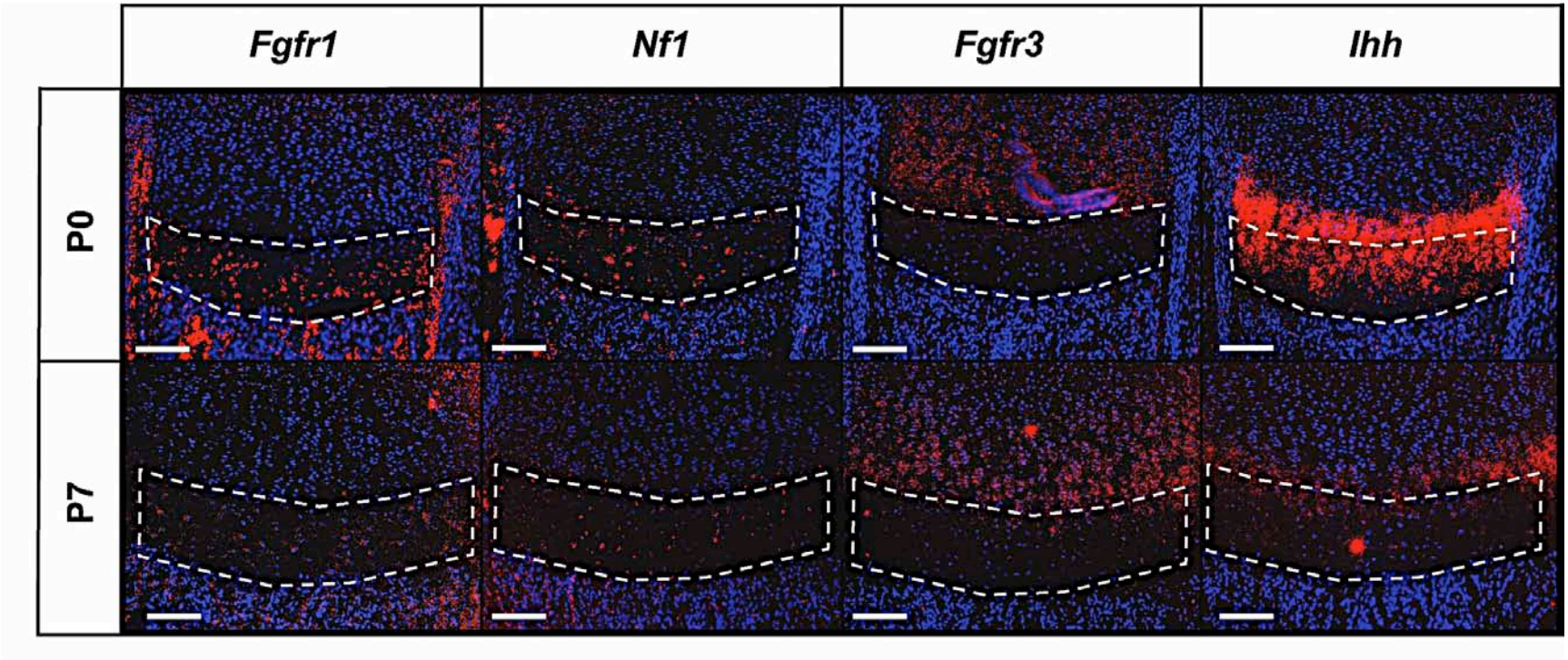

Figure 29. *Fgfr1*, *Fgfr3* and *Nf1* Expression Is Localized to Distinct Zones of the Growth Plate Across Early Postnatal Development

In situ hybridization (red signal) on serial distal femur sections shows that *Fgfr1* localizes to prehypertrophic and hypertrophic chondrocytes at P0 (top row) and P7 (bottom row). *Nf1* is expressed in prehypertrophic and hypertrophic chondrocytes at P0 and P7. *Fgfr3* localizes in the proliferation and prehypertrophic zones at P0 and P7. *Ihh* marks the prehypertrophic zone. Hoechst stained nuclei appear in blue. White boxes denote hypertrophic zones. Scale bar: 100 μm**.**

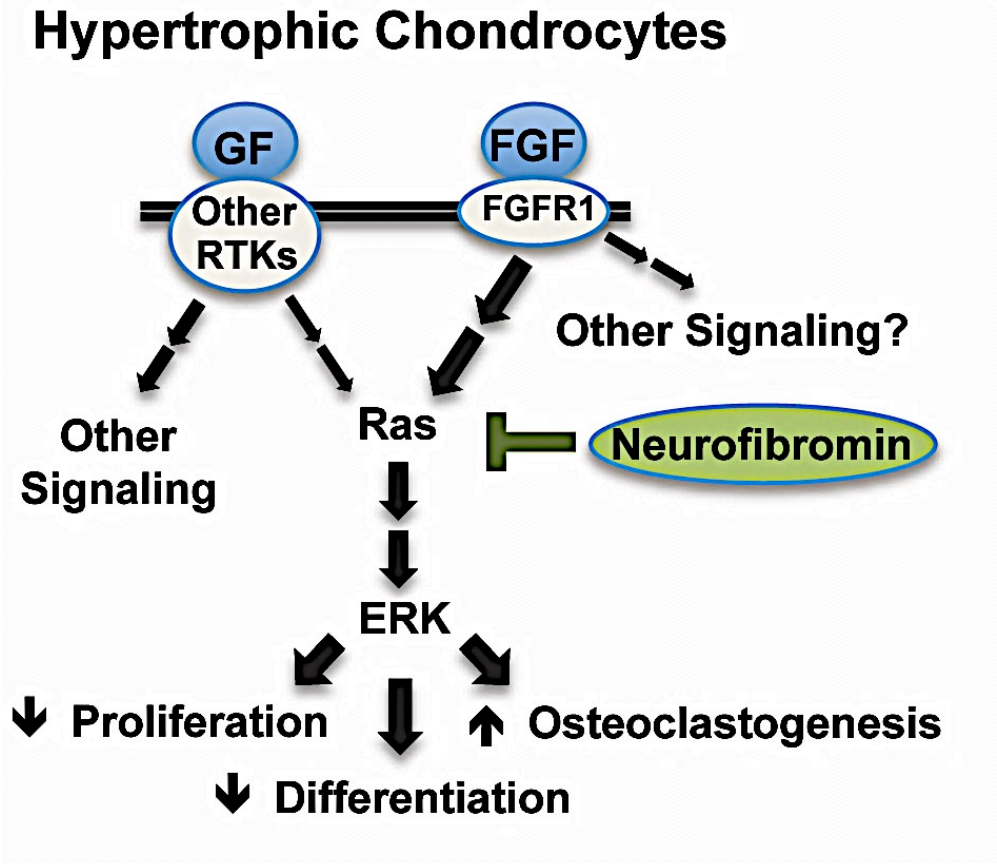

Figure 30. Schematic of FGFR1 Signaling in Hypertrophic Chondrocytes
We hypothesized that FGFR1 is a major Ras/ERK activator in hypertrophic chondrocytes that is tempered by the Ras-GAP activity of neurofibromin.

pathway activation (**Figures 11, 31**, bottom left and right), in *Fgfr1*-deficient chondrocytes (in which Ras-ERK1/2 signaling is reduced, **Figure 31**, top right), should reverse the growth plate phenotypes of $Fgfr1^{Col2cKO}$ mice. We thus generated double mutant mice lacking both *Nf1* and *Fgfr1* in *Col2a1*-expressing chondrocytes ($Dbl^{Col2cKO}$ mice) and compared their skeletal phenotypes to single knockout, $Fgfr1^{Col2cKO}$ and $Nf1^{Col2cKO}$ littermates. Gross examination over the period of rapid growth before weaning indicated that both body length (**Figure 32A**) and body weight (**Figure 32B**) were reduced in both $Nf1^{Col2cKO}$ and $Dbl^{Col2cKO}$ mice compared with WT littermates and $Fgfr1^{Col2cKO}$ mice. Similarly, both genotypes had significantly shorter bones than WT and $Fgfr1^{Col2cKO}$ mice at P18 (**Figure 32C**). $Fgfr1^{Col2cKO}$ mice also had reduced stature (by P4), body weight (by P9) and tibial length (P18) compared with WT littermates, despite the increased size of their hypertrophic zone (**Figure 33B**) (49). Body length, body weight and tibia length were not significantly different between $Nf1^{Col2cKO}$ and $Dbl^{Col2cKO}$ mice.

To assess changes in growth plate structure between WT, single mutant $Fgfr1^{Col2cKO}$ and $Nf1^{Col2cKO}$ and $Dbl^{Col2cKO}$ mice, growth plate histomorphometry was performed, analyzing the FGFR3-rich proliferation and FGFR1-rich hypertrophic zones longitudinally at P0, 7 and 14 days of age. The proliferation zones of all four groups of mice were not significantly different until P14, at which time the proliferation zones of $Nf1^{Col2cKO}$ and $Dbl^{Col2cKO}$ were significantly shorter than that of WT and $Fgfr1^{Col2cKO}$ mice, although they were not statistically different from each other (**Figure 33A**). In contrast, the hypertrophic zone across all three postnatal time points was elongated by inactivation of

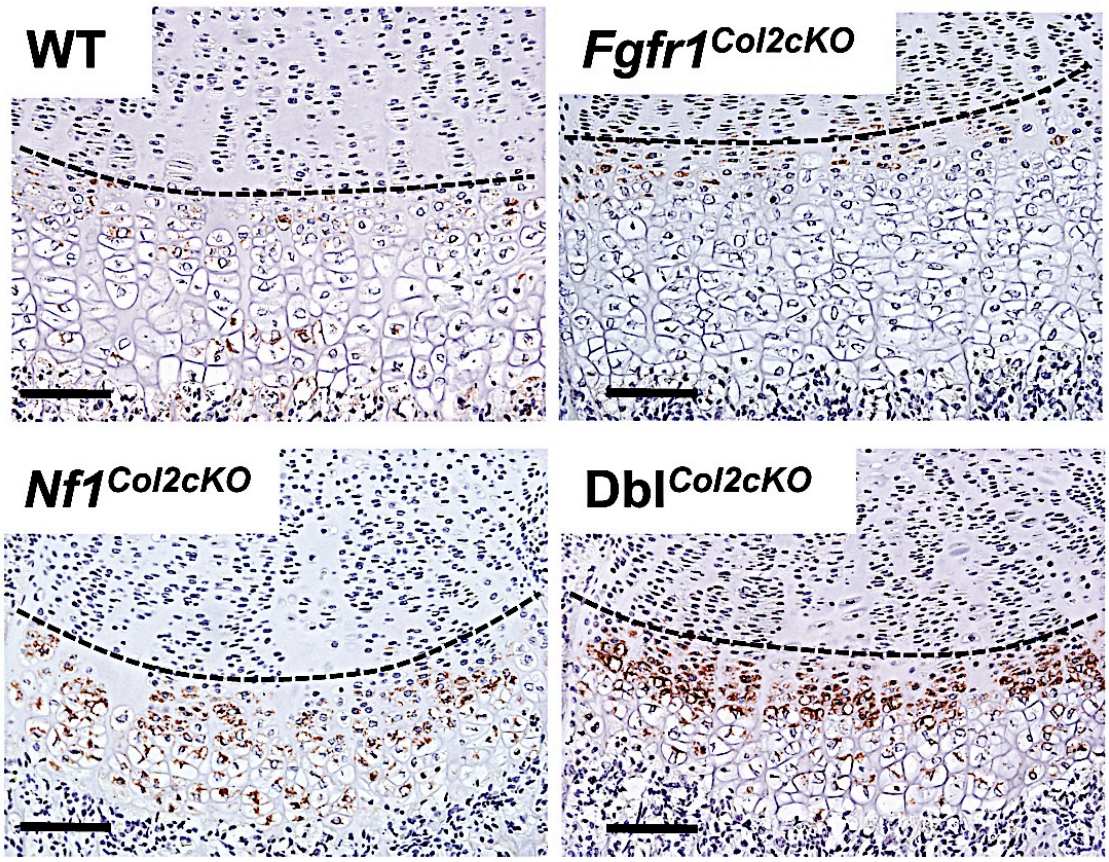

Figure 31. ERK Activation Status in WT, *Fgfr1*^{Col2cKO}, *Nf1*^{Col2cKO} and Dbl^{Col2cKO} Growth Plates

Immunohistochemistry for phospho-ERK revealed low basal activation of ERK in WT, reduced activation in *Fgfr1*^{Col2cKO} and elevated activation in *Nf1*^{Col2cKO} and Dbl^{Col2cKO} hypertrophic chondrocytes residing in P7 proximal tibial growth plates. Brown staining indicates phospho-ERK; sections are counterstained with hematoxylin. Black lines delineate proliferation and prehypertrophic zones. Scale Bar: 100 μm.

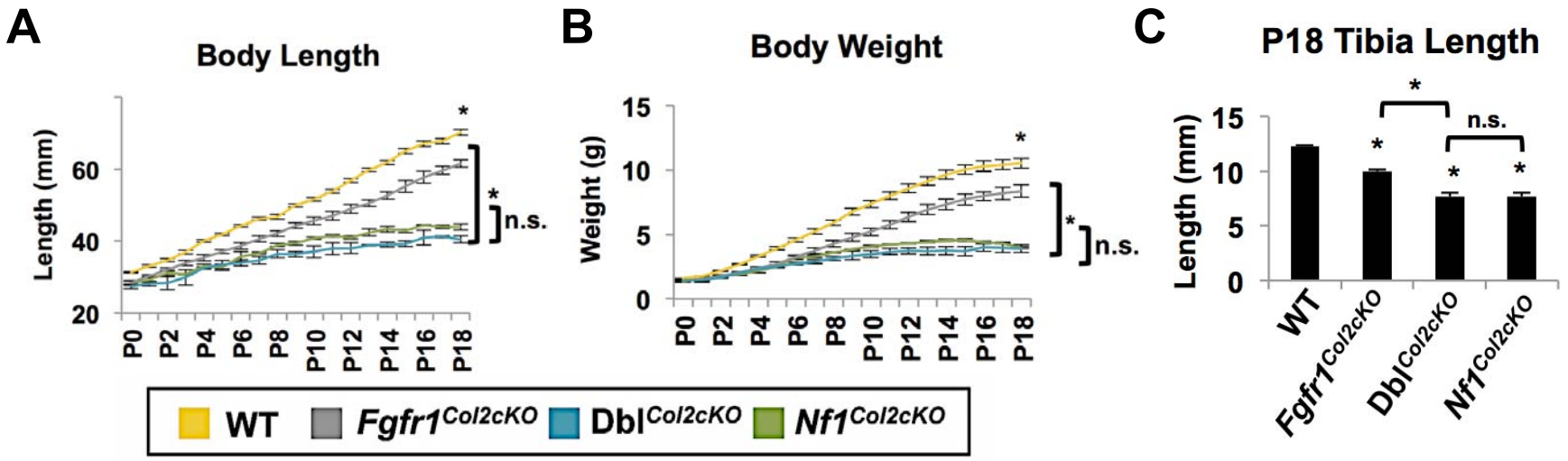

Figure 32. Ablation of *Nf1* in Chondrocytes Reduces Body Size and Long Bone Length in WT and *Fgfr1*^{Col2cKO} Mice
(**A, B**) Body length (A) and weight (B) was not significantly different between *Nf1*^{Col2cKO} and Dbl^{Col2cKO} mice, but both genotypes had body lengths and weights significantly less than WT and *Fgfr1*^{Col2cKO} mice (*:P<0.05 versus WT unless indicated, n=4 per group, repeated measure two-way ANOVA). (**C**) P18 tibial length of WT, *Fgfr1*^{Col2cKO}, *Nf1*^{Col2cKO} and Dbl^{Col2cKO} mice. Tibial length was not significantly different between *Nf1*^{Col2cKO} and Dbl^{Col2cKO} mice, but it was significantly less in Dbl^{Col2cKO} than in WT and *Fgfr1*^{Col2cKO} mice (*:P<0.05 versus WT unless indicated, n=7 per group, one-way ANOVA).

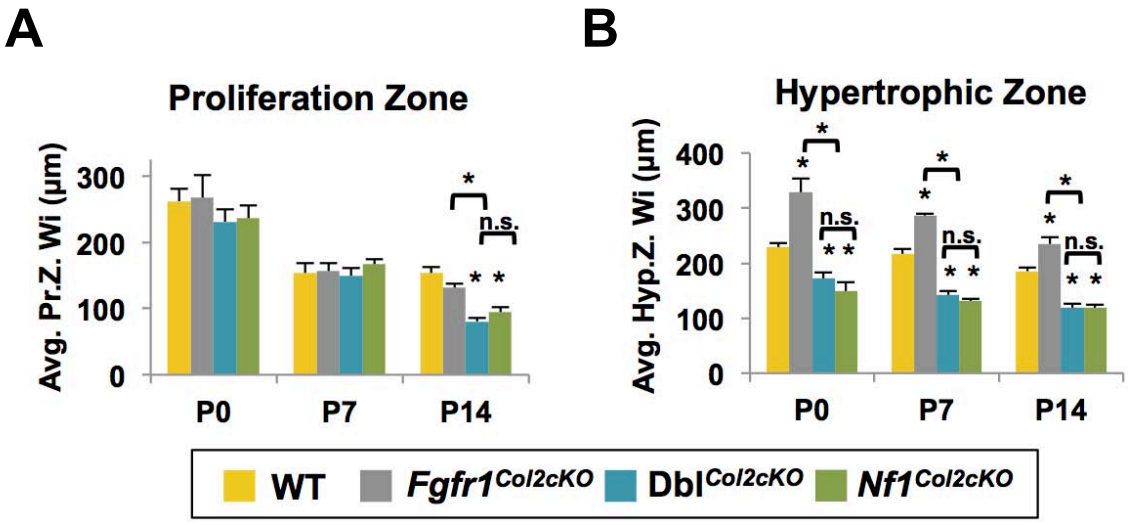

Figure 33. *Nf1* Ablation in *Fgfr1*^{Col2cKO} Mice Reverses their Growth Plate Hypertrophic Zone Expansion

(**A, B**) Length quantification of proximal tibial proliferation (A) and hypertrophic (B) zones of P0, 7 and 14 WT, *Fgfr1*^{Col2cKO}, *Nf1*^{Col2cKO} and Dbl^{Col2cKO} mice (*:P<0.05 versus WT unless indicated, n=4 per group, two-way ANOVA). The length of the proliferation zones in *Nf1*^{Col2cKO} and Dbl^{Col2cKO} mice were significantly shorter than WT but not significantly different from each other at P14. The same was true of the hypertrophic zone, but at P0, 7 and 14.

Fgfr1 in *Col2a1*-expressing chondrocytes (**Figure 33B**), in agreement with previous studies (49). Ablation of *Nf1* in double mutant Dbl^{Col2cKO} mice reduced the size of this zone compared with WT and *Fgfr1*^{Col2cKO} littermate mice at P0, 7 and 14, to values similar to the ones observed in *Nf1*^{Col2cKO} mice (the hypertrophic zones of *Nf1*^{Col2cKO} and Dbl^{Col2cKO} mice were not statistically different from each other). In addition, lack of *Nf1* in *Fgfr1*-deficient hypertrophic chondrocytes led to disturbances in the orderly stacking of the proliferative columns observed in WT and *Fgfr1*^{Col2cKO} mice (**Figure 34**). Increased phospho-ERK1/2 immunostaining in the growth plate prehypertrophic and hypertrophic regions in Dbl^{Col2cKO} mice compared with *Fgfr1*^{Col2cKO} mice (**Figure 31**, bottom right) confirmed reactivation of the Ras-ERK1/2 pathway upon *Nf1* ablation in these mice. In addition, *in situ* hybridization and quantitative RT-PCR (qPCR) verified efficient *Fgfr1* deletion in the growth plate of *Fgfr1*^{Col2cKO} mice and that *Fgfr1* expression was not affected with regard to level of expression or growth plate localization upon *Nf1* ablation (see **Figures 43 and 44B**) Thus, *Nf1* ablation in *Fgfr1*-deficient chondrocytes reverses the increase in growth plate hypertrophic zone observed in *Fgfr1*^{Col2cKO} mice, and has a deleterious effect on the stacking of the proliferative chondrocyte columns, which was not affected in mice lacking *Fgfr1* only.

Lack of Nf1 in Fgfr1-Deficient Chondrocytes Reverses the Reduction in Osteochondral Osteoclast Number Observed in Fgfr1^{Col2cKO} *Mice*

Osteoclast number at the chondro-osseous junction (the bone marrow zone immediately adjacent to the growth plate) was shown to be reduced in *Fgfr1*^{Col2cKO} mice, and *Mmp9* and *Opn* expression were lower in *Fgfr1* null chondrocytes compared with WT

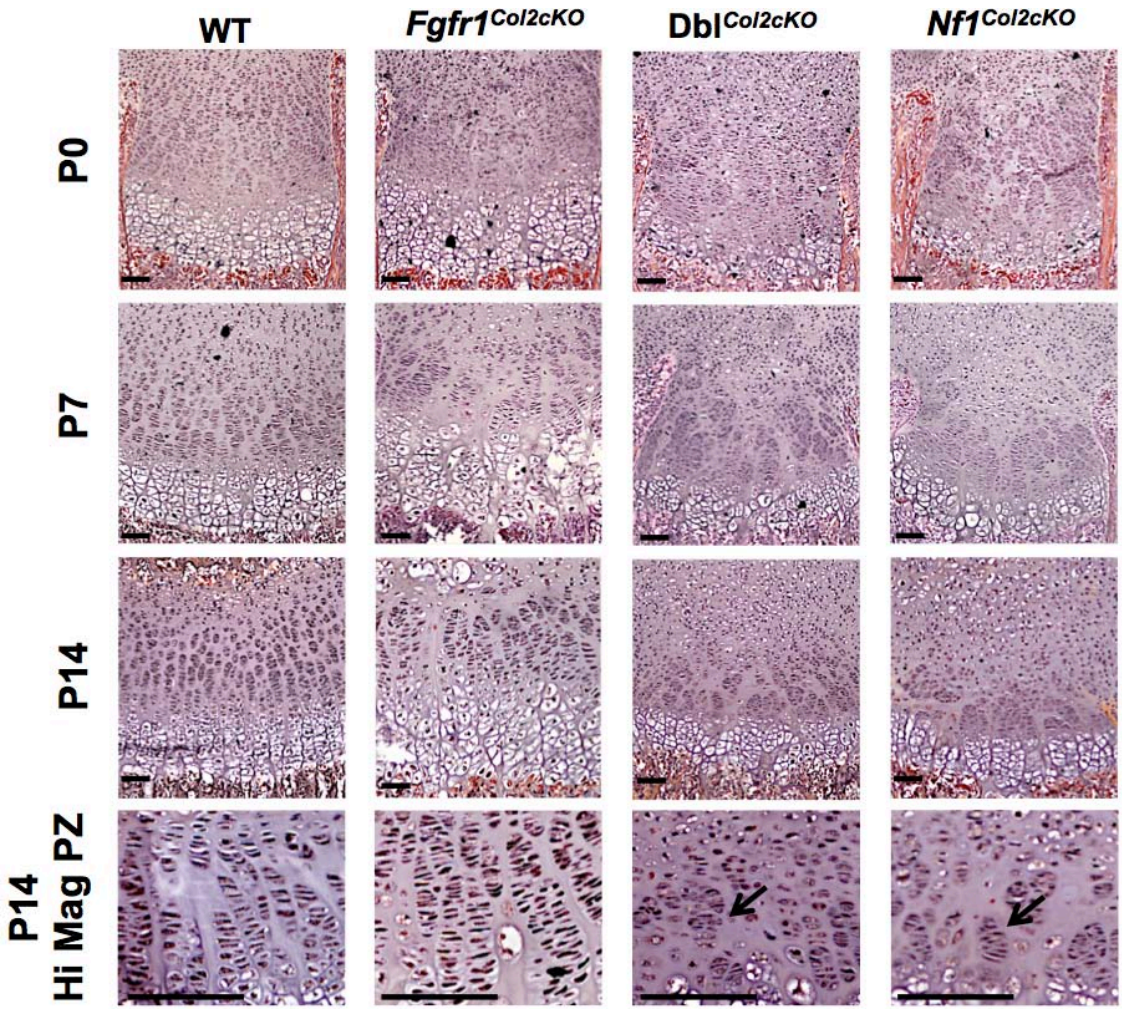

Figure 34. *Nf1* Ablation in *Fgfr1*^{Col2cKO} Mice Causes Growth Plate Disorganization
H&E staining of proximal tibial growth plates of WT, *Fgfr1*^{Col2cKO}, *Nf1*^{Col2cKO} and Dbl^{Col2cKO} mice at P0, 7 and 14 and high magnification of the proliferation zone (PZ) at P14 reveals abnormalities in *Fgfr1*^{Col2cKO}, Dbl^{Col2cKO} and *Nf1*^{Col2cKO} growth plates. Arrows indicate disorganized proliferative columns. Scale bar: 100 μm.

chondrocytes (49). In contrast, *Nf1*^{Col2cKO} mice display an increase in osteoclast number at the osteochondral border, and *Nf1* null chondrocytes have elevated expression of *Rankl*, *Opn*, *Mmp9* and *Mmp13*, all of which are important for extracellular bone matrix catabolism ((4, 134) and **Figures 16, 19, 20**). These observations suggested that FGFR1 and neurofibromin were both involved, in an antagonistic fashion, in the mechanism by which mature chondrocytes stimulate growth plate catabolism. To determine whether the reduced osteoclast density at the osteochondral border in *Fgfr1*^{Col2cKO} mice could be rescued by concurrent deletion of *Nf1* in growth plate chondrocytes, we quantified osteoclast number in TRAP-stained thin bone sections from WT, *Nf1*^{Col2cKO}, *Fgfr1*^{Col2cKO} and Dbl^{Col2cKO} mice. When *Fgfr1* was deleted in *Col2a1*-expressing chondrocytes of newborn and P14 mice, osteoclast number at the chondro-osseous junction was reduced (**Figure 35A-C**), in line with previous studies in *Fgfr1*^{Col2cKO} embryos (49). Upon *Nf1* deletion, a clear increase in the number of TRAP-positive multinucleated osteoclasts was observed in Dbl^{Col2cKO} growth plates versus WT mice. Osteoclast number was not significantly different between Dbl^{Col2cKO} and*Nf1*^{Col2cKO} growth plates at P0 and P14 (**Figure 35A-C**). Consistently, we found that *Rankl* expression was elevated in *Nf1*^{Col2cKO} and Dbl^{Col2cKO} growth plates, yet decreased in *Fgfr1*^{Col2cKO} growth plates taken from five-day-old pups, compared with WT animals (**Figure 36A).** *Opg* expression was unchanged in all four genotypes (**Figure 36B**). These findings suggest that neurofibromin attenuates FGFR1 signaling in hypertrophic growth plate chondrocytes to limit their osteoclastogenic-promoting function.

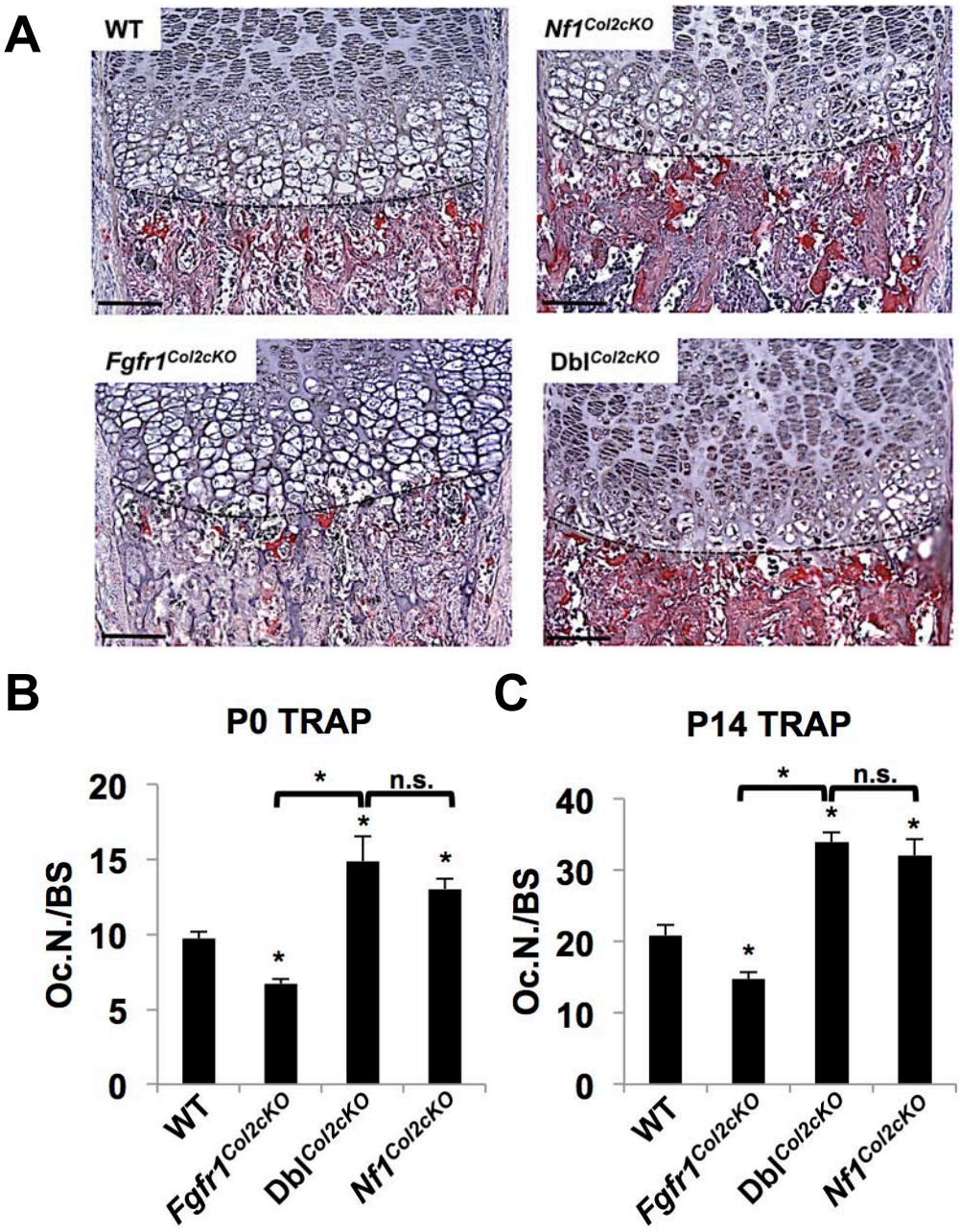

Figure 35. *Nf1* Ablation in *Fgfr1*^{Col2cKO} Mice Reverses their Decrease in Osteoclastogenesis at the Osteochondral Junction

(**A**) TRAP staining in distal femora from P0 pups and (**B, C**) quantification of the number of TRAP-positive (red) osteoclasts per osteochondral border surface (BS) in P0 (B) and P14 (C) distal femur sections showed that the number of osteoclasts at the chondro-osseous junction in *Fgfr1*^{Col2cKO} mice is reduced compared with WT littermates, and that *Nf1* deletion in Dbl^{Col2cKO} mice leads to higher number of osteoclasts compared with *Fgfr1*^{Col2cKO} mice. Osteoclast number in *Nf1*^{Col2cKO} and Dbl^{Col2cKO} was not significantly different from each other. The osteochondral border surface is indicated by a dotted line (*:P<0.05 versus WT unless indicated, n=4 per group, one-way ANOVA, Scale bar: 100 μm).

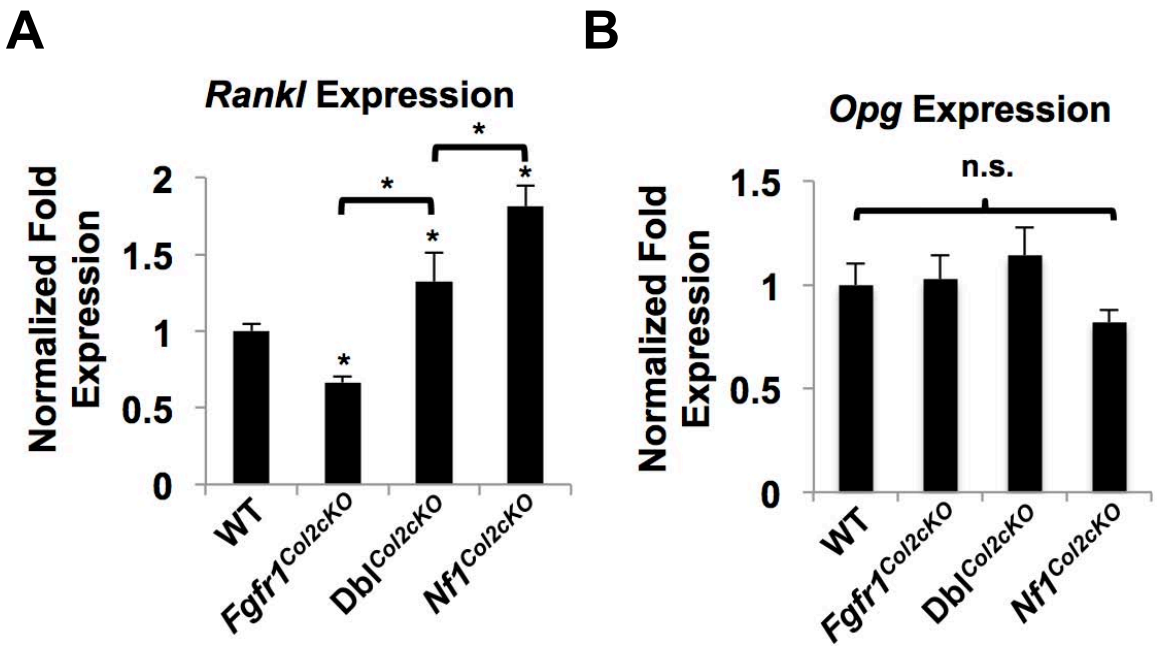

Figure 36. *Nf1* Ablation in *Fgfr1*^{Col2cKO} Mice Reverses their Reduction in Pro-Osteoclastogenic *Rankl* Expression

(**A**) *Rankl* expression is increased in P5 long bone growth plate cartilage from *Nf1*^{Col2cKO} and Dbl^{Col2cKO} mice and decreased in *Fgfr1*^{Col2cKO} P5 growth plate cartilage when compared with WT (*:$P<0.05$ versus WT unless indicated, n=4 per group, one-way ANOVA, qPCR). (**B**) *Opg* expression is not affected across the four genotypes (n=4 per group, one-way ANOVA, qPCR).

Nf1 Ablation in Col10a1-Positive Hypertrophic Chondrocytes Reduces Hypertrophic Zone Width but Does Not Impair Bone Elongation

The results above suggest a predominant role of neurofibromin, downstream of FGFR1, in hypertrophic chondrocytes, however, neurofibromin is also expressed in prehypertrophic chondrocytes (**Figures 9, 29**) and osteoblasts (4, 99). In an effort to determine the function of neurofibromin more specifically in hypertrophic growth plate chondrocytes, we generated $Nf1^{Col10cKO}$ mice lacking *Nf1* in *Col10a1*-positive cells, using the *Col10-Cre* driver transgenic mice (259). We first used these mice bred with *Rosa26*:*lacZ* reporter mice (260) to examine the activity of the *Col10-Cre* transgene specifically during the active phase of postnatal bone elongation at P0, P7 and P14, since prior studies only reported activity embryonically and shortly after birth (P1, P3) or 5 weeks after birth (230, 259, 264). We found, at all ages analyzed, that the *Col10-Cre* transgene is active in hypertrophic chondrocytes, as demonstrated by positive β -galactosidase staining in this area of the growth plate, but not in proliferative columns or prehypertrophic cells (**Figure 37A**). β -galactosidase activity was also observed in osteoblast-like cells in the primary spongiosa and embedded osteocyte-like cells (**Figure 37B,C**). $Nf1^{flox/flox}$ mice were then bred to *Col10-Cre* mice to generate, through two rounds of breeding, $Nf1^{Col10cKO}$ mice and WT littermates. Unexpectedly, $Nf1^{Col10cKO}$ mice had a normal stature, body weight, physical appearance and gross bone structure at all time points analyzed (from P0 to P18) compared with WT littermates (**Figures 38, 39**). Histologically, the proliferation zone length of $Nf1^{Col10cKO}$ growth plates was not significantly shorter than WT at P0 or P18 (**Figure 40A**). The hypertrophic zone of $Nf1^{Col10cKO}$ growth plates was, however, significantly shorter than WT at P0 and P18

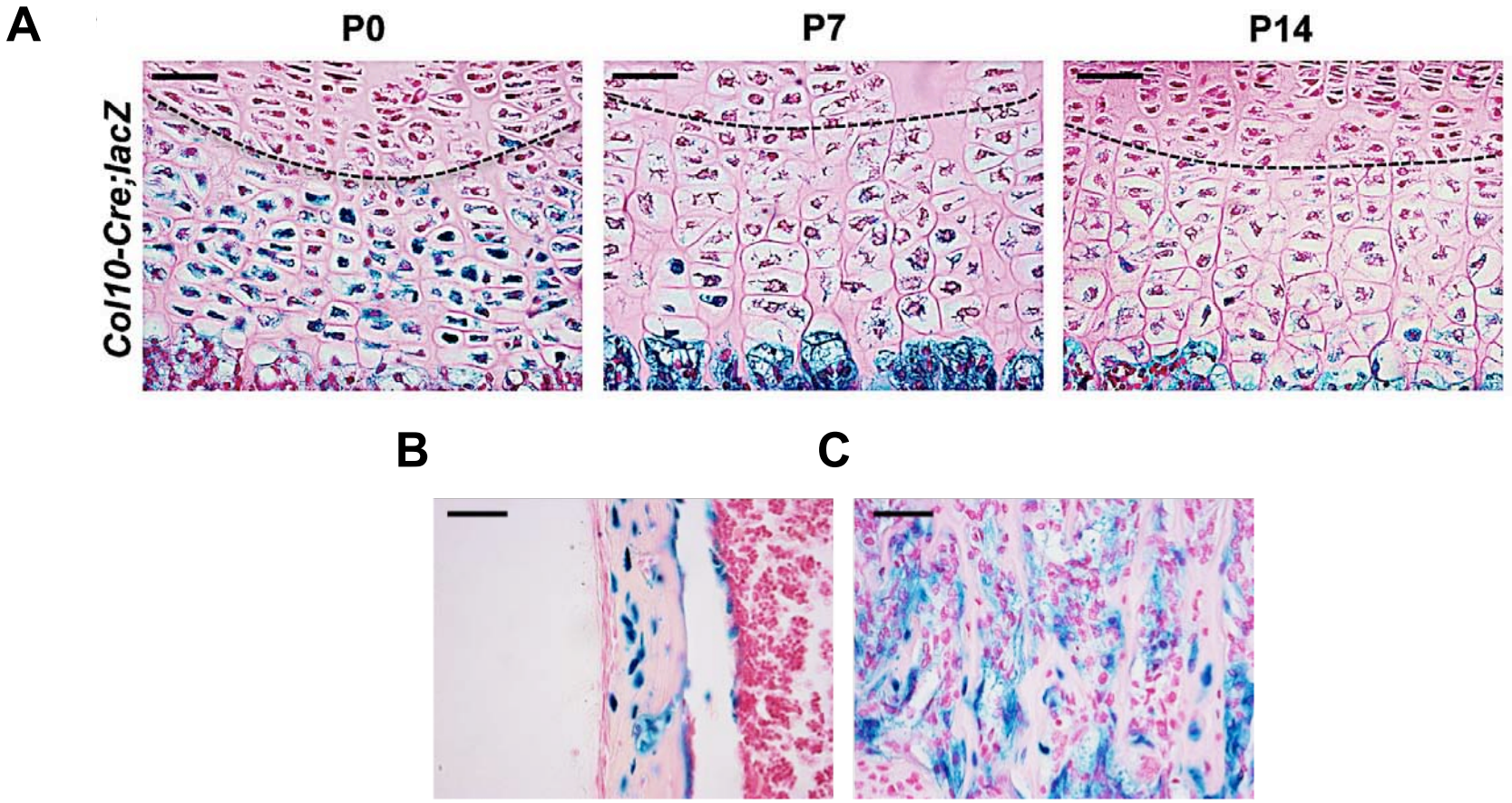

Figure 37. The *Col10-Cre* Transgene Is Active in Hypertrophic Chondrocytes During Early Postnatal Development
(**A**) β-galactosidase staining (blue) in P0, 7 and 14 proximal, *Col10-Cre*;*Rosa26*:*lacZ* tibiae show *Col10-Cre* transgene activity in hypertrophic chondrocytes, but not in prehypertrophic chondrocytes. (**B,C**) The transgene was also active in osteocyte-like cells of the cortex (B) and primary spongiosa osteoblasts (C). Sections were counterstained with nuclear fast red. Black dotted lines delineate prehypertrophic and hypertrophic zones. Scale bar: 50 μm.

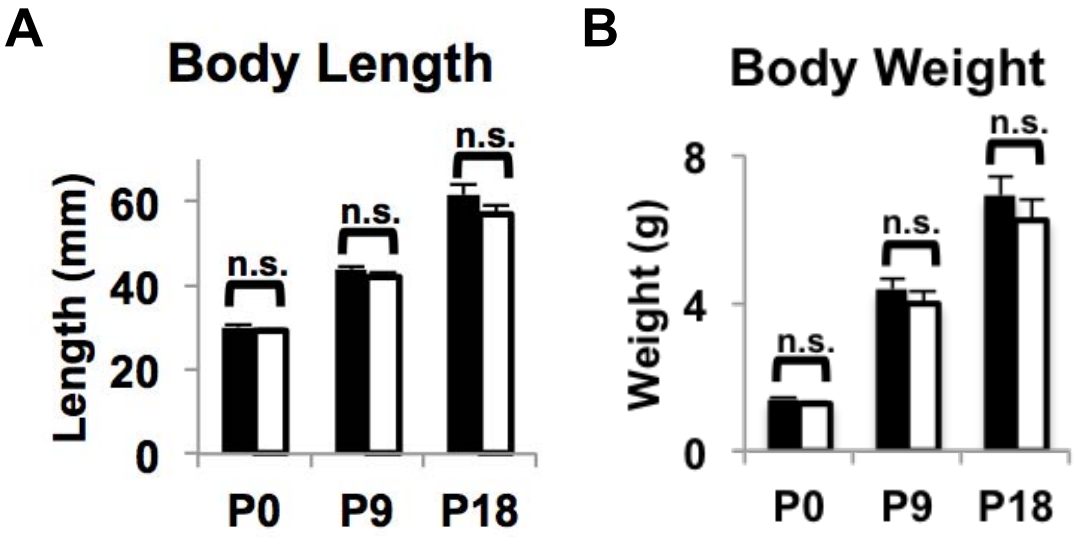

Figure 38. *Nf1* Ablation in Hypertrophic Chondrocytes Does Not Affect Body Size (**A, B**) The body length (A) and weight (B) of $Nf1^{Col10cKO}$ versus WT mice is not significantly different at P0, 9, or 18 (*:P<0.05 versus WT, n=5 per group, repeated measure two-way ANOVA).

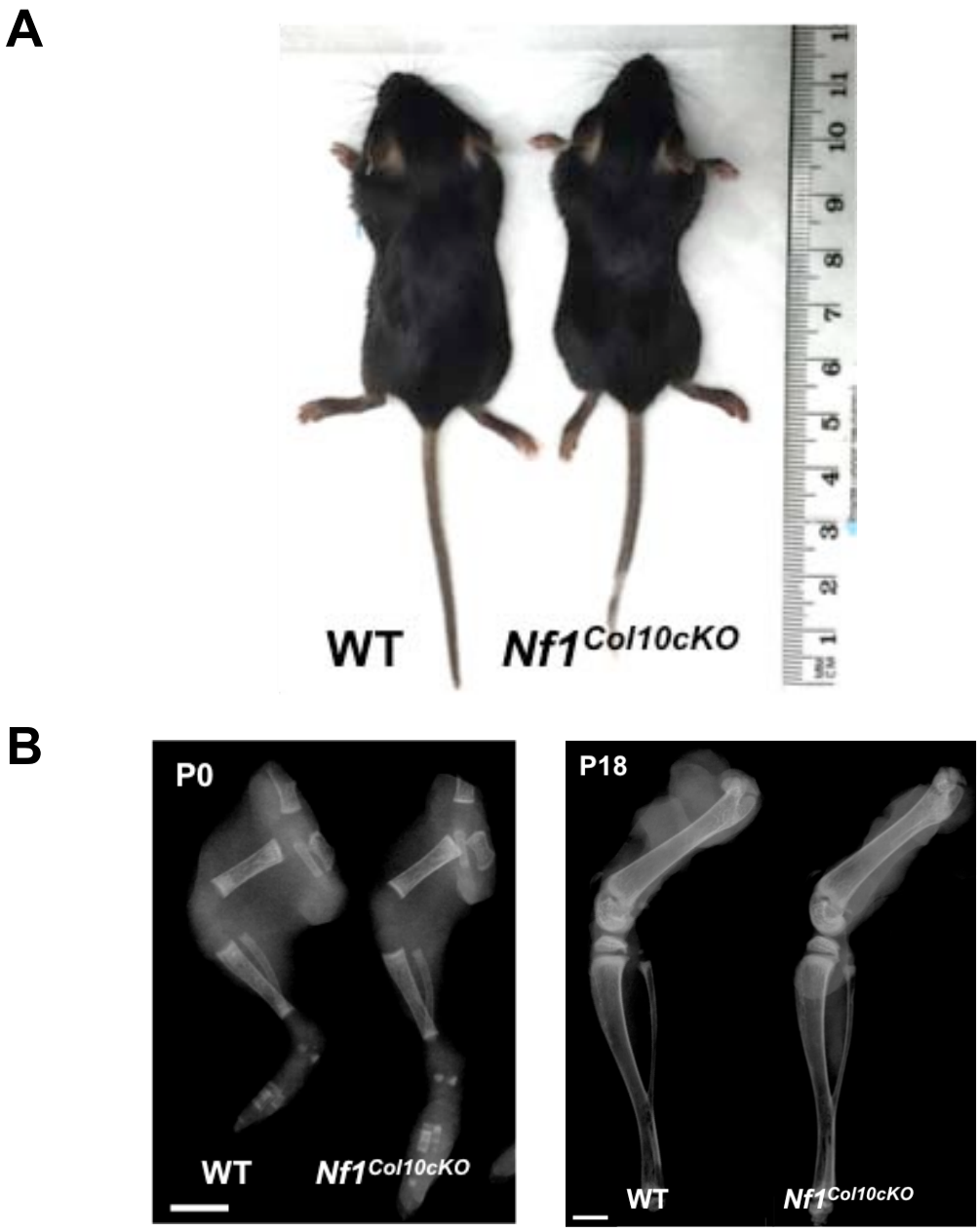

Figure 39. *Nf1*^{Col10cKO} Mice Have Normal Physical Appearance and Gross Bone Sturcture

(**A**) Visual assessment of WT and *Nf1*^{Col10cKO} mice revearled no overt differneces between the mice. (**B**) Radiographic assement of gross bone strucuture also revealed no overt differneces between WT and *Nf1*^{Col10cKO} mice. Scale bar: 1.5mm.

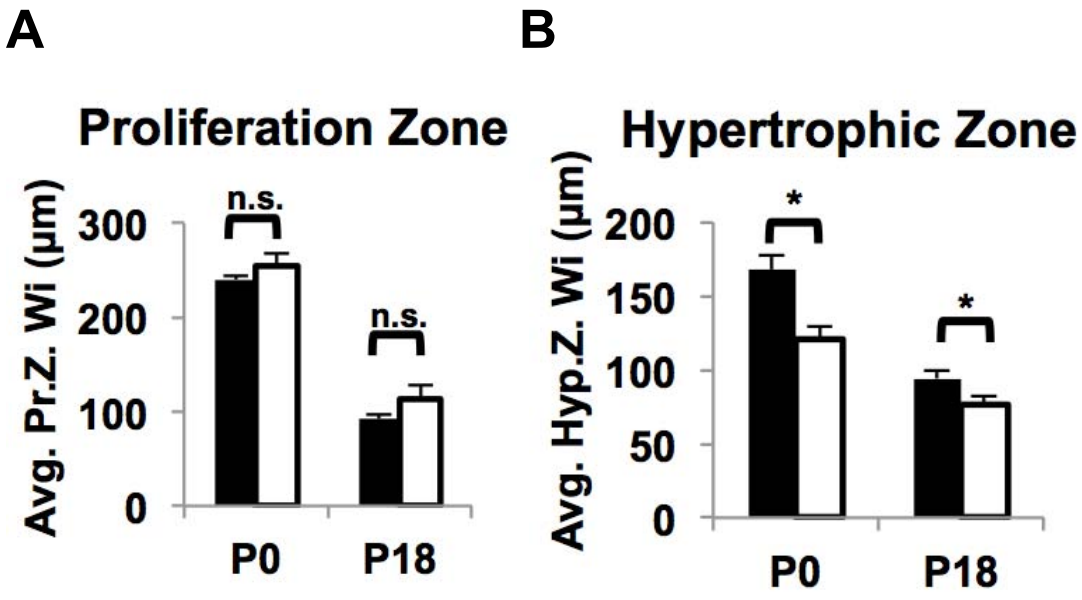

Figure 40. ***Nf1*^{Col10cKO} Mice Have Short Growth Plate Hypertrophic Zones**
(**A, B**) Length quantification of proximal tibial proliferation (A) and hypertrophic (B) zones of P0 and 18 WT and *Nf1*^{Col10cKO} mice (*:P<0.05 versus WT, n=5 per group, unpaired t-test). The length of the proliferation and hypertrophic zones between WT and *Nf1*^{Col10cKO} mice were not significantly different at P0 or P18.

(**Figure 40B**). We also found that the number of osteoclasts at the chondro-osseous border was significantly higher in $Nf1^{Col10cKO}$ compared with WT mice (**Figure 41)**. The growth plates of $Nf1^{Col10cKO}$ mice appeared normal with orderly proliferative chondrocyte columns as opposed to the disorganized columns seen in $Nf1^{Col2cKO}$ growth plates, in which *Nf1* is ablated in prehypertrophic and hypertrophic cells (**Figure 42**). Collectively, these results suggests that *Nf1* loss-of-function in prehypertrophic chondrocytes, where *Nf1* expression overlaps with both *Fgfr3* and *Fgfr1*, is responsible for the size and growth plate columnar defects observed in $Nf1^{Col2cKO}$ mice, whereas *Nf1* in hypertrophic chondrocytes primarily regulates osteoclastogenesis in the primary spongiosa.

Fgfr3 Expression Is Not Altered upon Fgfr1 Ablation in Col2a1-Positive Chondrocytes

Our analyses suggest that lack of *Nf1* in prehypertrophic chondrocytes causes alterations in the size and organization of *Nf1* and *Fgfr1*-negative, but *Fgfr3*-positive, proliferative chondrocyte columns. Ectopic *Fgfr3* upregulation was previously observed in *Fgfr1*-deficient osteoblasts (49), and it has been shown that chondrocytic FGFR and ERK1/2 signaling in general are inhibitory to chondrocyte proliferation/differentiation (although these effects were not attributed to an individual or specific FGF receptor) (44, 219). These observations prompted us to question whether *Nf1* or *Fgfr1* deficiency altered growth plate *Fgfr3* expression level or tissue localization, thus potentially causing some of the observed changes in proliferation and hypertrophic zones. Hence, we assessed *Fgfr3* expression by *in situ* hybridization and qPCR analyses in the growth plates of $Nf1^{Col2cKO}$ and $Dbl^{Col2cKO}$ mice. We found that *Fgfr3* expression was not mislocalized when *Nf1* was deleted alone or in combination with *Fgfr1,* and remained primarily in the

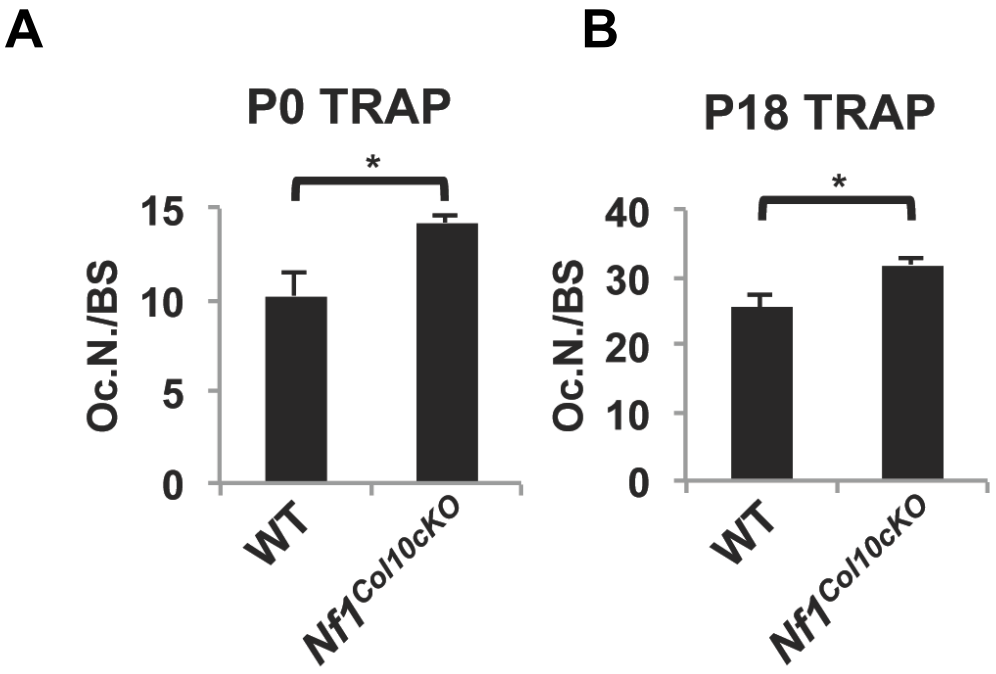

Figure 41. *Nf1* Ablation in Hypertrophic Chondrocytes Increasaes Osteoclastogenesis at the Osteochondral Border

(**A, B**) TRAP quantification of the number of TRAP-positive (red) osteoclasts per osteochondral border surface in P0 (A) and P18 (B) proximal tibial sections showed that the number of osteoclasts at the chondro-osseous junction in $Nf1^{Col10cKO}$ mice was significantly greater than WT (*:$P<0.05$ versus WT, n=5 per group, unpaired t-test, scale bar: 100 μm).

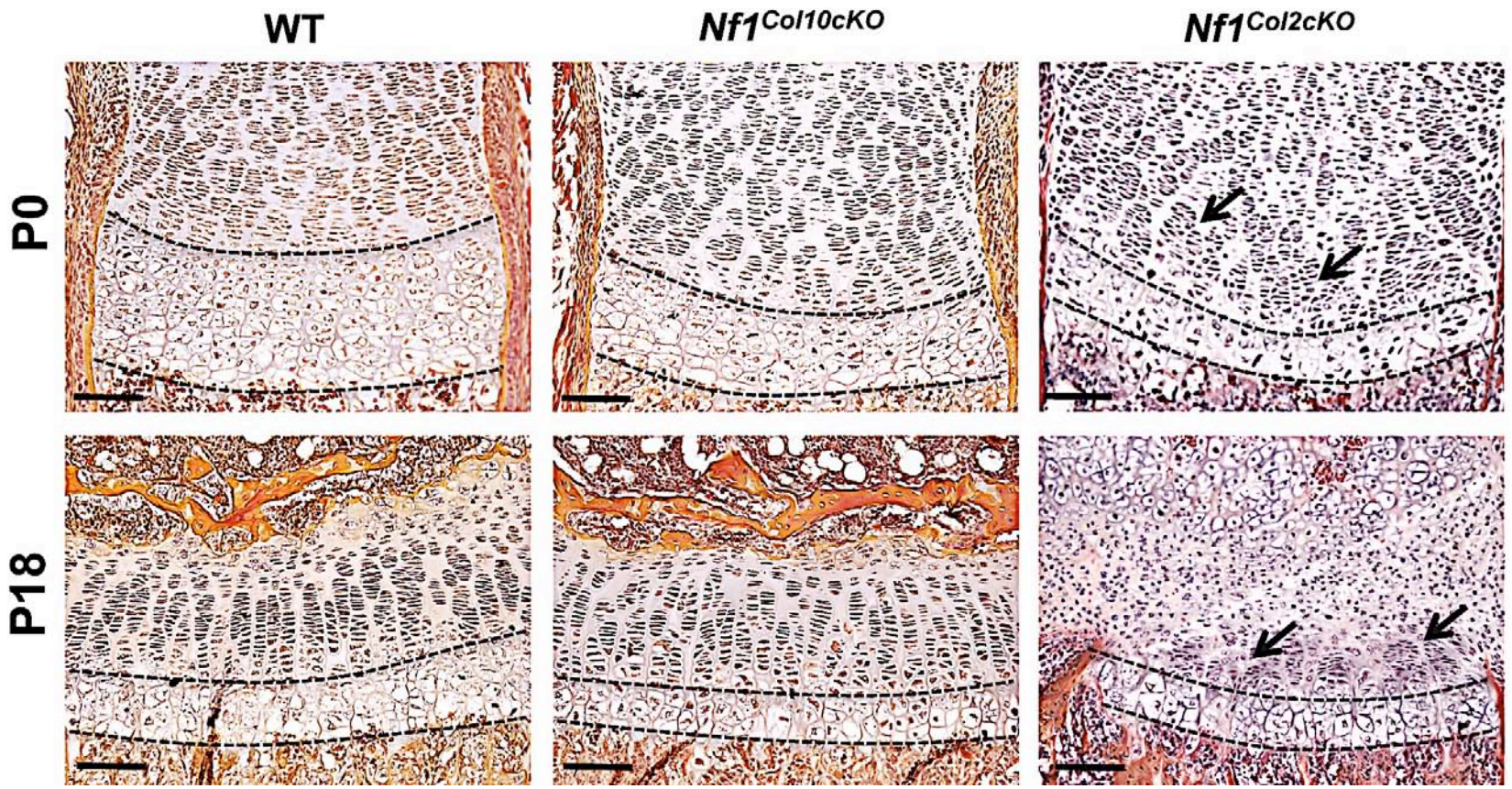

Figure 42. ***Nf1*^{Col10cKO} Mice Have Normal Organization of Growth Plate Proliferative Columns but Short Hypertrophic Zones**
H&E staining of proximal tibial growth plates of WT (left column) and *Nf1*^{Col10cKO} (middle column) littermates at P0 and 18. *Nf1*^{Col2cKO} proximal tibial growth plates (right column) are shown for comparison, with arrows highlighting disorganized proliferative columns and black dotted lines delineating the hypertrophic zones. Scale bar: 100 μm.

proliferation zone of the growth plate with some expression in the prehypertrophic zone at P0 (**Figure 43**). Furthermore, quantitative assessment by qPCR showed no significant differences in *Fgfr1*^{Col2cKO}, *Nf1*^{Col2cKO} and Dbl^{Col2cKO} growth plates versus WT growth plates 5 days after birth (**Figure 44A**).

We also sought to confirm whether expression levels and localization of *Fgfr1* were altered by *Nf1* deletion in *Nf1*^{Col2cKO} growth plates, and the efficiency of *Fgfr1* deletion with the *Col2-Cre* driver. We found that *Fgfr1* was normally localized in *Nf1*^{Col2cKO} growth plates and that *Fgfr1* expression was, as expected, reduced in Dbl^{Col2cKO} growth plates (**Figure 43**, left column). Quantitatively, *Fgfr1* expression was efficiently lowered using the *Col2*-Cre driver as assessed by qPCR on P5 growth plates and was not reduced in *Nf1*^{Col2cKO} growth plates (**Figure 44B**). Together, these results confirm efficient *Fgfr1* recombination in *Fgfr1*^{Col2cKO} and Dbl^{Col2cKO} mutant mice, and indicate that *Fgfr3* expression is not upregulated in response to *Fgfr1* ablation in these two models.

Pan-FGFR Inhibition with BGJ-398 Enhances Bone Growth in Nf1^{Col2cKO} *Mice*

The difference in bone size between *Nf1*^{Col2cKO} and *Nf1*^{Col10cKO} mice and the overlap of expression between *Nf1*, *Fgfr1* and *Fgfr3* in the prehypertrophic zone suggested that neurofibromin in prehypertrophic chondrocytes may control either FGFR3 or FGFR1 signaling and the mechanism by which these cells inhibit chondrocyte proliferation and column stacking. To address this question and because of the difficulties in generating triple conditional knockout mice lacking *Fgfr1*, *Fgfr3* and *Nf1* with proper littermate controls, we chose to administer the pan-FGFR inhibitor BGJ-398 (261) or vehicle

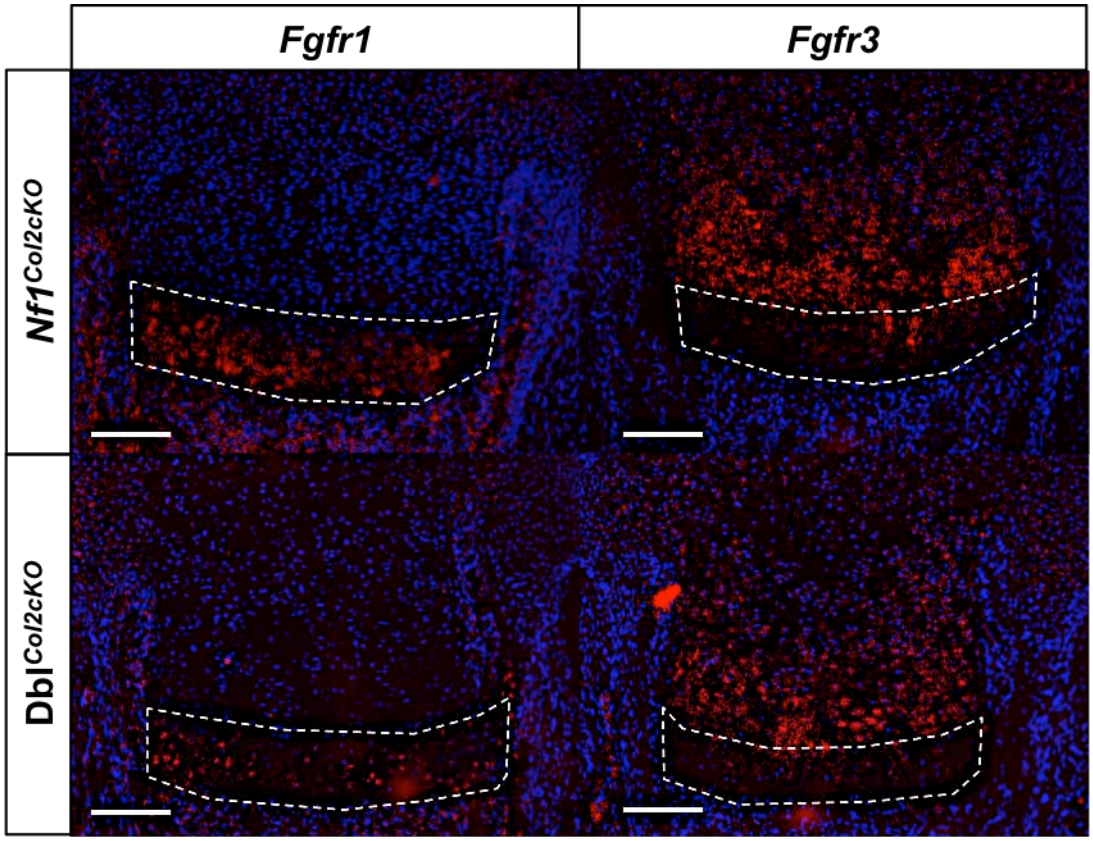

Figure 43. *Nf1* Loss-of-Function Does Not Cause Mislocalization of *Fgfr1* or *Fgfr3* Expression

Fgfr3 expression is not altered upon *Fgfr1* ablation in *Col2a1*-positive chondrocytes. *In situ* hybridization (red signal) in P0 distal femora shows that *Fgfr1* expression (left column) remains localized to hypertrophic chondrocytes in *Nf1*-null chondrocytes and has a less intense signal in $Dbl^{Col2cKO}$ growth plates (bottom left). *Fgfr3* expression (right column) remains restricted to the proliferation and prehypertrophic zones in $Nf1^{Col2cKO}$ (upper right) and $Dbl^{Col2cKO}$ (lower right) growth plates. Hoechst stained nuclei appear in blue. White boxes denote hypertrophic zones. Scale bar: 100 μm.

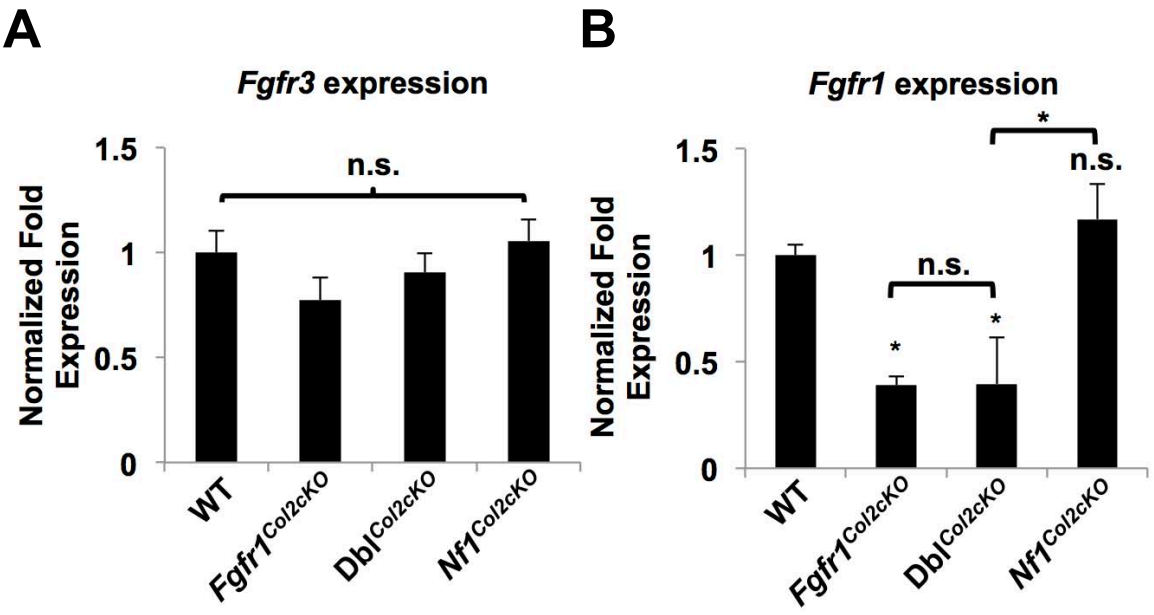

Figure 44. Genetic Compensation for the Loss of *Fgfr1* and/or *Nf1* Expression Does Not Occur *In Vivo*

(**A**) *Fgfr3* expression is not significantly different in P5 long bone growth plate cartilage between WT, $Fgfr1^{Col2cKO}$, $Dbl^{Col2cKO}$ and $Nf1^{Col2cKO}$ mice (one-way ANOVA, n=4 per group, qPCR). (**B**) *Fgfr1* expression is reduced in P5 long bone growth plate cartilage from $Fgfr1^{Col2cKO}$ and $Dbl^{Col2cKO}$ mice when compared with WT, while there is no significant difference in *Fgfr1* expression between $Nf1^{Col2cKO}$ and WT mice (*:$P<0.05$ versus WT unless indicated, n=4 per group, one-way ANOVA, qPCR).

subcutaneously and daily to WT and *Nf1*^{Col2cKO} newborns. We assessed body weight, body length, tibial length and growth plate parameters at the termination of the experiment 18 days later. Pan-FGFR inhibition by this dose of BGJ-398 reduced ERK1/2 activation in both prehypertrophic and hypertrophic chondrocytes in *Nf1*^{Col2cKO} mice when compared with vehicle-treated animals (**Figure 45**), confirming that the drug was active and acting, at least in part, directly on growth plate chondrocytes. BGJ-398 significantly improved tibial length and the lengths of the proliferation and hypertrophic zones in the growth plates of *Nf1*^{Col2cKO} mice (**Figure 46**), whereas it did not impact bone growth in WT mice. Body length and weight in *Nf1*^{Col2cKO} mice treated by BGJ-398 were improved compared with vehicle control, but the differences did not reach statistical significance for these two parameters **(Figure 47)**. Noticeably, BGJ-398 treatment improved the columnar organization of the proliferation zone (**Figure 48**). Since the proliferation zone in *Fgfr1*^{Col2cKO} mice is normal in terms of width and organization, and since *Nf1* is not detectable in this zone, this effect of pan-FGFR inhibition on the width and organization of the proliferation zone in *Nf1*^{Col2cKO} mice suggests that neurofibromin in prehypertrophic chondrocytes, and downstream of FGFR3 mainly, is required for normal chondrocyte proliferation and column stacking.

Discussion

The respective role of FGFs and FGFRs in the growth plate, the intracellular machinery mediating their activity, and in particular the potential contribution of Ras-GAP proteins to these activities remain incompletely characterized, in part because of the dynamic and

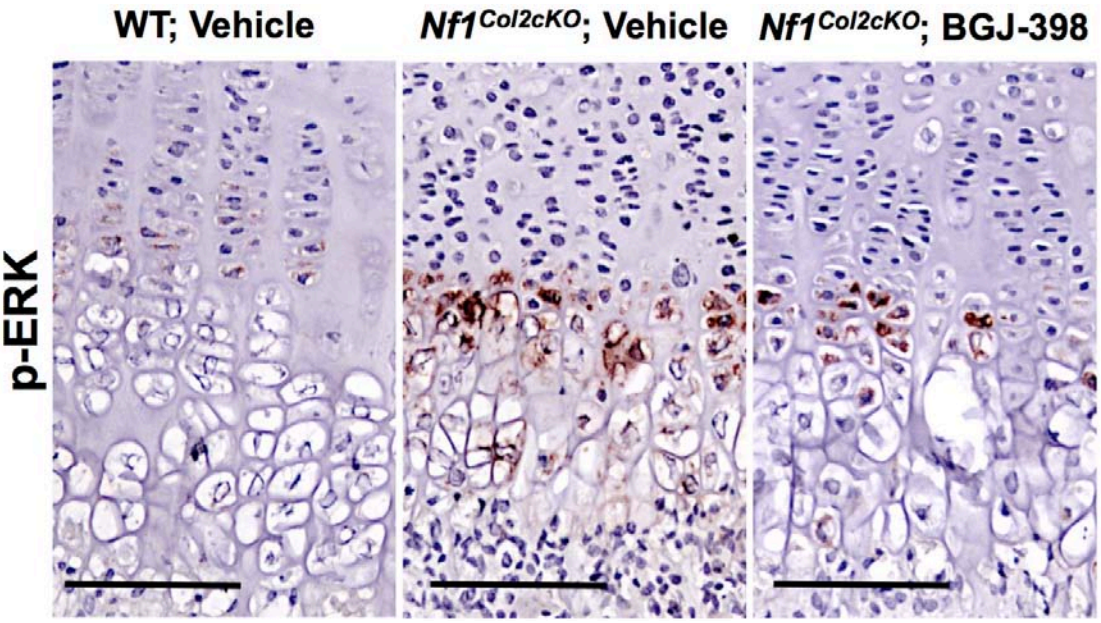

Figure 45. FGFR Inhibition with BGJ-398 Reduces ERK Activation in Growth Plate Chondrocytes *In Vivo.*

Immunohistochemistry for phospho-ERK in proximal tibial growth plates P18 WT, vehicle-treated and $Nf1^{Col2cKO}$ mice treated with vehicle or 10mg/kg BGJ-398. Basal staining is observed in WT, vehicle-treated mice. Staining intensity was high in $Nf1^{Col2cKO}$, vehicle-treated mice and comparatively reduced in $Nf1^{Col2cKO}$, BGJ-treated prehypertrophic and hypertrophic chondrocytes. Brown signal indicates phospho-ERK; sections are counterstained with hematoxylin. Scale Bar: 100 μm.

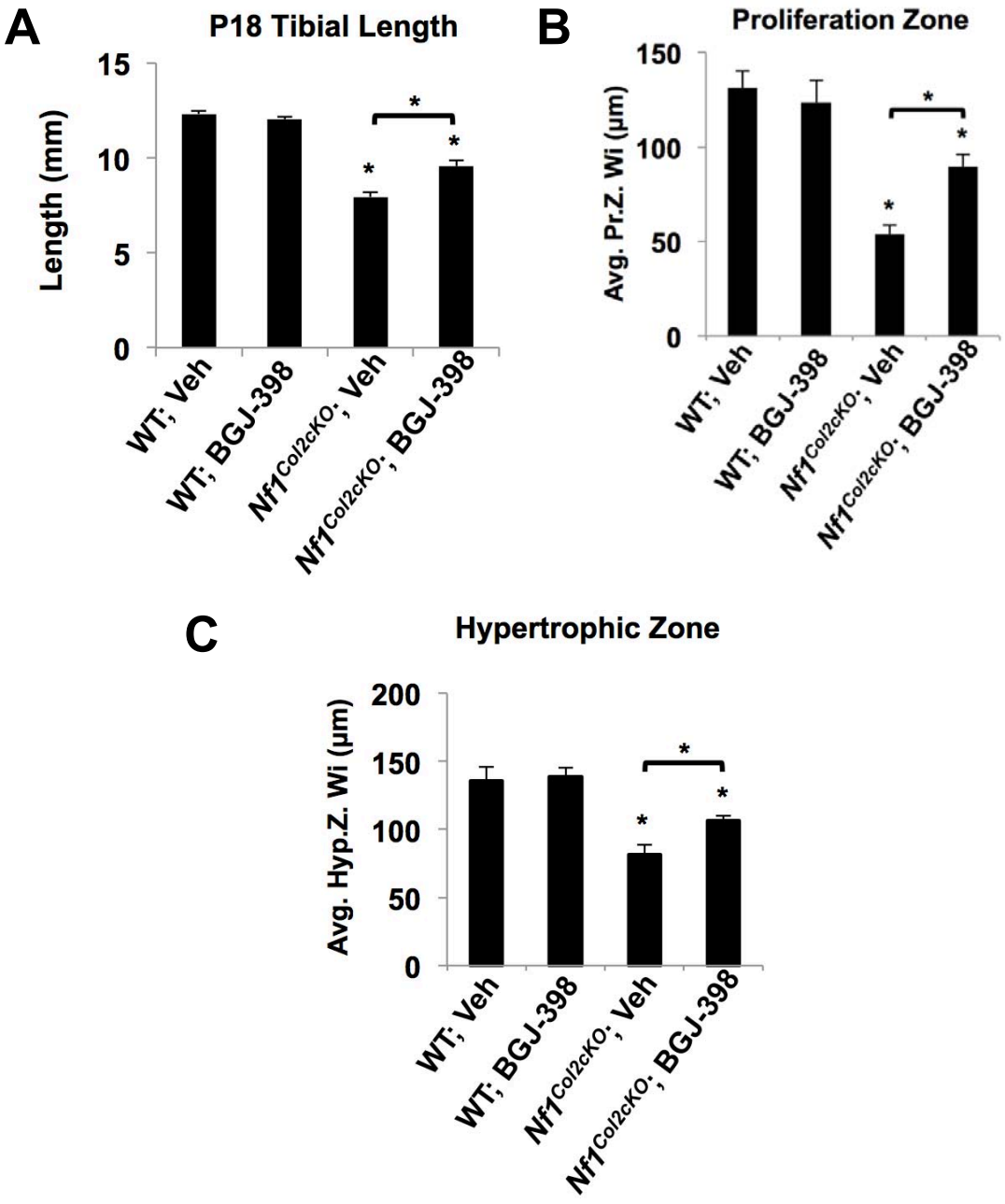

Figure 46. FGFR Inhibition with BGJ-398 Enhances Bone Growth in *Nf1*^{Col2cKO} Mice

(**A**) P18 tibial length of *Nf1*^{Col2cKO} mice treated daily with 10mg/kg BGJ-398 for 18 days after birth were improved compared with *Nf1*^{Col2cKO} mice treated with vehicle, while there was no difference between WT animals treated with vehicle or BGJ-398 (*:P<0.05 versus WT, vehicle unless indicated, n=6 per group, one-way ANOVA). (**B, C**) Length quantification of proliferation (B) and hypertrophic (C) zone lengths at P18 in WT and *Nf1*^{Col2cKO} littermates treated daily with BGJ-398 or vehicle. BGJ-398 treatment significantly improved both proliferation and hypertrophic zone width in *Nf1*^{Col2cKO} mice but not in WT mice (*:P<0.05 versus WT, vehicle unless indicated, n=6 per group, one-way ANOVA).

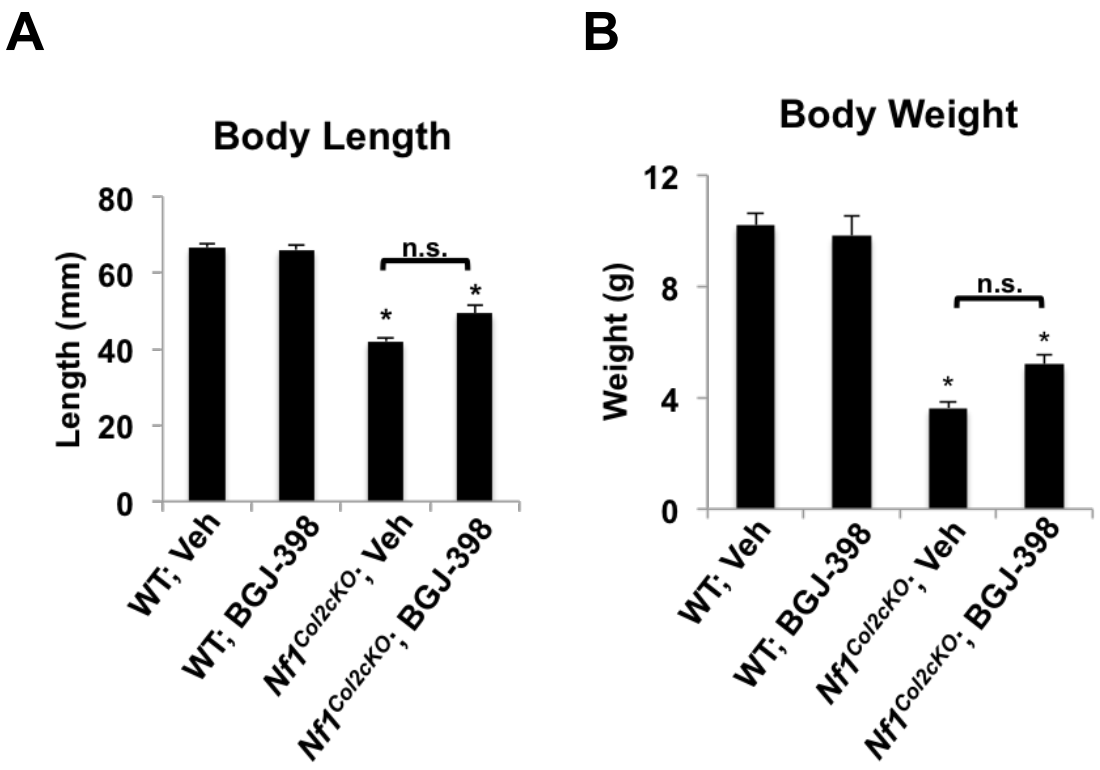

Figure 47. FGFR Inhibition with BGJ-398 Slightly Improves Body Size of *Nf1*^{Col2cKO} Mice

(**A, B**) P18 body length (A) and weight (B) of *Nf1*^{Col2cKO} mice treated with BGJ-398 for 18 days after birth trended towards improvement compared with *Nf1*^{Col2cKO} mice treated with vehicle, while there was no difference between WT animals treated with vehicle or BGJ-398. (*:P<0.05 versus WT, vehicle unless indicated, n=6 per group, one-way ANOVA).

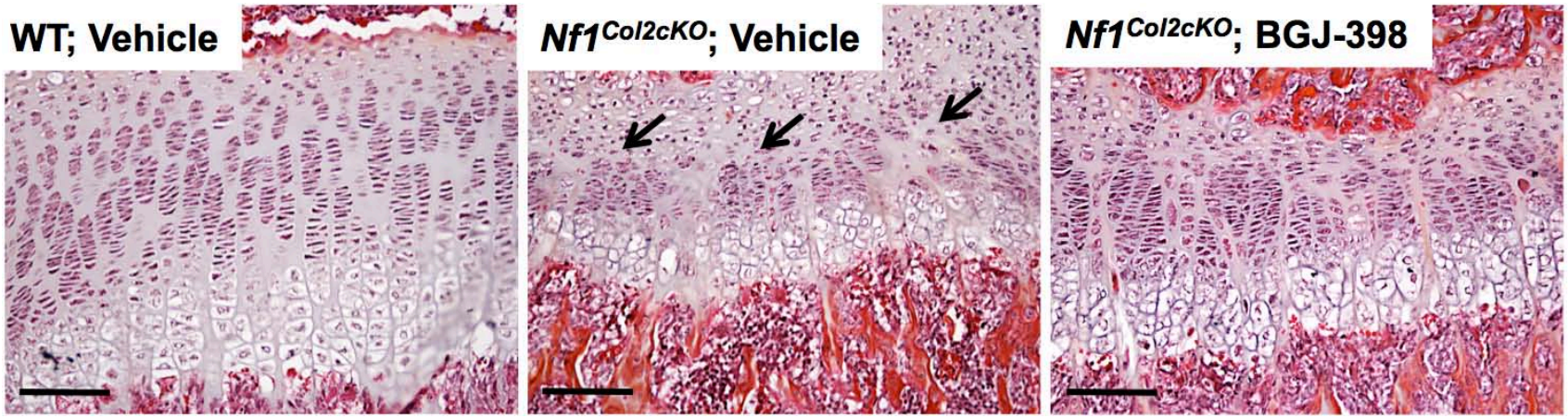

Figure 48. FGFR Inhibition with BGJ-398 Improves Growth Plate Columnar Organization and Zone Length in *Nf1*^{Col2cKO} Mice

H&E staining of P18 distal femora growth plates of WT, vehicle-treated (left), *Nf1*^{Col2cKO}, vehicle-treated (middle) and *Nf1*^{Col2cKO}, BGJ-398-treated (right) littermates showing improved columnar structure and zone lengths in *Nf1*^{Col2cKO}, BGJ-398-treated mice versus *Nf1*^{Col2cKO} vehicle-treated growth plates. Arrows indicate disorganized proliferative columns. Scale bar: 100 μm.

complex nature of the process of endochondral bone formation and the overlap in expression and signaling pathways downstream of FGFRs. This study provides the first comprehensive experimental and genetic evidence indicating that the Ras-GAP activity of neurofibromin is required in prehypertrophic chondrocytes for normal growth plate elongation and proliferative column organization, and is required in hypertrophic chondrocytes, downstream of FGFR1, to attenuate the osteoclastogenic properties of these cells in contact with the bone marrow environment, and thus the coupling between chondrogenesis and bone modeling during development.

A number of signaling proteins are phosphorylated in response to chondrocyte FGF stimulation, including Shc, PLCγ, STAT1, Gab1 and FRS2α (39, 265, 266). These signaling events lead to the activation of intracellular signaling pathways that control cell shape, proliferation, differentiation, migration and survival. In particular, the docking proteins FRS2α and FRS2β are major mediators of the Ras/MAPK and PI3K/AKT signaling pathways that fine-tune the signal initiated by FGFR stimulation by negative feedback mechanisms. This study identifies the Ras-GAP neurofibromin as one critical intracellular components controlling FGFR signaling, specifically in postmitotic growth plate chondrocytes, and further supports the notion that the intensity or duration of FGFR signals during endochondral bone formation must be tightly controlled for harmonious development of the growth plate.

Dwarfism in humans is primarily attributed to FGFR3-activating mutations and the resulting lack of extension of the growth plate proliferating zone (24, 25, 27, 267), where

FGFR3 is highly expressed. FGFR3 activation affects chondrocyte proliferation in a cell-autonomous fashion but also indirectly via repression of IHH signaling (32). Global knockout of *Fgfr3* on the other hand produces mice with a bone overgrowth phenotype including expanded proliferation and hypertrophic zones, enhanced osteoblast differentiation and enhanced osteoclastogenesis (34, 35). In contrast to $Fgfr3^{-/-}$ mice, chondrocyte proliferation is reduced in $Nf1^{Col2cKO}$ mice (**Figure 14**), but the width of the proliferation zone is not affected in $Nf1^{Col10cKO}$ mice. Because *Nf1* is expressed in prehypertrophic and hypertrophic chondrocytes, but not detectable in proliferating chondrocytes, these results suggest that neurofibromin is required in prehypertrophic chondrocytes to indirectly control the extension and organization of proliferative chondrocyte columns. This conclusion is further supported by the beneficial effect of pan-FGFR inhibition on bone size, proliferation zone width and columnar organization in growing $Nf1^{Col2cKO}$ mice (although other systemic or indirect effects of the drug cannot be excluded). These results, along with the fact that $Fgfr1^{Col2cKO}$ mice have no proliferation zone size phenotype and that *Fgfr1*-null prehypertrophic chondrocytes still are positive for phospho-ERK (**Figure 31**), suggest that in prehypertrophic chondrocytes, neurofibromin controls FGFR3 signaling to regulate, in an indirect, paracrine fashion, chondrocyte proliferation zone length and their typical columnar organization. This does not rule out a role of FGFR1 signaling in prehypertrophic chondrocytes, based on the severe dwarfism of individuals with osteoglophonic dysplasia and mice with *Fgfr1*-activating mutations.

Concurrent ablation of *Nf1* and *Fgfr1* in the same *Col2a1*-expressing cell population and at the same time during development reversed the increase in hypertrophic zone width observed in $Fgfr1^{Col2cKO}$ mice. These findings suggest that reactivation of the Ras-Raf-MEK-ERK1/2 pathway by *Nf1* deletion in *Fgfr1*-deficient hypertrophic chondrocytes functionally compensated for the reduced FGFR1 signaling in these cells *in vivo*. We have shown in a previous study that neurofibromin controls *Rankl* expression and osteoclastogenesis in mature chondrocytes, and thus that increased growth plate catabolism likely contributes to the reduction in hypertrophic width observed in $Nf1^{Col2cKO}$ mice (**Figures 20, 21**). The observation that chondrocytic ablation of *Nf1* reverses the reduction in osteoclast number observed in $Fgfr1^{Col2cKO}$ mice, and the fact that the hypertrophic zone width in $Nf1^{Col10cKO}$ mice is shorter (while being longer in $Fgfr1^{Col2cKO}$ mice) compared with WT mice, suggests that neurofibromin, in hypertrophic chondrocytes, inhibits the pro-osteoclastogenic signals from FGFR1 in the chondrocytic lineage. It remains to be determined if activation of the transcription factor ATF4, resulting from the lack of *Nf1,* contributes to the observed increase in *Rankl* expression, as was previously shown in mature osteoblasts (135). Though RANKL and OPG are obvious candidates to mediate changes in osteoclastogenesis at the osteochondral border, and though we indeed observed significant changes in *Rankl* expression *in vivo*, we cannot rule out the possibility that other pro- or anti-osteoclastogenic molecules are differentially regulated in the presence/absence of neurofibromin or FGFR1. It must also be emphasized that the relatively modest increase in *Rankl* expression in the growth plates of $Nf1^{Col2cKO}$ mice is to be expected based on the nature of the cells expressing this cytokine in the growth plate, i.e. hypertrophic chondrocytes. This area of the growth plate

indeed represents a small volume of the growth plate, is under constant catabolism, and contains cells that are hypertrophic (hence less cell number per volume) and rapidly eliminated. These characteristics are even more pronounced in the shortened growth plates of $Nf1^{Col2cKO}$ mice, hence the increase in *Rankl* expression in this transient area cannot be of high amplitude.

The width of the hypertrophic zone depends on multiple factors, including growth plate maturation, catabolism but also chondrocyte apoptosis. However, no difference in chondrocyte apoptosis has been detected between WT mice and mice lacking FGFR1 ($Fgfr1^{Col2cKO}$) (49) or B-Raf (268) or ERK1/2 (269) in *Col2a1*-expressing chondrocytes. On the other hand, we have shown in previous studies that *Nf1*-null chondrocytes have enhanced sensitivity to phosphate-mediated apoptosis *in vitro*, although we failed to detect increased *in vivo* apoptosis in the growth plate of $Nf1^{Col2cKO}$ mice (**Figure 18**). Chondrocyte forced expression of *Spry1*, which leads to ERK activation, as well as an FGFR3-activating mutation, did result in premature chondrocyte apoptosis *in vivo* (41, 270), although constitutive activation of MEK1 in these cells did not affect apoptosis (44). Therefore, *Fgfr1* loss-of-function does not alter the rate of chondrocyte apoptosis, but ERK constitutive activation promotes it, via mechanism(s) that remains to be characterized.

Recalcitrant bone healing (pseudarthrosis) affects about 5% of children with NF1. In this condition, tibial bowing is followed by fracture and absence of repair, accumulation of myofibroblasts, non-mineralized matrix and hyaline cartilage, suggesting that the early

stages of endochondral bone repair are blocked (2). The developmental phenotypes observed in this study suggest that pan-FGFR inhibition might have a beneficial effect in the setting of NF1 pseudarthrosis on the formation of the cartilaginous callus and its transition to a calcified callus required for bone union following fracture, as bone development recapitulates the main stages of adult bone repair. Although very speculative, this hypothesis can be addressed in preclinical models of NF1 pseudarthrosis, particularly if treatment can be applied locally to avoid undesirable effects on other tissues.

Collectively, our findings thus indicate that neurofibromin in postmitotic growth plate chondrocytes plays an integral part in the Ras-ERK-dependent regulatory mechanisms that allow these cells to integrate and balance signaling from various growth factors via RTKs like FGFRs. These findings are relevant to genetic diseases affecting embryonic development of the skeleton like chondrodysplasias, but also to genetic conditions impacting bone healing in adults, like neurofibromatosis type 1 and RASopathies in general.

Summary

Aberrant Fibroblast Growth Factor Receptor 3 (FGFR3) signaling disrupts chondrocyte proliferation and growth plate size and architecture, leading to various chondrodysplasias or bone overgrowth. These observations suggest that the duration, intensity and cellular context of FGFR signaling during growth plate chondrocyte maturation require tight,

regulated control for proper bone elongation. However, the machinery fine-tuning FGFR signaling in chondrocytes is incompletely defined. We report here that neurofibromin, a Ras-GAP encoded by *Nf1*, has an overlapping expression pattern with FGFR1 and FGFR3 in prehypertrophic chondrocytes, and with FGFR1 in hypertrophic chondrocytes during endochondral ossification. Based on previous evidence that neurofibromin inhibits Ras-ERK signaling in chondrocytes and phenotypic analogies between mice with constitutive FGFR1 activation and *Nf1* deficiency in *Col2a1*-positive chondrocytes, we asked whether neurofibromin is required to control FGFR1-Ras-ERK signaling in maturing chondrocytes *in vivo*. Genetic *Nf1* ablation in *Fgfr1*-deficient chondrocytes reactivated Ras-ERK1/2 signaling in hypertrophic chondrocytes and reversed the expansion of the hypertrophic zone observed in mice lacking *Fgfr1* in *Col2a1*-positive chondrocytes. Histomorphometric and gene expression analyses suggested that neurofibromin, by inhibiting *Rankl* expression, attenuates pro-osteoclastogenic FGFR1 signaling in hypertrophic chondrocytes. We also provide evidence suggesting that neurofibromin in prehypertrophic chondrocytes, downstream of FGFRs and via an indirect mechanism, is required for normal extension and organization of proliferative columns. Collectively, this study indicates that FGFR signaling provides an important input into the Ras-Raf-MEK-ERK1/2 signaling axis in chondrocytes, and that this input is differentially regulated during chondrocyte maturation by a complex intracellular machinery, of which neurofibromin is a critical component.

CHAPTER IV

DISCUSSION, FUTURE DIRECTIONS AND CONCLUSION

Part I: General Discussion – Basic Science

In an effort to study the function of neurofibromin in osteoprogenitors as opposed to mature osteoblasts, we conditionally deleted *Nf1* in osteochondroprogenitors using the Collagen Type 2 cre recombinase ($Nf1_{Col2}^{-/-}$) (4). In addition to defects in $Nf1^{-/-}$ osteoprogenitors, we found that $Nf1_{Col2}^{-/-}$ mice are dwarf suggesting a major function of neurofibromin in growth plate chondrocytes.

The hypothesis guiding this dissertation was that the gene product of *NF1*, neurofibromin, is not only necessary for proper growth plate formation but also negatively regulates FGFR1 signaling during endochondral bone formation. In Chapter II, I addressed the general function of neurofibromin in growth plate chondrocytes, and in Chapter III, I refined the model of neurofibromin's function and the signaling it regulates in growth plate chondrocytes during endochondral ossification. Furthermore, both of these studies have significant implications for the clinical treatment of NF1 pseudarthrosis. In combination with other studies from our laboratory of the function of neurofibromin in osteoblasts and their progenitors, my studies set the stage for a series of preclinical *in*

vitro and *in vivo* fracture healing studies in mouse models of NF1 fracture healing; these will be discussed in Part II of this chapter.

Neurofibromin Regulates Chondrocyte Proliferation Indirectly

Neurofibromin was found by immunohistochemistry to be expressed in prehypertrophic and hypertrophic chondrocytes (**Figure 9**). As such, we examined all aspects of growth plate chondrocytes' proliferation and maturation, rather than chondrogenesis due to this expression pattern and as $Nf1_{Col2}^{-/-}$ mice did not seem to have overt problems with chondrogenesis.

We found that though *Nf1* is not expressed in proliferative chondrocytes, $Nf1^{-/-}$ growth plates had significantly shorter proliferation zones versus littermate, WT growth plates (**Figure 13**). Furthermore, $Nf1^{-/-}$ chondrocytes in the proliferation zone of the growth plate were significantly less proliferative than their WT counterparts (**Figure 14**). We found that $Nf1^{-/-}$ chondrocytes expressed significantly lower levels of *Ihh*, a critical paracrine factor promoting chondrocyte proliferation, both *in vitro* and *in vivo* (**Figure 15**). This suggests a potential mechanism whereby $Nf1^{-/-}$ prehypertrophic chondrocytes, via impaired synthesis of *Ihh*, negatively influence chondrocyte proliferation in the proliferation zone, in a non-cell-autonomous, paracrine fashion. These findings agree with others' findings that MAPK signaling is an important regulator of *Ihh* gene transcription (45). *In vivo* Smoothened agonism (with purmorphamine) or expression of a *Col2a1* driven *Ihh* transgene would be necessary to demonstrate conclusively that IHH is responsible for the defects in chondrocyte proliferation in $Nf1_{Col2}^{-/-}$ growth plates.

Neurofibromin Regulates Chondrocyte Hypertrophic Maturation

The hypertrophic zone of growth plates from $Nf1_{Col2}^{-/-}$ mice was also shorter than in WT littermates (**Figure 13**). To test whether $Nf1^{-/-}$ chondrocytes had an inherent deficit in maturing to hypertrophy rather than this being secondary to defective proliferation or excessive osteoclast mediated removal of hypertrophic chondrocytes, we assessed the maturation of WT and $Nf1^{-/-}$ chondrocytes *in vitro* by relative *Col10a1* expression, a marker of hypertrophic chondrocytes. We found that $Nf1^{-/-}$ chondrocytes do not mature to hypertrophy as shown by decreased *Col10a1* expression compared to WT chondrocytes *in vitro* (**Figure 17**). These results agree with others' findings that excessive MEK activity impairs chondrocytes' hypertrophic maturation (44).

Chondrocytic Neurofibromin Regulates Matrix Catabolism at the Osteochondral Border

Because the hypertrophic zone of $Nf1_{Col2}^{-/-}$ growth plates was short compared to WT littermates, we assessed whether matrix catabolism and osteoclastogenesis at the chondro-osseous junction was dysregulated. We found that there was increased osteoclast number at the osteochondral border in $Nf1_{Col2}^{-/-}$ mice compared to WT littermates (**Figure 20**) and furthermore, that $Nf1^{-/-}$ chondrocytes *in vitro* were capable of stimulating osteoclastogenesis more so than WT chondrocytes (**Figure 21**). Finally, we also found that $Nf1^{-/-}$ chondrocytes synthesize more *Mmp9/13* and *Opn* than WT chondrocytes (**Figures 16, 19, 20**), which could further contribute to the excessive cartilage matrix catabolic phenotype (due to enhanced osteoclastogenesis) in $Nf1_{Col2}^{-/-}$ mice.

The relative contribution of *Nf1*-null osteoblasts versus chondrocytes to this dysregulated matrix catabolic phenotype remains unknown. *In vitro* evidence from chondrocytes suggests that chondrocytes are sufficient to mediate this phenotype (**Figure 21**). However, because my *in vivo* studies used the *Col2a1* and *Col101a* driven cre recombinases which target both chondrocytes and some subpopulations of osteoblasts ((4) and **Figure 37**), it cannot be concluded whether the phenotype *in vivo* is primarily attributable to chondrocytes or osteoblasts or whether chondrocytes are sufficient to mediate this phenotype *in vivo*. There are currently no available Cre-transgenes that are specific to chondrocytes (or hypertrophic chondrocytes) without also targeting a subpopulation of osteoblasts, making this question difficult to address. The lack of chondrocyte specific promoters, however, may support the rather provocative hypothesis that some hypertrophic chondrocytes do not undergo apoptotic cell death at the osteochondral border, but instead persist and transdifferentiate into osteoblasts.

Finally, these data regarding *Nf1*-null chondrocyte maturation deficits in combination with enhanced matrix catabolic properties suggest that perhaps *Nf1*-null chondrocytes may not necessarily posses an inherent inability to mature to hypertrophy, but rather follow an accelerated "maturation" program or may skip conventional hypertrophy (marked by *Col10a1* expression) altogether. More precise longitudinal *in vitro* characterization of WT versus $Nf1^{-/-}$ chondrocyte gene expression is required to adequately address this unknown.

Neurofbromin Attenuates FGFR1 Signaling in Hypertrophic Chondrocytes

To elucidate the function of neurofibromin in specific zones of the growth plate (prehypertrophic and hypertrophic) and whether neurofibromin attenuated FGFR1 in these zones, I generated double conditional knockout mice ($Fgfr1_{Col2}^{-/-}$;$Nf1_{Col2}^{-/-}$ or $Dbl^{Col2cKO}$) to determine whether the phenotypes of $Fgfr1^{Col2cKO}$ mice were reversed and thus indicating that neurofibromin indeed regulates FGFR1 signaling in growth plate chondrocytes. I found that $Dbl^{Col2cKO}$ mice had opposite phenotypes of $Fgfr1^{Col2cKO}$ with respect to the length of the hypertrophic zone and osteoclastogenesis at the osteochondral border (**Figures 33, 35**). Furthermore, conditional deletion of *Nf1* in hypertrophic chondrocytes alone, led to no overt body size abnormalities of $Nf1^{Co10cKO}$ mice (**Figures 38, 39**), though they did exhibit dysregulated osteoclastogenesis and shorter hypertrophic zones (**Figures 40-42**).

Taken together, these results indicate that FGFR1 signaling is attenuated neurofibromin in the hypertrophic zone and that FGFR1 signaling in the hypertrophic zone is important in regulating hypertrophic zone length and matrix catabolism at the osteochondral border. These results also suggest that $Nf1^{-/-}$ hypertrophic chondrocytes play a relatively minor role in the bone length defect of $Nf1_{Col2}^{-/-}$ mice.

Neurofibromin Likely Attenuates Both FGFRs 1 and 3 in Prehypertrophic Chondrocytes

Finally, these studies indicate that neurofibromin likely attenuates both FGFR1 and FGFR3 signaling in prehypertrophic growth plate chondrocytes. We come to this conclusion based on the cumulative weight of four distinct lines of evidence. First,

because $Nf1^{Col10cKO}$ mice were not dwarf but $Nf1^{Col2cKO}$ were, we concluded that the function of neurofibromin in prehypertrophic chondrocytes is crucial to overall length of long bones, and that this is possibly due to indirect regulation of chondrocytes proliferation regulated by IHH produced by prehypertrophic chondrocytes. When we treated $Nf_{Col2}^{-/-}$ mice with the pan-FGFR inhibitor BGJ-398 we found that both proliferation and hypertrophic zone lengths were significantly improved and body length also improved. Because both *Fgfr1* and *Fgfr3* are expressed in the prehypertrophic zone (**Figure 29**), these results suggest that FGFRs 1 and 3 in the prehypertrophic zone were inhibited to improve proliferation zone length; indeed, we showed that ERK activation was decreased by immunohistochemistry following BGJ-398 administration (**Figure 45**). Secondly, this conclusion is supported by the fact that FGFR1 constitutive activation in osteoglophonic dysplasia patients is sufficient to cause dwarfism (50, 258), and FGFR3 constitutive activation is also sufficient to cause dwarfism (27, 28) (though this is effect is both direct as FGFR3 is expressed in proliferative chondrocytes and indirect via IHH mediated effects in prehypertrophic chondrocytes (32, 38-43)). Third, because our BGJ-398 treatment had no effect on body length or growth plate zone lengths in WT animals but was effective in $Nf1_{Col2}^{-/-}$ (**Figures 46, 47**), this suggests that the effect of the BGJ-398, a pan-FGFR inhibitor, was specific to the $Nf1_{Col2}^{-/-}$ phenotype, and as discussed above, prehypertrophic chondrocytes are responsible for bone/body length in $Nf1_{Col2}^{-/-}$ animals. Finally, because *Fgfr1* inactivation in $Nf1^{-/-}$ chondrocytes did not rescue $Nf1_{Col2}^{-/-}$ induced dwarfism (**Figure 32**) and ERK activity was still dysregulated with *Fgfr1* ablation in *Nf1*-null chondrocytes (**Figure 31**), we hypothesize that another dysregulated receptor(s), most likely FGFR3 based on established precedent, is sufficient

to cause a dwarfism phenotype. An informative but technically challenging experiment to study this genetic interaction further would be the generation of triple conditional *Fgfr1*, *Fgfr3*, *Nf1* knockout mice. The refinement of the role of neurofibromin in growth plate chondrocytes is shown in **Figure 49**.

Part II: Clinically Oriented Discussion – Future Directions

The goal of these studies, in addition to the pursuit of determining the function of neurofibromin in growth plate chondrocytes, is to inform clinically relevant studies of neurofibromin's function in chondrocytes during fracture healing and the ways in which NF1 pseudarthroses can be treated given these findings.

New Pharmacological Strategies for Treating NF1 Pseudarthrosis

These studies highlight several pharmacological targets that may be targeted during fracture healing in NF1. These include modulation of IHH signaling, the MAPK cascade directly, and FGFR inhibition.

Purmorphamine

My studies showed that *Ihh* expression was decreased in $Nf1^{-/-}$ chondrocytes. Though not performed, it remains to be explored whether agonism of the hedgehog pathway with the smoothened agonist purmorphamine *in vivo* would improve the body size of $Nf1_{Col2}^{-/-}$ mice via stimulation of chondrocyte proliferation or whether purmorphamine would enhance differentiation of $Nf1^{-/-}$ osteoprogenitors into functional osteoblasts *in vitro* or *in*

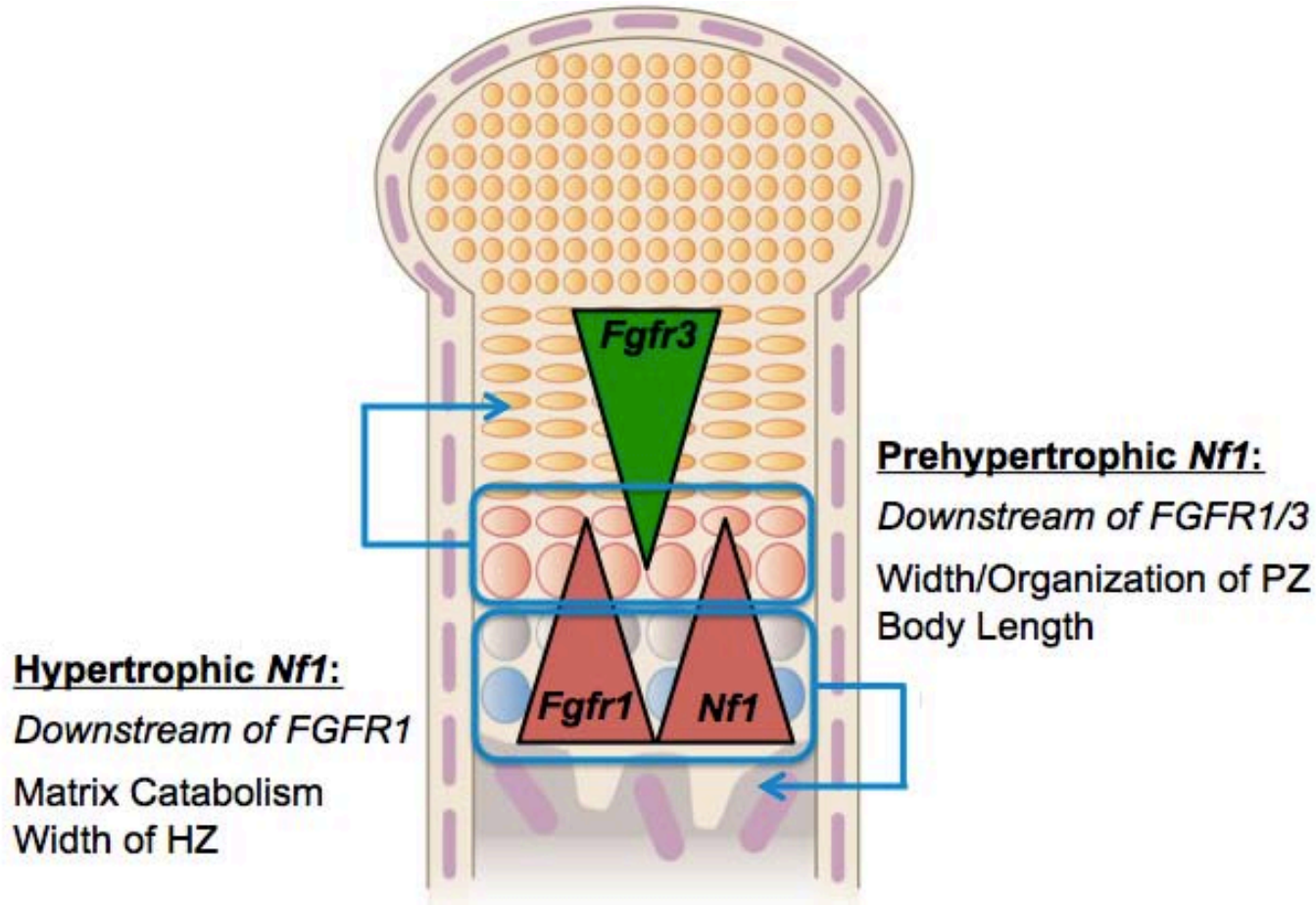

Figure 49. Model of the Role of Neurofibromin in Growth Place Chondrocytes Considering Spatial Expression of *Nf1*, *Fgfr1*, and *Fgfr3*

In hypertrophic chondrocytes, neurofibromin attenuates FGFR1 signaling to regulate width of the growth plate hypertrophic zone and matrix catabolism at the osteochondral border. In prehypertrophic chondrocytes, neurofibromin attenuates both FGFR1 and FGFR3 signaling to regulate body/long bone length and width/organization of the growth plate proliferation zone.

vivo. If purmorphamine proved to be effective on these developmental parameters in mice, it may also be useful in the setting of NF1 pseudarthrosis as chondrocyte function is hypothesized to also be impaired in fracture healing and others in our group have showed that osteoprogenitor/osteoblast differentiation and function are also impaired with *Nf1* loss-of-function (134, 144). There are potential caveats to this approach however. Given that we observed non-*Ihh* dependent effects of *Nf1* loss-of-function, namely defects in chondrocytic matrix catabolism (**Figures 16, 19-21**) and excessive production of pyrophosphate in osteoblasts and chondrocytes (134), we hypothesize that purmorphamine treatment would not correct these defects in matrix catabolism/mineralization.

CNP

My studies showed the efficacy of C-type natriuretic peptide to improves body length and proliferation/hypertrophic zone lengths in $Nf1^{-/-}$ growth plate chondrocytes (**Figures 25, 26**) and that ERK activation in chondrocytes was decreased with treatment (**Figures 24, 25**). These studies, performed in the developmental context suggest that CNP may also be useful in chondrocytes in the fracture healing setting. Furthermore, molecular confirmation of rescue of aberrant gene expression and function in chondrocytes with CNP treatment (such as *Ihh*, *Col10a1,* and *Rankl*) is warranted.

Of note, our laboratory recently published studies showing the efficacy of dual treatment of $Nf1^{-/-}$ osteoprogenitors with the MEK inhibitor trametinib and BMP-2 in promoting osteoprogenitor differentiation into osteoblasts and improving fracture healing in $Nf1_{Osx}^{-/-}$

mice (144). Because WT and *Nf1*$^{-/-}$ bone marrow osteoprogenitors also express the CNP receptor *Npr-b*, regardless of their differentiation stage (**Figure 50**), we hypothesize that treating *Nf1*$^{-/-}$ osteoprogenitors with CNP and BMP-2 together may prove to be as equally efficacious as trametinib and BMP-2 treatment since CNP, like trametinib, inhibits the MAPK cascade, though via indirect Raf inhibition, not MEK inhibition (197). These studies are in the very preliminary *in vitro* stages, but are ongoing. Use of CNP as opposed to trametinib in osteoblasts may be advantageous for several reasons. First, if CNP is also effective in normalizing *Nf1*$^{-/-}$ callus chondrocyte function as well as being effective in promoting callus osteoblast differentiation and function, simplification of the treatment paradigm from three drugs, CNP (to target chondrocytes), trametinib and BMP-2 (to target osteoblasts/progenitors), to only two drugs, CNP (to target both chondrocytes and osteoblasts/progenitors) and BMP-2 (to target osteoblasts/progenitors), would be possible and desirable. Secondly, CNP can be administered systemically with limited adverse effects as opposed to trametinib whose target (MEK) is ubiquitously expressed. CNP's receptor, NPR-B, has a much more limited expression pattern. This is known because CNP is in currently in clinical trials for achondroplasia in children and thus has known safety, pharmacokinetic and dosing profiles in children with bone abnormalities.

BGJ-398

I also addressed the effects of FGFR inhibition during endochondral bone formation in my studies. I found that the pan-FGFR inhibitor BGJ-398 significantly improved bone length and growth plate zone length parameters in *Nf1*$_{Col2}^{-/-}$ mice and in a manner that suggested it was specific to the defects caused by *Nf1* deficiency (**Figures 46-48**). Use of

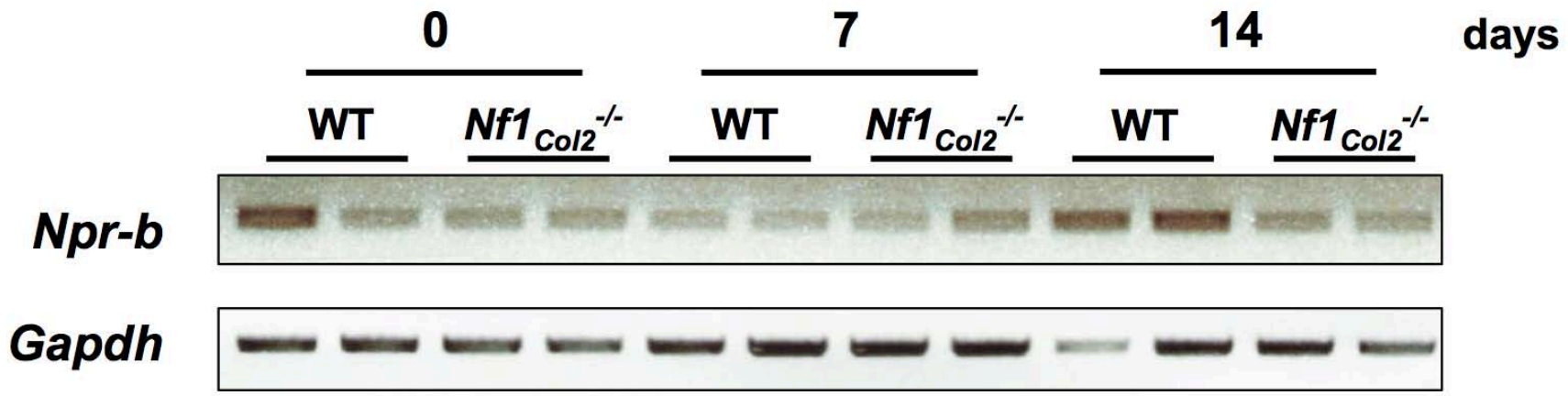

Figure 50. *Npr-b* Expression in WT and *Nf1*$^{-/-}$ Bone Marrow Derived Osteoblasts
RT-PCR for *Npr-b* shows that *Npr-b* is expressed in WT and *Nf1*$^{-/-}$ osteoblasts and progenitors throughout their differentiation in osteogenic media and does not depend on the mutation status of *Nf1*.

BGJ-398 in a fracture healing setting in a NF1 mouse model to target chondrocytes is an attractive thought to address whether BGJ-398 would improve the function of *Nf1*$^{-/-}$ chondrocytes in this setting as well and whether this significantly improves bone union/fracture healing callus parameters that are defective in NF1 fracture healing mouse models.

We hypothesize that inhibition of FGFRs may be beneficial for *Nf1*$^{-/-}$ chondrocyte function and maturation in the soft callus of NF1 patients, however the therapeutic potential of FGFR inhibition in other cells types contributing to fracture healing (MSCs and osteoblasts/progenitors) remains unclear as does whether neurofibromin attenuates FGFRs in these cell populations. Regardless of whether neurofibromin attenuates FGFRs in MSCs, FGFRs have been implicated in MSC proliferation during fracture healing (71, 72). Therefore, it is possible that FGFR inhibition could prevent MSC proliferation necessary for callus formation resulting in an atrophic callus, or, on the opposite end of the spectrum, that FGFR inhibition could prevent aberrant proliferation of *Nf1*$^{-/-}$ progenitors that may contribute to the hamartomatous accumulation of cells at the NF pseudarthrosis site.

FGFR1 and FGFR3 signaling in osteoblasts/progenitors during development and fracture healing remains poorly understood in addition to whether neurofibromin regulates these signaling molecules in these contexts. Both gain- and loss-of-function mutations in *Fgfr3* in mice cause a low bone mass phenotype (225, 271-273). *Fgfr1* conditional knockout studies indicated that the effects of FGFR1 signaling in osteoblast/progenitors was

dependent on the stage of differentiation (49). Therefore, the effect of FGFR inhibition in osteoblasts/progenitors during fracture healing also remains difficult to predict.

Thus, the effect of FGFR inhibition during fracture healing may have diverging effects at different stages of fracture healing, the worst case scenario being that BGJ-398 has negative effects on MSCs, positive effects on chondrocytes, and negative effects on osteoblasts/progenitors. For these reasons, we believe that FGFR inhibition therapy for NF1 pseudarthrosis may only target soft callus formation and its transition to the hard callus. This would make the timing of BGJ-398 delivery crucial to its potential as an NF1 pseudarthrosis therapy. It may also be necessary to deliver BGJ-398 locally in children to avoid off-target effects of systemic FGFR inhibition. The timing of delivery and delivery formation are both parameters that can be addressed in NF1 fracture healing mouse models. Finally, identification of the NF1 pseudarthrosis cell of origin, and thus knowing which cells to target, would lend insight into these significant unknowns; this will be discussed below.

Is Proper Chondrocyte Function Necessary for NF1 Fracture Healing?

Fracture healing is a regenerative process that resembles both endochondral and intramembranous bone formation processes at its core with intramembranous ossification occurring distal to the fracture site (adjacent to the vascularized bone) and endochondral ossification occurring distal to the bone fragments (the avascular zone) (65-68). One thought experiment that should be undertaken in the case of the NF1 pseudarthrosis and these proposed therapies is whether fractures can heal via intramembranous bone repair

alone. If intramembranous bone repair can be sufficient for bone healing, therapies that sufficiently targeted the NF1 pseudarthrosis cell of origin to differentiate into a functional osteoblast, not a functional chondrocyte, may be sufficient to promote fracture healing in NF1 patients.

Lessons from achondroplasia patients electing to undergo limb-lengthening surgeries may be informative to this question. Limb-lengthening surgeries involve surgically induced fracture (osteomy) followed by Ilizarov distraction osteogenesis (274). One study compared the limb-lengthening bone healing times in patients with syndromic short stature (such as achondroplasia) versus non-syndromic patients with limb length discrepancy (due to physeal trauma, etc.) found that the bone healing index (days to bone consolidation per cm lengthened) was no different in non-syndromic patients versus achondroplasia patients (275). Similarly, there were no significant differences in the complication rate between these two populations. Because it remains debated whether Ilizarov distraction osteogenesis promotes bone healings via both intramembranous and endochondral bone repair or solely intramembranous bone repair (276-281), two slightly different interpretations can be drawn from this study. If intramembranous ossification is solely responsible for the repair, this suggests that patients with chondrocyte defects can repair bone solely via intramembranous repair mechanisms, without chondrocytes. If both endochondral and intramembranous bone repair are at work in Ilizarov distraction osteogenesis, the lack of healing index differences between non-syndromic patients versus achondroplasia patients suggests that functionally impaired chondrocytes, at least in achondroplasia patients, do not negatively affect fracture repair. It must be noted that

mouse models of achondroplasia suggest that chondrocyte proliferation and maturation to hypertrophy are severely affected (30, 32, 33). There is limited evidence for defects in matrix catabolic defects (some reported increased osteoclastogenesis dependent on FGFR3 activity in osteoblast precursors (33)) at the osteochondral border in $Fgfr3^{ACH}$ mice as there are in $Nf1_{Col2}^{-/-}$ mice. This zone is critical in coordinating the transition from cartilage to bone at the osteochondral border during development and suspected to be equally critical during fracture healing (68, 282-284). Functional defects in hypertrophic chondrocytes may therefore not be completely addressed by fracture studies in achondroplasia patients and thus may not speak to the necessity of hypertrophic chondrocytes for successful fracture healing.

Studies from our laboratory indicate that $Nf1^{-/-}$ osteoprogenitor function and differentiation can be rescued via combined MEK inhibitor and BMP-2 treatment *in vitro* and in the $Nf1_{Osx}^{-/-}$ model of fracture repair (144). Though it remains unknown whether the $Nf1_{Osx}^{-/-}$ model of fracture healing has defects in callus chondrocytes (the transgene is developmentally active in hypertrophic chondrocytes (145)), it is clear that the osteoprogenitor is severely affected in this model and fracture healing is similarly impaired (134, 144). Therefore, if targeting $Nf1^{-/-}$ osteoprogenitors/osteoblasts was sufficient to promote bone healing in these $Nf1_{Osx}^{-/-}$ fracture studies and the NF1 cell of origin in humans were not ontogenetically prior to the osteoprogenitors genetically targeted in the *Osx*-Cre model or even if therapies could promote an ontogenetically earlier cell of origin to differentiate into an $Nf1^{-/-}$ osteoprogenitor that could then be treated with MEK inhibitors and BMP-2 dual treatment, therapies that exclusively

targeted chondrocytes may be irrelevant. In this scenario, it should be noted, that therapies targeting *Nf1*$^{-/-}$ chondrocytes during fracture healing, such as BGJ-398, would not necessarily be detrimental to fracture healing, but may not be critical to promoting bone union.

Modeling NF1 Skeletal Dysplasia: The Search for the Cell of Origin

This discussion leads to a central question that remains unanswered in the field of NF1 skeletal manifestations: what is the cell of origin of the disease and is it the same for all of the skeletal manifestations? Many genetic mouse models of NF1 skeletal dysplasia have been developed which have greatly helped us understand the function of neurofibromin in various skeletal cell types and have improved our understanding of the etiology/pathogenesis of the NF1 pseudarthrosis and other skeletal manifestations. Each of the current models, however, is not without shortcoming. For example the *Nf1*$_{Col1}^{-/-}$ mouse model showed that mature osteoblasts are not responsible for the majority of the NF1 skeletal pathologies since the bone phenotypes of this mouse deviated significantly from patients (135). However, studies in this mouse model revealed several important functions of neurofibromin in mature osteoblasts and as a result, this was model was still useful in a proof-of-concept study using nanoparticles encapsulating statins to target Ras activity in the fracture callus osteoblasts (137).

Generation of new mouse models that more closely recapitulate fracture healing, skeletal development, and the genetic context as in NF1 patients will, however, help more fully understand the NF1 skeletal etiology/pathogenesis as well as aid in the development of

novel therapies. New mouse models of NF1 skeletal dysplasia that further elucidate and model the cell of origin for each skeletal manifestation and how it contributes to these disease manifestations will significantly progress the field. One of the current limitations of the existing mouse models of NF1 skeletal dysplasia using Cre/*loxP* conditional knockout technologies is that enormous populations of cells and their progeny are targeted for genetic *Nf1* ablation. In NF1 patients however, it is hypothesized that focal bone manifestations arise via the local loss of *NF1* heterozygosity in a small number of cells, presumably arising from a single cell (5-7). Thus, in NF1 patients not all skeletal cells are *NF1*-null, but in the $Nf1_{Col2}^{-/-}$ model, almost all long bone skeletal cells are $Nf1^{-/-}$. This results in mouse skeletal phenotypes that are much stronger and different than in NF1 patients (i.e. dwarfism via targeting growth plate chondrocytes, and *Nf1* deficiency in all long bones), and though this is useful in studying the pathology of NF1 skeletal manifestations because finding a phenotype is relatively simple, it is not always clinically relevant.

$\underline{Nf1_{PrxERT}^{-/-}}$ Mouse Model Development

We have begun some preliminary work to generate a new mouse model of NF1 skeletal dysplasia that we hope will ameliorate many of the shortcomings of the existing mouse models of NF1 skeletal dysplasia. This entails using mice harboring a tamoxifen inducible *Prx*-Cre transgene to target *Nf1* for deletion. The expression of this cre-transgene in adult mice is limited to the periosteum, where it is believed the osteochondroprogenitors responsible for both intramembranous and endochondral bone repair following fracture reside (285). Initial characterization of this transgenic cre line

by cell lineage tracing experiments show that the periosteal cells expressing *Prx* in adult life give rise to both osteoblasts and chondrocytes within the fracture callus (285). We believe given this tracing pattern, that adult *Prx* expressing cells within the periosteum may be the cell of origin of the NF1 pseudarthrosis and other skeletal phenotypes.

Furthermore, to address the hypothesis that a small number of cells within the periosteum loose *NF1* heterozygosity, we are experimenting with injecting the active tamoxifen metabolite, 4-OH tamoxifen in a local fashion, against the periosteum with the goal of recombining only a small number of *Prx* expressing, $Nf1^{f/f}$ cells within the periosteum. We will then fracture these locally recombined mice and assess whether fracture healing delays manifest. These mice can also be bred onto an $Nf1^{+/-}$ global genetic background to assess the role of the $Nf1^{+/-}$ microenvironment in developing NF1 pseudarthrosis. This model could recapitulate what occurs in NF1 pseudarthrosis patients and identify the cell of origin as a periosteal residing, *Prx* expressing $Nf1^{-/-}$ cell.

Other possible applications of this model could include the study of NF1 related scoliosis and tibial bowing. Potential experiments could include local injection of 4-OH tamoxifen against the periosteum during early postnatal development to study whether $Nf1^{-/-}$ periosteal cells expand aggressively (similar to cancer cells) and are capable of inducing bone bowing or cortical porosity leading to bone fragility and spontaneous fracture. Likewise, local injection of 4-OH tamoxifen adjacent to spinal vertebral periosteum during development and subsequent assessment of whether scoliosis develops is another possible use of this model. These studies would require confirmation of axial skeletal

expression of the *Prx* transgene in the periosteum since *Prx* is not expressed the axial skeleton during embryonic development (286).

Identifying an NF1 Skeletal Cell Surface Marker or Secreted Factor

A provocative way of thinking of the NF1 pseudarthrosis, its treatment, and identifying its cell of origin is to think of NF1 focal manifestations as a neoplasm. Initially, cells that have lost *NF1* heterozygosity most likely represents a very small portion of the total populations of $NF1^{+/-}$ cells in bone. These cells, however, can have a profound effect on the bone and contribute to significant skeletal morbidity. Two approaches that have been employed in the pursuit against cancer have been to (i) identify cancer cell derived biomarkers in bodily fluids that are diagnostic, predictive, and informative of disease course as well as to (ii) identify unique cell surface markers on cancer cells that can be exploited either by small molecules or biologics to eliminate these cells.

Approaching the NF1 cell of origin as a cancer cell and thus targeting it by similar approaches may prove fruitful. Existing mouse models of NF1 skeletal dysplasia that target large numbers of cells may be used in combination with novel proteomic or metabolomic approaches with bodily fluids to initially identify secreted factors unique to $Nf1^{-/-}$ skeletal cells and comparison between models may be helpful to identify cell type specific markers. If the $Nf1_{PrxERT}^{-/-}$ model proves useful, this model could also be used to refine such markers and determine whether such biomarkers exist in $Nf1^{-/-}$ periosteal progenitor cells and can be detected when only a small number of $Nf1^{-/-}$ cells are present after local recombination. These searches can also be performed and/or refined using

bodily fluids from NF1 patients with or without obvious skeletal disease. Identification of cell surface markers can theoretically be pursued in a similar manner via proteomic approaches with mouse and human *NF1*-null cells

Conservation of Neurofibromin Function: From Mice to Humans

To date, most studies of the function of neurofibromin in bone have been performed in mice and in cells derived from the mouse. Questions related to the conservation of the function of neurofibromin in mouse bone cells versus human bone cells remain largely unaddressed. This gap in knowledge has primarily arisen due to the difficulty of isolating $NF1^{-/-}$ bone cells from pseudarthrotic sites. It was not until recently where *NF1* loss of heterozygosity was identified in a significant number of pseudarthrotic sites in NF1 patients (6, 7). This discovery was made in large part due to the use of novel sequencing approaches (whole exome sequencing). Similar to efforts in identifying $NF1^{-/-}$ cells in pseudarthrotic sites, efforts to culture and study $NF1^{-/-}$ bone cells have been hindered by isolating $NF1^{-/-}$ pseudarthrosis cells and furthermore by getting the $NF1^{-/-}$ pseudarthrosis cells to thrive in culture for experimentation. Use of iPS technologies are currently being explored by our collaborators to take $NF1^{-/-}$ cells from non-pseudarthrotic sites that may grow better than $NF1^{-/-}$ pseudarthrosis cells (*i.e.* neurofibroma, etc.) as well as using $NF1^{+/-}$ skin cells transformed into $NF1^{-/-}$ by genome editing *Crispr/Cas9* technologies. $NF1^{-/-}$ iPS cells along with autogenous $NF1^{+/-}$ iPS cells (generated from other anatomical sites within the same patient) could then theoretically be grown in osteogenic media to study to function of neurofibromin in human bone cells with identical genetic

backgrounds. These studies will be informative of the function of neurofibromin in human cells and whether knowledge gained from the mouse is conserved across species.

Conclusion

In closing, the research described in this dissertation has shown several critical functions of neurofibromin and cell surface receptors that it regulates in the developing mouse growth plate and in chondrocytes. This research lays the groundwork for numerous preclinical applications and future research directions towards the goal of treating the NF1 skeletal dysplasia and particularly NF1 pseudarthrosis.

REFERENCES

1. Elefteriou, F., Kolanczyk, M., Schindeler, A., Viskochil, D.H., Hock, J.M., Schorry, E.K., Crawford, A.H., Friedman, J.M., Little, D., Peltonen, J., *et al.* (2009) Skeletal abnormalities in neurofibromatosis type 1: Approaches to therapeutic options. *Am. J. Med. Genet.*, **149A**, 2327–2338.

2. Stevenson, D.A., Little, D., Armstrong, L., Crawford, A.H., Eastwood, D., Friedman, J.M., Greggi, T., Gutierrez, G., Hunter-Schaedle, K., Kendler, D.L., *et al.* (2013) Approaches to Treating NF1 Tibial Pseudarthrosis: Consensus From the Children's Tumor Foundation NF1 Bone Abnormalities Consortium. *J. Pediatr. Orthop.*, **33**, 269–275.

3. Khassawna, El,T., Toben, D., Kolanczyk, M., Schmidt-Bleek, K., Koennecke, I., Schell, H., Mundlos, S. and Duda, G.N. (2012) Deterioration of fracture healing in the mouse model of NF1 long bone dysplasia. *Bone*, **51**, 651–660.

4. Wang, W., Nyman, J.S., Ono, K., Stevenson, D.A., Yang, X. and Elefteriou, F. (2011) Mice lacking Nf1 in osteochondroprogenitor cells display skeletal dysplasia similar to patients with neurofibromatosis type I. *Hum. Mol. Gen.*, **20**, 3910–3924.

5. Stevenson, D.A., Zhou, H., Ashrafi, S., Messiaen, L.M., Carey, J.C., D'Astous, J.L., Santora, S.D. and Viskochil, D.H. (2006) Double inactivation of NF1 in tibial pseudarthrosis. *Am. J. Hum. Genet.*, **79**, 143–148.

6. Sant, D.W., Margraf, R.L., Stevenson, D.A., Grossmann, A.H., Viskochil, D.H., Hanson, H., Everitt, M.D., Rios, J.J., Elefteriou, F., Hennessey, T., *et al.* (2015) Evaluation of somatic mutations in tibial pseudarthrosis samples in neurofibromatosis type 1. *J. Med. Genet.*, **0**, 1–6.

7. Paria, N., Cho, T.-J., Choi, I.H., Kamiya, N., Kayembe, K., Mao, R., Margraf, R.L., Obermosser,G., Oxendine,I., Sant,D.W., *et al.* (2014) Neurofibromin deficiency-associated transcriptional dysregulation suggests a novel therapy for tibial pseudoarthrosis in NF1. *J. Bone Miner. Res.*, **29**, 2636–2642.

8. Sakamoto, A., Yoshida, T., Yamamoto, H., Oda, Y., Tsuneyoshi, M. and Iwamoto, Y. (2007) Congenital pseudarthrosis of the tibia: analysis of the histology and the NF1 gene. *J. Orthop. Sci.*, **12**, 361–365.

9. Olsen, B.R., Reginato, A.M. and Wang, W. (2000) Bone development. *Annu. Rev. Cell Dev. Biol.*, **16**, 191–220.

10. Lefebvre, V. and Bhattaram, P. (2010) Vertebrate Skeletogenesis. *Curr Top Dev Biol*, **90**, 291–317.

11. Spranger, J. (2006) Skeletal Dysplasias. In Stevenson,R.E., Hall,J.G. (eds), *Human Malformations and Related Abnormalities*. Oxford University Press, New York, Vol. 160C, pp. 997–1020.

12. Wellik, D.M. (2009) Hox genes and vertebrate axial pattern. *Curr Top Dev Biol*, **88**, 257–278.

13. Hermanns, P. and Lee, B. (2001) Transcriptional dysregulation in skeletal malformation syndromes. *Am. J. Med. Genet.*, **106**, 258–271.

14. Kronenberg, H.M. (2003) Developmental regulation of the growth plate. *Nature*, **423**, 332–336.

15. Karsenty, G., Kronenberg, H.M. and Settembre, C. (2009) Genetic Control of Bone Formation. *Annu. Rev. Cell Dev. Biol.*, **25**, 629–648.

16. Olney, R.C. (2009) Mechanisms of impaired growth: effect of steroids on bone and cartilage. *Horm. Res.*, **72 Suppl 1**, 30–35.

17. Gao, B. and He, L. (2004) Answering a century old riddle: brachydactyly type A1. *Cell Res*, **14**, 179–187.

18. Minina,E., Wenzel,H.M., Kreschel,C., Karp,S., Gaffield,W., McMahon,A.P. and Vortkamp,A. (2001) BMP and Ihh/PTHrP signaling interact to coordinate chondrocyte proliferation and differentiation. *Development*, **128**, 4523–4534.

19. Minina, E., Kreschel, C., Naski, M.C., Ornitz, D.M. and Vortkamp, A. (2002) Interaction of FGF, Ihh/Pthlh, and BMP signaling integrates chondrocyte proliferation and hypertrophic differentiation. *Dev. Cell*, **3**, 439–449.

20. Kobayashi, T. and Kronenberg, H. (2005) Minireview: Transcriptional Regulation in Development of Bone. *Endocrinology*, **146**, 1012–1017.

21. Pogue, R. and Lyons, K. (2006) BMP signaling in the cartilage growth plate. *Curr Top Dev Biol*, **76**, 1–48.

22. Ornitz, D.M. and Marie, P.J. (2002) FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes Dev.*, **16**, 1446–1465.

23. Ornitz, D.M. (2005) FGF signaling in the developing endochondral skeleton. *Cytokine Growth Factor Rev.*, **16**, 205–213.

24. Rousseau, F., Bonaventure, J., Legeai-Mallet, L., Pelet, A., Rozet, J.M., Maroteaux, P., Le Merrer, M. and Munnich, A. (1994) Mutations in the gene encoding fibroblast growth factor receptor-3 in achondroplasia. *Nature*, **371**, 252–254.

25. Shiang, R., Thompson, L.M., Zhu, Y.Z., Church, D.M., Fielder, T.J., Bocian, M., Winokur, S.T. and Wasmuth, J.J. (1994) Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. *Cell*, **78**, 335–342.

26. Bellus, G.A., McIntosh, I., Smith, E.A., Aylsworth, A.S., Kaitila, I., Horton, W.A., Greenhaw, G.A., Hecht, J.T. and Francomano, C.A. (1995) A recurrent mutation in the tyrosine kinase domain of fibroblast growth factor receptor 3 causes hypochondroplasia. *Nat. Genet.*, **10**, 357–359.

27. Naski, M.C., Wang, Q., Xu, J. and Ornitz, D.M. (1996) Graded activation of fibroblast growth factor receptor 3 by mutations causing achondroplasia and thanatophoric dysplasia. *Nat. Genet.*, **13**, 233–237.

28. Webster, M.K. and Donoghue, D.J. (1996) Constitutive activation of fibroblast growth factor receptor 3 by the transmembrane domain point mutation found in achondroplasia. *EMBO J.*, **15**, 520–527.

29. Pagon, R.A., Bird, T.D., Dolan, C.R., Stephens, K., Adam, M.P. and Pauli, R.M. (1998) Achondroplasia. *NCBI Bookshelf*.

30. Iwata, T., Chen, L., Li, C., Ovchinnikov, D.A., Behringer, R.R., Francomano, C.A. and Deng, C.X. (2000) A neonatal lethal mutation in FGFR3 uncouples proliferation and differentiation of growth plate chondrocytes in embryos. *Hum. Mol. Gen.*, **9**, 1603–1613.

31. Kaufman, R.L., Rimoin, D.L., McAlister, W.H. and Kissane, J.M. (1970) Thanatophoric dwarfism. *Am. J. Dis. Child.*, **120**, 53–57.

32. Naski, M.C., Colvin, J.S., Coffin, J.D. and Ornitz, D.M. (1998) Repression of hedgehog signaling and BMP4 expression in growth plate cartilage by fibroblast growth factor receptor 3. *Development*, **125**, 4977–4988.

33. Chen, L., Adar, R., Yang, X., Monsonego, E.O., Li, C., Hauschka, P.V., Yayon, A. and Deng, C.X. (1999) Gly369Cys mutation in mouse FGFR3 causes achondroplasia by affecting both chondrogenesis and osteogenesis. *J. Clin. Invest.*, **104**, 1517–1525.

34. Deng, C., Wynshaw-Boris, A., Zhou, F., Kuo, A. and Leder, P. (1996) Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell*, **84**, 911–921.

35. Colvin, J.S., Bohne, B.A., Harding, G.W., McEwen, D.G. and Ornitz, D.M. (1996) Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nat. Genet.*, **12**, 390–397.

36. Su, N., Xu, X., Li, C., He, Q., Zhao, L., Li, C., Chen, S., Luo, F., Yi, L., Du, X., *et al.* (2010) Generation of Fgfr3 conditional knockout mice. *Int. J. Biol. Sci.*, **6**, 327–332.

37. Peters, K., Ornitz, D., Werner, S. and Williams, L. (1993) Unique expression pattern of the FGF receptor 3 gene during mouse organogenesis. *Dev. Biol.*, **155**, 423–430.

38. Su, W.C., Kitagawa, M., Xue, N., Xie, B., Garofalo, S., Cho, J., Deng, C., Horton, W.A. and Fu, X.Y. (1997) Activation of Stat1 by mutant fibroblast growth-factor receptor in thanatophoric dysplasia type II dwarfism. *Nature*, **386**, 288–292.

39. Sahni, M., Ambrosetti, D.C., Mansukhani, A., Gertner, R., Levy, D. and Basilico, C. (1999) FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway. *Genes Dev.*, **13**, 1361–1366.

40. Li, C., Chen, L., Iwata, T., Kitagawa, M., Fu, X.Y. and Deng, C.X. (1999) A Lys644Glu substitution in fibroblast growth factor receptor 3 (FGFR3) causes dwarfism in mice by activation of STATs and ink4 cell cycle inhibitors. *Hum. Mol. Gen.*, **8**, 35–44.

41. Legeai-Mallet, L., Benoist-Lasselin, C., Delezoide, A.L., Munnich, A. and Bonaventure, J. (1998) Fibroblast growth factor receptor 3 mutations promote apoptosis but do not alter chondrocyte proliferation in thanatophoric dysplasia. *J. Biol. Chem.*, **273**, 13007–13014.

42. Murakami, S., Kan, M., McKeehan, W.L. and de Crombrugghe, B. (2000) Up-regulation of the chondrogenic Sox9 gene by fibroblast growth factors is mediated by the mitogen-activated protein kinase pathway. *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 1113–1118.

43. Chen, L., Li, C., Qiao, W., Xu, X. and Deng, C. (2001) A Ser(365)-->Cys mutation of fibroblast growth factor receptor 3 in mouse downregulates Ihh/PTHrP signals and causes severe achondroplasia. *Hum. Mol. Gen.*, **10**, 457–465.

44. Murakami, S., Balmes, G., McKinney, S., Zhang, Z., Givol, D. and de Crombrugghe, B. (2004) Constitutive activation of MEK1 in chondrocytes causes Stat1-independent achondroplasia-like dwarfism and rescues the Fgfr3-deficient mouse phenotype. *Genes Dev.*, **18**, 290–305.

45. Lai, L.P., DaSilva, K.A. and Mitchell, J. (2005) Regulation of Indian hedgehog mRNA levels in chondrocytic cells by ERK1/2 and p38 mitogen-activated protein kinases. *J. Cell. Physiol.*, **203**, 177–185.

46. Bundschu, K. (2005) Gene Disruption of Spred-2 Causes Dwarfism. *J. Biol. Chem.*, **280**, 28572–28580.

47. Cohen, M.M. (1998) Achondroplasia, hypochondroplasia and thanatophoric dysplasia: clinically related skeletal dysplasias that are also related at the molecular level. *Int. J. Oral Maxillofac. Surg.*, **27**, 451–455.

48. Peters, K.G., Werner ,S., Chen, G. and Williams, L.T. (1992) Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. *Development*, **114**, 233–243.

49. Jacob, A.L., Smith, C., Partanen, J. and Ornitz, D.M. (2006) Fibroblast growth factor receptor 1 signaling in the osteo-chondrogenic cell lineage regulates sequential steps of osteoblast maturation. *Dev. Biol.*, **296**, 315–328.

50. White, K.E., Cabral, J.M., Davis, S.I., Fishburn, T., Evans, W.E., Ichikawa, S., Fields, J., Yu, X., Shaw, N.J., McLellan, N.J., *et al.* (2005) Mutations that cause osteoglophonic dysplasia define novel roles for FGFR1 in bone elongation. *Am. J. Hum. Genet.*, **76**, 361–367.

51. Sklower Brooks, S., Kassner, G., Qazi, Q., Keogh, M.J. and Gorlin, R.J. (1996) Osteoglophonic dysplasia: review and further delineation of the syndrome. *Am. J. Med. Genet.*, **66**, 154–162.

52. Zhou, Y.X., Xu, X., Chen, L., Li, C., Brodie, S.G. and Deng, C.X. (2000) A Pro250Arg substitution in mouse Fgfr1 causes increased expression of Cbfa1 and premature fusion of calvarial sutures. *Hum. Mol. Gen.*, **9**, 2001–2008.

53. Kouhara, H., Hadari, Y.R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I. and Schlessinger, J. (1997) A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell*, **89**, 693–702.

54. Hadari, Y.R., Gotoh, N., Kouhara, H., Lax, I. and Schlessinger, J. (2001) Critical role for the docking-protein FRS2 alpha in FGF receptor-mediated signal transduction pathways. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 8578–8583.

55. Hoch, R.V. and Soriano, P. (2006) Context-specific requirements for Fgfr1 signaling through Frs2 and Frs3 during mouse development. *Development*, **133**, 663–673.

56. Beighton, P. (1989) Osteoglophonic dysplasia. *J. Med. Genet.*, **26**, 572–576.

57. Beighton, P., Cremin, B. and Kozlowski, K. (1980) Osteoglophonic dwarfism. *Pediatr. Radiol.*, **10**, 46–50.

58. Delezoide, A.L., Benoist-Lasselin, C., Legeai-Mallet, L., Le Merrer, M., Munnich, A., Vekemans, M. and Bonaventure, J. (1998) Spatio-temporal expression of FGFR 1, 2 and 3 genes during human embryo-fetal ossification. *Mech. Dev.*, **77**, 19–30.

59. Wang, Y., Xiao, R., Yang ,F., Karim, B.O., Iacovelli, A.J., Cai, J., Lerner, C.P., Richtsmeier, J.T., Leszl, J.M., Hill, C.A., *et al.* (2005) Abnormalities in cartilage and bone development in the Apert syndrome FGFR2(+/S252W) mouse. *Development*, **132**, 3537–3548.

60. Eswarakumar, V.P., Monsonego-Ornan, E., Pines, M., Antonopoulou, I., Morriss-Kay, G.M. and Lonai, P. (2002) The IIIc alternative of Fgfr2 is a positive regulator of bone formation. *Development*, **129**, 3783–3793.

61. Chen, L., Li, D., Li, C., Engel, A. and Deng, C.X. (2003) A Ser250Trp substitution in mouse fibroblast growth factor receptor 2 (Fgfr2) results in craniosynostosis. *Bone*, **33**, 169–178.

62. Yu, K., Xu, J., Liu, Z., Sosic, D., Shao, J., Olson, E.N., Towler, D.A. and Ornitz, D.M. (2003) Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth. *Development*, **130**, 3063–3074.

63. Minina, E., Schneider, S., Rosowski, M., Lauster, R. and Vortkamp, A. (2005) Expression of Fgf and Tgfbeta signaling related genes during embryonic endochondral ossification. *Gene Expr. Patterns*, **6**, 102–109.

64. Weinstein, M., Xu, X., Ohyama, K. and Deng, C.X. (1998) FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. *Development*, **125**, 3615–3623.

65. Gerstenfeld, L.C., Cullinane, D.M., Barnes, G.L., Graves, D.T. and Einhorn, T.A. (2003) Fracture healing as a post-natal developmental process: Molecular, spatial, and temporal aspects of its regulation. *J. Cell. Biochem.*, **88**, 873–884.

66. Einhorn, T.A. (1998) The cell and molecular biology of fracture healing. *Clin. Orthop. Relat. Res.*, **355 Suppl**, S7–S21.

67. Einhorn, T.A. (2005) The science of fracture healing. *J. Orthop. Trauma*, **106**, 258–271.

68. Yuasa, M., Mignemi, N.A., Barnett, J.V., Cates, J.M.M., Nyman, J.S., Okawa, A., Yoshii, T., Schwartz, H.S., Stutz, C.M. and Schoenecker, J.G. (2014) The temporal and spatial development of vascularity in a healing displaced fracture. *Bone*, **67**, 208–221.

69. Rundle, C.H., Miyakoshi, N., Ramirez, E., Wergedal, J.E., Lau, K.H.W. and Baylink, D.J. (2002) Expression of the fibroblast growth factor receptor genes in fracture repair. *Clin. Orthop. Relat. Res.*, **403**, 253–263.

70. Schmid, G.J., Kobayashi, C., Sandell, L.J. and Ornitz, D.M. (2009) Fibroblast growth factor expression during skeletal fracture healing in mice. *Dev. Dyn.*, **238**, 766–774.

71. Nakamura, T., Hara, Y., Tagawa, M., Tamura, M., Yuge, T., Fukuda, H. and Nigi, H. (1998) Recombinant human basic fibroblast growth factor accelerates fracture healing by enhancing callus remodeling in experimental dog tibial fracture. *J. Bone Miner. Res.*, **13**, 942–949.

72. Jingushi, S., Heydemann, A., Kana, S.K., Macey, L.R. and Bolander, M.E. (1990) Acidic fibroblast growth factor (aFGF) injection stimulates cartilage enlargement and inhibits cartilage gene expression in rat fracture healing. *J. Orthop. Res.*, **8**, 364–371.

73. Su, N., Yang, J., Xie, Y., Du, X., Lu, X., Yin, Z., Yin, L., Qi, H., Zhao, L., Feng, J., *et al.* (2008) Gain-of-function mutation of FGFR3 results in impaired fracture healing due to inhibition of chondrocyte differentiation. *Biochem. Biophys. Res. Commun.*, **376**, 454–459.

74. Nakajima, A., Nakajima, F., Shimizu, S., Ogasawara, A., Wanaka, A., Moriya, H., Einhorn, T.A. and Yamazaki, M. (2001) Spatial and temporal gene expression for fibroblast growth factor type I receptor (FGFR1) during fracture healing in the rat. *Bone*, **29**, 458–466.

75. Barker, D., Wright, E., Nguyen, K., Cannon, L., Fain, P., Goldgar, D., Bishop, D.T., Carey, J., Baty, B. and Kivlin, J. (1987) Gene for von Recklinghausen neurofibromatosis is in the pericentromeric region of chromosome 17. *Science*, **236**, 1100–1102.

76. Seizinger, B.R., Rouleau, G.A., Ozelius, L.J., Lane, A.H., Faryniarz, A.G., Chao, M.V., Huson, S., Korf, B.R., Parry, D.M. and Pericak-Vance, M.A. (1987) Genetic linkage of von Recklinghausen neurofibromatosis to the nerve growth factor receptor gene. *Cell*, **49**, 589–594.

77. Goldgar, D.E., Green, P., Parry, D.M. and Mulvihill, J.J. (1989) Multipoint linkage analysis in neurofibromatosis type I: an international collaboration. *Am. J. Hum. Genet.*, **44**, 6–12.

78. O'Connell, P., Leach, R.J., Ledbetter, D.H., Cawthon, R., Culver, M., Eldridge, J.R., Frej, A.K., Holm, T.R., Wolff, E. and Thayer, M.J. (1989) Fine structure DNA mapping studies of the chromosomal region harboring the genetic defect in neurofibromatosis type I. *Am. J. Hum. Genet.*, **44**, 51–57.

79. Huson, S.M., Compston, D.A., Clark, P. and Harper, P.S. (1989) A genetic study of von Recklinghausen neurofibromatosis in south east Wales. I. Prevalence, fitness, mutation rate, and effect of parental transmission on severity. *J. Med. Genet.*, **26**, 704–711.

80. Riccardi, V.M. (1981) Von Recklinghausen neurofibromatosis. *N. Engl. J. Med.*, **305**, 1617–1627.

81. Riccardi, V.M. and Lewis, R.A. (1988) Penetrance of von Recklinghausen neurofibromatosis: a distinction between predecessors and descendants. *Am. J. Hum. Genet.*, **42**, 284–289.

82. Shannon, K.M., O'Connell, P., Martin, G.A., Paderanga, D., Olson, K., Dinndorf, P. and McCormick, F. (1994) Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders. *N. Engl. J. Med.*, **330**, 597–601.

83. Sawada, S., Florell, S., Purandare, S.M., Ota, M., Stephens, K. and Viskochil, D. (1996) Identification of NF1 mutations in both alleles of a dermal neurofibroma. *Nat. Genet.*, **14**, 110–112.

84. Brannan, C.I., Perkins, A.S., Vogel, K.S., Ratner, N., Nordlund, M.L., Reid, S.W., Buchberg, A.M., Jenkins, N.A., Parada, L.F. and Copeland, N.G. (1994) Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. *Genes Dev.*, **8**, 1019–1029.

85. Schmidt, M.A., Michels, V.V., Dewald, G.W., Opitz, J.M. and Reynolds, J.F. (1987) Cases of neurofibromatosis with rearrangements of chromosome 17 involving band 17q11.2. *Am. J. Med. Genet.*, **28**, 771–777.

86. Ledbetter, D.H., Rich, D.C., O'Connell, P., Leppert, M. and Carey, J.C. (1989) Precise localization of NF1 to 17q11.2 by balanced translocation. *Am. J. Hum. Genet.*, **44**, 20–24.

87. Menon, A.G., Ledbetter, D.H., Rich, D.C., Seizinger, B.R., Rouleau, G.A., Michels, V.F., Schmidt, M.A., Dewald, G., DallaTorre, C.M. and Haines, J.L. (1989) Characterization of a translocation within the von Recklinghausen neurofibromatosis region of chromosome 17. *Genomics*, **5**, 245–249.

88. Cawthon, R., Weiss, R., Xu, G.F., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R. and O'Connell, P. (1990) A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. *Cell*, **62**, 609–201.

89. Viskochil, D., Xu, G., Cawthon, R., Stevens, J., Wolff, R.K., Culver, M. and Carey, J.C. (1990) Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. *Cell*, **62**, 187–192.

90. Wallace, M.R., Marchuk, D.A., Andersen, L.B., Letcher, R., Odeh, F.M., Saulino, A.M., Fountain, J.W., Brereton, A., Nicholson, J., Mitchell, A.L., *et al.* (1990) Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 Patients. *Science*, **249**, 181–186.

91. Xu, G.F., O'Connell, P., Viskochil, D., Cawthon, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R. and White, R. (1990) The neurofibromatosis type 1 gene encodes a protein related to GAP. *Cell*, **62**, 599–608.

92. Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M. and Collins, F.S. (1990) The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. *Cell*, **63**, 851–859.

93. Martin, G.A., Viskochil, D., Bollag, G., McCabe, P.C., Crosier, W.J., Haubruck, H., Conroy, L., Clark, R., O'Connell, P. and Cawthon, R. (1990) The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. *Cell*, **63**, 843–849.

94. Basu, T.N., Gutmann, D.H., Fletcher, J.A., Glover, T.W., Collins, F.S. and Downward, J. (1992) Aberrant regulation of ras proteins in malignant tumour cells from type 1 neurofibromatosis patients. *Nature*, **356**, 713–715.

95. Daston, M.M., Scrable, H., Nordlund, M., Sturbaum, A.K. and Nissen, L.M. (1992) The protein product of the neurofibromatosis type 1 gene is expressed at highest abundance in neurons, Schwann cells, and oligodendrocytes. *Neuron*, **8**, 415–428.

96. Gutmann, D.H., Wood, D.L. and Collins, F.S. (1991) Identification of the neurofibromatosis type 1 gene product. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 9658–9662.

97. DeClue, J.E., Cohen, B.D. and Lowy, D.R. (1991) Identification and characterization of the neurofibromatosis type 1 protein product. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 9914–9918.

98. Huynh, D.P., Nechiporuk, T. and Pulst, S.M. (1994) Differential expression and tissue distribution of type I and type II neurofibromins during mouse fetal development. *Dev. Biol.*, **161**, 538–551.

99. Kuorilehto, T., Nissinen, M., Koivunen, J., Benson, M.D. and Peltonen, J. (2004) NF1 Tumor Suppressor Protein and mRNA in Skeletal Tissues of Developing and Adult Normal Mouse and NF1-Deficient Embryos. *J. Bone Miner. Res.*, **19**, 983–989.

100. Gregory, P.E., Gutmann, D.H., Mitchell, A., Park, S., Boguski, M., Jacks, T., Wood, D.L., Jove, R. and Collins, F.S. (1993) Neurofibromatosis type 1 gene product (neurofibromin) associates with microtubules. *Somat. Cell Mol. Genet.*, **19**, 265–274.

101. Xu, H. and Gutmann, D.H. (1997) Mutations in the GAP-related domain impair the ability of neurofibromin to associate with microtubules. *Brain Res.*, **759**, 149–152.

102. The, I., Hannigan, G.E., Cowley, G.S., Reginald, S., Zhong, Y., Gusella, J.F., Hariharan, I.K. and Bernards, A. (1997) Rescue of a Drosophila NF1 mutant phenotype by protein kinase A. *Science*, **276**, 791–794.

103. Tong, J., Hannan, F., Zhu, Y., Bernards, A. and Zhong, Y. (2002) Neurofibromin regulates G protein-stimulated adenylyl cyclase activity. *Nat. Neurosci.*, **5**, 95–96.

104. Dasgupta, B., Dugan, L.L. and Gutmann, D.H. (2003) The neurofibromatosis 1 gene product neurofibromin regulates pituitary adenylate cyclase-activating polypeptide-mediated signaling in astrocytes. *J. Neurosci.*, **23**, 8949–8954.

105. Guo, H.F., The, I., Hannan, F., Bernards, A. and Zhong, Y. (1997) Requirement of Drosophila NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides. *Science*, **276**, 795–798.

106. Ismat, F.A., Xu, J., Lu, M.M. and Epstein,J.A. (2006) The neurofibromin GAP-related domain rescues endothelial but not neural crest development in Nf1 mice. *J. Clin. Invest.*, **116**, 2378–2384.

107. Crawford, A.H. and Schorry, E.K. (1999) Neurofibromatosis in children: the role of the orthopaedist. *J. Am. Acad. Orthop. Surg.*, **7**, 217–230.

108. Crawford, A.H. and Bagamery, N. (1986) Osseous manifestations of neurofibromatosis in childhood. *J. Pediatr. Orthop.*, **6**, 72–88.

109. Kuorilehto, T., Pöyhönen, M., Bloigu, R., Heikkinen, J., Väänänen, K. and Peltonen, J. (2004) Decreased bone mineral density and content in neurofibromatosis type 1: Lowest local values are located in the load-carrying parts of the body. *Osteoporos. Int.*, **16**, 928–936.

110. Stevenson, D.A., Moyer-Mileur, L.J., Murray, M., Slater, H., Sheng, X., Carey, J.C., Dube, B. and Viskochil, D.H. (2007) Bone Mineral Density in Children and Adolescents with Neurofibromatosis Type 1. *J. Pediatr.*, **150**, 83–88.

111. Vitale, M.G., Guha, A. and Skaggs, D.L. (2002) Orthopaedic Manifestations of Neurofibromatosis in Children: An Update. *Clin. Orthop. Relat. Res.*, **401**, 107–118.

112. Brunetti-Pierri, N., Doty, S.B., Hicks, J., Phan, K., Mendoza-Londono, R., Blazo, M., Tran, A., Carter, S., Lewis, R.A., Plon, S.E., *et al.* (2008) Generalized metabolic bone disease in Neurofibromatosis type I. *Mol. Genet. Metab.*, **94**, 105–111.

113. Tucker, T., Schnabel, C., Hartmann, M., Friedrich, R.E., Frieling, I., Kruse, H.-P., Mautner, V.F. and Friedman, J.M. (2009) Bone health and fracture rate in individuals with neurofibromatosis 1 (NF1). *J. Med. Genet.*, **46**, 259–265.

114. Coleman, S.S. and Coleman, D.A. (1994) Congenital pseudarthrosis of the tibia: treatment by transfer of the ipsilateral fibula with vascular pedicle. *J. Pediatr. Orthop.*, **14**, 156–160.

115. Stevenson, D.A., Birch, P.H., Friedman, J.M., Viskochil, D.H., Balestrazzi, P., Boni, S., Buske, A., Korf, B.R., Niimura, M., Pivnick, E.K., *et al.* (1999) Descriptive analysis of tibial pseudarthrosis in patients with neurofibromatosis 1. *Am. J. Med. Genet.*, **84**, 413–419.

116. Wientroub, S. and Grill, F. (2000) Congenital pseudarthrosis of the tibia: Part 1. European Pediatric Orthopaedic Society multicenter study of congenital pseudoarthrosis. *J. Pediatr. Orthop. B*, **9**, 1–2.

117. Le, L.Q. and Parada, L.F. (2007) Tumor microenvironment and neurofibromatosis type I: connecting the GAPs. *Oncogene*, **26**, 4609–4616.

118. Seitz, S., Schnabel, C., Busse, B., Schmidt, H.U., Beil, F.T., Friedrich, R.E., Schinke, T., Mautner, V.F. and Amling, M. (2009) High bone turnover and accumulation of osteoid in patients with neurofibromatosis 1. *Osteoporos. Int.*, **21**, 119–127.

119. Kuorilehto, T., Ekholm, E., Nissinen, M., Hietaniemi, K., Hiltunen, A., Paavolainen, P., Penttinen, R. and Peltonen, J. (2006) NF1 Gene Expression in Mouse Fracture Healing and in Experimental Rat Pseudarthrosis. *J. Histochem. Cytochem.*, **54**, 363–370.

120. Elefteriou, F. and Yang, X. (2011) Genetic mouse models for bone studies—Strengths and limitations. *Bone*, **49**, 1242–1254.

121. Jacks, T., Shih, T.S., Schmitt, E.M., Bronson, R.T., Bernards, A. and Weinberg, R.A. (1994) Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. *Nat. Genet.*, **7**, 353–361.

122. Costa, R.M., Yang, T., Huynh, D.P., Pulst, S.M., Viskochil, D.H., Silva, A.J. and Brannan, C.I. (2001) Learning deficits, but normal development and tumor predisposition, in mice lacking exon 23a of Nf1. *Nat. Genet.*, **27**, 399–405.

123. Yu, X., Chen, S., Potter, O.L., Murthy, S.M., Li, J., Pulcini, J.M., Ohashi, N., Winata, T., Everett, E.T., Ingram, D., *et al.* (2005) Neurofibromin and its inactivation of Ras are prerequisites for osteoblast functioning. *Bone*, **36**, 793–802.

124. Yang, F.C., Chen, S., Robling, A.G., Yu, X., Nebesio, T.D., Yan, J., Morgan, T., Li, X., Yuan, J., Hock, J., *et al.* (2006) Hyperactivation of p21ras and PI3K cooperate to alter murine and human neurofibromatosis type 1-haploinsufficient osteoclast functions. *J. Clin. Invest.*, **116**, 2880–2891.

125. Wu, X. (2006) Neurofibromin plays a critical role in modulating osteoblast differentiation of mesenchymal stem/progenitor cells. *Hum. Mol. Gen.*, **15**, 2837–2845.

126. Yan, J., Chen, S., Zhang, Y., Li, X., Li, Y., Wu, X., Yuan, J., Robling, A.G., Karpur, R., Chan, R.J., *et al.* (2007) Rac1 mediates the osteoclast gains-in-function induced by haploinsufficiency of Nf1. *Hum. Mol. Gen.*, **17**, 936–948.

127. Schindeler, A., Morse, A., Harry, L., Godfrey, C., Mikulec, K., McDonald, M., Gasser, J.A. and Little, D.G. (2008) Models of tibial fracture healing in normal and Nf1-deficient mice. *J. Orthop. Res.*, **26**, 1053–1060.

128. Zhu, Y., Romero, M.I., Ghosh, P., Ye, Z., Charnay, P., Rushing, E.J., Marth, J.D. and Parada, L.F. (2001) Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain. *Genes Dev.*, **15**, 859–876.

129. Wu, X., Chen, S., He, Y., Rhodes, S.D., Mohammad, K.S., Li, X., Yang, X., Jiang, L., Nalepa, G., Snider, P., *et al.* (2011) The Haploinsufficient Hematopoietic Microenvironment Is Critical to the Pathological Fracture Repair in Murine Models of Neurofibromatosis Type 1. *PLoS ONE*, **6**, e24917.

130. Yang, F.C., Ingram, D.A., Chen, S., Zhu, Y., Yuan, J., Li, ., Yang, X., Knowle S., Horn, W., Li, Y., *et al.* (2008) Nf1-dependent tumors require a microenvironment containing Nf1+/- - and c-kit-dependent bone marrow. *Cell*, **135**, 437–448.

131. Le, L.Q., Shipman, T., Burns, D.K. and Parada, L.F. (2009) Cell of Origin and Microenvironment Contribution for NF1-Associated Dermal Neurofibromas. *Stem Cell*, **4**, 453–463.

132. Daginakatte, G.C. and Gutmann, D.H. (2007) Neurofibromatosis-1 (Nf1) heterozygous brain microglia elaborate paracrine factors that promote Nf1-deficient astrocyte and glioma growth. *Hum. Mol. Gen.*, **16**, 1098–1112.

133. Kolanczyk, M., Kossler, N., Kuhnisch, J., Lavitas, L., Stricker, S., Wilkening, U., Manjubala, I., Fratzl, P., Sporle, R., Herrmann, B.G., *et al.* (2007) Multiple roles for neurofibromin in skeletal development and growth. *Hum. Mol. Gen.*, **16**, 874–886.

134. Ndong, J.D.L.C., Makowski, A.J., Uppuganti, S., Vignaux, G., Ono, K., Perrien, D.S., Joubert, S., Baglio, S.R., Granchi, D., Stevenson, D.A., *et al.* (2014) Asfotase-α improves bone growth, mineralization and strength in mouse models of neurofibromatosis type-1. *Nat. Med.*, **20**, 904–910.

135. Elefteriou, F., Benson, M.D., Sowa, H., Starbuck, M., Liu, X., Ron, D., Parada, L.F. and Karsenty, G. (2006) ATF4 mediation of NF1 functions in osteoblast reveals a nutritional basis for congenital skeletal dysplasiae. *Cell Metabolism*, **4**, 441–451.

136. Dacquin, R., Starbuck, M., Schinke, T. and Karsenty, G. (2002) Mouse alpha1(I)-collagen promoter is the best known promoter to drive efficient Cre recombinase expression in osteoblast. *Dev. Dyn.*, **224**, 245–251.

137. Wang, W., Nyman, J.S., Moss, H.E., Gutierrez, G., Mundy, G.R., Yang, X. and Elefteriou, F. (2010) Local low-dose lovastatin delivery improves the bone-healing defect caused by Nf1 loss of function in osteoblasts. *J. Bone Miner. Res.*, **25**, 1658–1667.

138. Zhang, W., Rhodes, S.D., Zhao, L., He, Y., Zhang, Y., Shen, Y., Yang, D., Wu, X., Li, X., Yang, X., *et al.* (2011) Primary osteopathy of vertebrae in a neurofibromatosis type 1 murine model. *Bone*, **48**, 1378–1387.

139. Rhodes, S.D., Zhang, W., Yang, D., Yang, H., Chen, S., Wu, X., Li, X., Yang, X., Mohammad, K.S., Guise, T.A., *et al.* (2015) Dystrophic Spinal Deformities in a Neurofibromatosis Type 1 Murine Model. *PLoS ONE*, **10**, e0119093–19.

140. Kolanczyk, M., Kühnisch, J., Kossler, N., Osswald, M., Stumpp, S., Thurisch, B., Kornak, U. and Mundlos, S. (2008) Modelling neurofibromatosis type 1 tibial dysplasia and its treatment with lovastatin. *BMC Med.*, **6**, 21–11.

141. Kossler, N., Stricker, S., Rodelsperger, C., Robinson, P.N., Kim, J., Dietrich, C., Osswald, M., Kuhnisch, J., Stevenson, D.A., Braun, T., *et al.* (2011) Neurofibromin (Nf1) is required for skeletal muscle development. *Hum. Mol. Gen.*, **20**, 2697–2709.

142. Sullivan, K., El-Hoss, J., Quinlan, K.G.R., Deo, N., Garton, F., Seto, J.T.C., Gdalevitch, M., Turner, N., Cooney, G.J., Kolanczyk, M., *et al.* (2014) NF1 is a critical regulator of muscle development and metabolism. *Hum. Mol. Gen.*, **23**, 1250–1259.

143. Ovchinnikov, D.A., Deng, J.M., Ogunrinu, G. and Behringer, R.R. (2000) Col2a1-directed expression of Cre recombinase in differentiating chondrocytes in transgenic mice. *genesis*, **26**, 145–146.

144. Ndong, J.D.L.C., Stevens, D.M., Vignaux, G., Uppuganti, S., Perrien, D.S., Yang, X., Nyman, J.S., Harth, E. and Elefteriou, F. (2015) Combined MEK inhibition and BMP2 treatment promotes osteoblast differentiation and bone healing in Nf1Osx -/- mice. *J. Bone Miner. Res.*, **30**, 55–63.

145. Rodda, S.J. and McMahon, A.P. (2006) Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development*, **133**, 3231–3244.

146. Ndong, J.D.L.C., Stevens, D.M., Vignaux, G., Uppuganti, S., Perrien, D.S., Yang, X., Nyman, J.S., Harth, E. and Elefteriou, F. (2014) Combined MEK Inhibition and BMP2 Treatment Promotes Osteoblast Differentiation and Bone Healing in Nf1Osx−/−Mice. *J. Bone Miner. Res.*, **30**, 55–63.

147. Chen, J., Shi, Y., Regan, J., Karuppaiah, K., Ornitz, D.M. and Long, F. (2014) Osx-Cre Targets Multiple Cell Types besides Osteoblast Lineage in Postnatal Mice. *PLoS ONE*, **9**, e85161.

148. Alanne, M.H., Siljamäki, E., Peltonen, S., Väänänen, K., Windle, J.J., Parada, L.F., Määttä, J.A. and Peltonen, J. (2012) Phenotypic characterization of transgenic mice harboring Nf1+/- or Nf1-/- osteoclasts in otherwise Nf1+/+ background. *J. Cell. Biochem.*, **113**, 2136–2146.

149. Crawford, A.H. and Schorry, E.K. (2006) Neurofibromatosis update. *J. Pediatr. Orthop.*, **26**, 413–423.

150. Bara, T., Sibiński, M. and Synder, M. (2007) Own clinical experience with functional bracing for treatment of pseudarthrosis and delayed union of the tibia. *Ortop. Traumatol. Rehabil.*, **9**, 259–263.

151. Ofluoglu, O., Davidson, R.S. and Dormans, J.P. (2008) Prophylactic bypass grafting and long-term bracing in the management of anterolateral bowing of the tibia and neurofibromatosis-1. *J. Bone Joint Surg. Am.*, **90**, 2126–2134.

152. Dobbs, M.B., Rich, M.M., Gordon, J.E., Szymanski, D.A. and Schoenecker,P.L. (2005) Use of an intramedullary rod for the treatment of congenital pseudarthrosis of the tibia. Surgical technique. *J. Bone Joint Surg. Am.*, **87 Suppl 1**, 33–40.

153. Richards, B.S., Oetgen, M.E. and Johnston, C.E. (2010) The use of rhBMP-2 for the treatment of congenital pseudarthrosis of the tibia: a case series. *J. Bone Joint Surg. Am.*, **92**, 177–185.

154. Tan, J.S., Roach, J.W. and Wang, A.A. (2011) Transfer of ipsilateral fibula on vascular pedicle for treatment of congenital pseudarthrosis of the tibia. *J. Pediatr. Orthop.*, **31**, 72–78.

155. Morrissy, R.T., Riseborough, E.J. and Hall, J.E. (1981) Congenital pseudarthrosis of the tibia. *J. Bone Joint Surg. Br.*, **63-B**, 367–375.

156. Johnston, C.E. (2002) Congenital pseudarthrosis of the tibia: results of technical variations in the charnley-williams procedure. *J. Bone Joint Surg. Am.*, **84-A**, 1799–1810.

157. Khan, T. and Joseph, B. (2013) Controversies in the management of congenital pseudarthrosis of the tibia and fibula. *Bone Joint J.*, **95-B**, 1027–1034.

158. Schindeler, A., Birke, O., Yu, N.Y.C., Morse, A., Ruys, A., Baldock, P.A. and Little,D.G. (2011) Distal tibial fracture repair in a neurofibromatosis type 1-deficient mouse treated with recombinant bone morphogenetic protein and a bisphosphonate. *J. Bone Joint Surg. Br.*, **93**, 1134–1139.

159. Lee, F.Y.I., Sinicropi, S.M., Lee, F.S., Vitale, M.G., Roye, D.P. and Choi, I.H. (2006) Treatment of congenital pseudarthrosis of the tibia with recombinant human bone morphogenetic protein-7 (rhBMP-7). A report of five cases. *J. Bone Joint Surg. Am.*, **88**, 627–633.

160. Van Beek, E.R., Cohen ,L.H., Leroy, I.M., Ebetino, F.H., Löwik, C.W.G.M. and Papapoulos, S.E. (2003) Differentiating the mechanisms of antiresorptive action of nitrogen containing bisphosphonates. *Bone*, **33**, 805–811.

161. Das, S.P., Ganesh, S., Pradhan, S., Singh, D. and Mohanty, R.N. (2014) Effectiveness of recombinant human bone morphogenetic protein-7 in the management of congenital pseudoarthrosis of the tibia: a randomised controlled trial. *Int. Orthop.*, **38**, 1987–1992.

162. Birke, O., Schindeler, A., Ramachandran, M., Cowell, C.T., Munns, C.F., Bellemore, M. and Little, D.G. (2010) Preliminary experience with the combined use of recombinant bone morphogenetic protein and bisphosphonates in the treatment of congenital pseudarthrosis of the tibia. *J. Child. Orthop.*, **4**, 507–517.

163. Grill, F., Bollini, G., Dungl, P., Fixsen, J., Hefti, F., Ippolito, E., Romanus, B., Tudisco, C. and Wientroub, S. (2000) Treatment approaches for congenital pseudarthrosis of tibia: results of the EPOS multicenter study. European Paediatric Orthopaedic Society (EPOS). *J. Pediatr. Orthop. B*, **9**, 75–89.

164. Choi, I.H., Lee, S.J., Moon, H.J., Cho, T.J., Yoo, W.J., Chung, C.Y. and Park, M.S. (2011) '4-in-1 osteosynthesis' for atrophic-type congenital pseudarthrosis of the tibia. *J. Pediatr. Orthop.*, **31**, 697–704.

165. Cho, T.J., Choi, I.H., Lee, S.M., Chung, C.Y., Yoo, W.J., Lee, D.Y. and Lee, J.W. (2008) Refracture after Ilizarov osteosynthesis in atrophic-type congenital pseudarthrosis of the tibia. *J. Bone Joint Surg. Br.*, **90-B**, 488–493.

166. Pataki, A., Muller, K., Green, J.R., Ma, Y.F., Li, Q.N. and Jee, W.S. (1997) Effects of short-term treatment with the bisphosphonates zoledronate and pamidronate on rat bone: a comparative histomorphometric study on the cancellous bone formed before, during, and after treatment. *Anat. Rec.*, **249**, 458–468.

167. Erdogan, M., Bereket, C., Ozkan, N., Alici, O., Sener, I., Desteli, E.E. and Ilkaya, F. (2014) The effect of zoledronic acid on growth plates and high turnover bones. *Bratisl Lek Listy*, **115**, 131–135.

168. Zhu, E.D., Louis, L., Brooks, D.J., Bouxsein, M.L. and Demay, M.B. (2014) Effect of bisphosphonates on the rapidly growing male murine skeleton. *Endocrinology*, **155**, 1188–1196.

169. Oetgen, M.E. and Richards, B.S. (2010) Complications associated with the use of bone morphogenetic protein in pediatric patients. *J. Pediatr. Orthop.*, **30**, 192–198.

170. Steib, J.P., Jean-Paul, S., Bouchaïb, J., Julia, B., Walter, A., Axel, W., Schuller, S., Sébastien, S., Charles, Y.P. and Philippe, C. (2010) Could an osteoinductor result in degeneration of a neurofibroma in NF1? *Eur. Spine J.*, **19 Suppl 2**, S220–5.

171. Millán, J.L., Lemire, I., McKee, M.D., Crine, P. and Whyte, M.P. (2008) Enzyme replacement therapy for murine hypophosphatasia. *J. Bone Miner. Res.*, **23**, 777–787.

172. Park, J.S., Chu, J.S., Tsou, A.D., Diop, R., Tang, Z., Wang, A. and Li, S. (2011) The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-β. *Biomaterials*, **32**, 3921–3930.

173. Hempel, U., Preissler, C., Vogel, S., Möller, S., Hintze, V., Becher, J., Schnabelrauch, M., Rauner, M., Hofbauer, L.C. and Dieter, P. (2014) Artificial extracellular matrices with oversulfated glycosaminoglycan derivatives promote the differentiation of osteoblast-precursor cells and premature osteoblasts. *Biomed Res. Int.*, **2014**, 938368–10.

174. Shih, Y.R.V., Tseng, K.F., Lai, H.Y., Lin, C.H. and Lee, O.K. (2011) Matrix stiffness regulation of integrin-mediated mechanotransduction during osteogenic differentiation of human mesenchymal stem cells. *J. Bone Miner. Res.*, **26**, 730–738.

175. Whyte, M.P., Greenberg, C.R., Salman, N.J., Bober, M.B., McAlister, W.H., Wenkert, D., Van Sickle, B.J., Simmons, J.H., Edgar, T.S., Bauer, M.L., *et al.* (2012) Enzyme-replacement therapy in life-threatening hypophosphatasia. *N. Engl. J. Med.*, **366**, 904–913.

176. Massagué, J. (2012) TGFβ signalling in context. *Nat. Rev. Mol. Cell Biol.*, **13**, 616–630.

177. Tang, Y., Wu, X., Lei, W., Pang, L., Wan, C., Shi, Z., Zhao, L., Nagy, T.R., Peng, X., Hu, J., *et al.* (2009) TGF-beta1-induced migration of bone mesenchymal stem cells couples bone resorption with formation. *Nat. Med.*, **15**, 757–765.

178. Koseki, T., Gao, Y., Okahashi, N., Murase, Y., Tsujisawa, T., Sato, T., Yamato, K. and Nishihara, T. (2002) Role of TGF-beta family in osteoclastogenesis induced by RANKL. *Cell. Signal.*, **14**, 31–36.

179. Fuller, K., Lean, J.M., Bayley, K.E., Wani, M.R. and Chambers, T.J. (2000) A role for TGFbeta(1) in osteoclast differentiation and survival. *J. Cell Sci.*, **113**, 2445–2453.

180. Itonaga, I., Sabokbar, A., Sun, S.G., Kudo, O., Danks, L., Ferguson, D., Fujikawa, Y. and Athanasou, N.A. (2004) Transforming growth factor-β induces osteoclast formation in the absence of RANKL. *Bone*, **34**, 57–64.

181. Quinn, J.M., Itoh, K., Udagawa, N., Hausler, K., Yasuda, H., Shima, N., Mizuno, A., Higashio,K., Takahashi,N., Suda,T., *et al.* (2001) Transforming growth factor beta affects osteoclast differentiation via direct and indirect actions. *J. Bone Miner. Res.*, **16**, 1787–1794.

182. Rhodes, S.D., Wu, X., He, Y., Chen, S., Yang, H., Staser, K.W., Wang, J., Zhang, P., Jiang, C., Yokota, H., *et al.* (2013) Hyperactive Transforming Growth Factor-β1 Signaling Potentiates Skeletal Defects in a Neurofibromatosis Type 1 Mouse Model. *J. Bone Miner. Res.*, **28**, 2476–2489.

183. Jakowlew, S.B. (2006) Transforming growth factor-beta in cancer and metastasis. *Cancer Metastasis Rev.*, **25**, 435–457.

184. Li, W., Cui, Y., Kushner, S.A., Brown, R.A.M., Jentsch, J.D., Frankland, P.W., Cannon, T.D. and Silva, A.J. (2005) The HMG-CoA reductase inhibitor lovastatin reverses the learning and attention deficits in a mouse model of neurofibromatosis type 1. *Curr. Biol.*, **15**, 1961–1967.

185. Ohnaka, K., Shimoda, S., Nawata, H., Shimokawa, H, Kaibuchi, K., Iwamoto, Y. and Takayanagi, R. (2001) Pitavastatin enhanced BMP-2 and osteocalcin expression by inhibition of Rho-associated kinase in human osteoblasts. *Biochem. Biophys. Res. Commun.*, **287**, 337–342.

186. Skoglund, B., Forslund, C. and Aspenberg, P. (2002) Simvastatin improves fracture healing in mice. *J. Bone Miner. Res.*, **17**, 2004–2008.

187. Skoglund, B. and Aspenberg, P. (2007) Locally applied Simvastatin improves fracture healing in mice. *BMC Musculoskelet. Disord.*, **8**, 98.

188. Garrett, I.R., Gutierrez, G.E., Rossini, G., Nyman, J., McCluskey, B., Flores, A. and Mundy, G.R. (2007) Locally delivered lovastatin nanoparticles enhance fracture healing in rats. *J. Orthop. Res.*, **25**, 1351–1357.

189. Samatar, A.A. and Poulikakos, P.I. (2014) Targeting RAS–ERK signalling in cancer: promises and challenges. *Nat. Rev. Drug Discov.*, **13**, 928–942.

190. El-Hoss, J., Cheng, T., Carpenter, E.C., Sullivan, K., Deo, N., Mikulec, K., Little, D.G. and Schindeler, A. (2014) A Combination of rhBMP-2 (Recombinant Human Bone Morphogenetic Protein-2) and MEK (MAP Kinase/ERK Kinase) Inhibitor PD0325901 Increases Bone Formation in a Murine Model of Neurofibromatosis Type I Pseudarthrosis. *J. Bone Joint Surg. Am.*, **96**, e117 (1–11).

191. Yasoda, A., Komatsu, Y., Chusho, H., Miyazawa, T., Ozasa, A., Miura, M., Kurihara, T., Rogi, T., Tanaka, S., Suda, M., *et al.* (2004) Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. *Nat. Med.*, **10**, 80–86.

192. Chusho, H., Tamura, N., Ogawa, Y., Yasoda, A., Suda, M., Miyazawa, T., Nakamura, K., Nakao, K., Kurihara, T., Komatsu, Y., *et al.* (2001) Dwarfism and early death in mice lacking C-type natriuretic peptide. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 4016–4021.

193. Moncla, A., Missirian, C., Cacciagli, P., Balzamo, E., Legeai-Mallet, L., Jouve, J.L., Chabrol, B., Le Merrer, M., Plessis, G., Villard, L., *et al.* (2007) A cluster of translocation breakpoints in 2q37 is associated with overexpression of NPPC in patients with a similar overgrowth phenotype. *Hum. Mutat.*, **28**, 1183–1188.

194. Bocciardi, R., Giorda, R., Buttgereit, J., Gimelli, S., Divizia, M.T., Beri, S., Garofalo, S., Tavella, S., Lerone, M., Zuffardi, O., *et al.* (2007) Overexpression of the C-type natriuretic peptide (CNP) is associated with overgrowth and bone anomalies in an individual with balanced t(2;7) translocation. *Hum. Mutat.*, **28**, 724–731.

195. Bartels, C.F., Bükülmez, H., Padayatti, P., Rhee, D.K., van Ravenswaaij-Arts, C., Pauli, R.M., Mundlos, S., Chitayat, D., Shih, L.Y., Al-Gazali, L.I., *et al.* (2004) Mutations in the Transmembrane Natriuretic Peptide Receptor NPR-B Impair Skeletal Growth and Cause Acromesomelic Dysplasia, Type Maroteaux. *Am. J. Hum. Genet.*, **75**, 27–34.

196. Tsuji, T. and Kunieda, T. (2005) A Loss-of-Function Mutation in Natriuretic Peptide Receptor 2 (Npr2) Gene Is Responsible for Disproportionate Dwarfism in cn/cn Mouse. *J. Biol. Chem.*, **280**, 14288–14292.

197. Krejci, P., Masri, B., Fontaine, V., Mekikian, P.B., Weis, M., Prats, H. and Wilcox, W.R. (2005) Interaction of fibroblast growth factor and C-natriuretic peptide signaling in regulation of chondrocyte proliferation and extracellular matrix homeostasis. *J. Cell Sci.*, **118**, 5089–5100.

198. Suga, S., Nakao, K., Hosoda, K., Mukoyama, M., Ogawa, Y., Shirakami, G., Arai, H., Saito, Y., Kambayashi, Y. and Inouye, K. (1992) Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. *Endocrinology*, **130**, 229–239.

199. Hagiwara, H., Sakaguchi, H., Itakura, M., Yoshimoto, T., Furuya, M., Tanaka, S. and Hirose, S. (1994) Autocrine regulation of rat chondrocyte proliferation by natriuretic peptide C and its receptor, natriuretic peptide receptor-B. *J. Biol. Chem.*, **269**, 10729–10733.

200. Lorget, F., Kaci, N., Peng, J., Benoist-Lasselin, C., Mugniery, E., Oppeneer, T., Wendt, D.J., Bell, S.M., Bullens, S., Bunting, S., *et al.* (2012) Evaluation of the therapeutic potential of a CNP analog in a Fgfr3 mouse model recapitulating achondroplasia. *Am. J. Hum. Genet.*, **91**, 1108–1114.

201. Nagase, M., Katafuchi, T., Hirose, S. and Fujita, T. (1997) Tissue distribution and localization of natriuretic peptide receptor subtypes in stroke-prone spontaneously hypertensive rats. *J. Hypertens.*, **15**, 1235–1243.

202. Mackie, E.J., Tatarczuch, L. and Mirams, M. (2011) The skeleton: a multi-functional complex organ: the growth plate chondrocyte and endochondral ossification. *J. Endocrinol.*, **211**, 109–121.

203. Farnum, C.E., Lee, R., O'Hara, K. and Urban, J.P.G. (2002) Volume increase in growth plate chondrocytes during hypertrophy: the contribution of organic osmolytes. *Bone*, **30**, 574–581.

204. Muenke, M., Schell, U., Hehr, A., Robin, N.H., Losken, H.W., Schinzel, A., Pulleyn, L.J., Rutland, P., Reardon, W. and Malcolm, S. (1994) A common mutation in the fibroblast growth factor receptor 1 gene in Pfeiffer syndrome. *Nat. Genet.*, **8**, 269–274.

205. Oldridge, M., Wilkie, A.O., Slaney, S.F., Poole, M.D., Pulleyn, L.J., Rutland, P., Hockley, A.D., Wake, M.J., Goldin, J.H. and Winter, R.M. (1995) Mutations in the third immunoglobulin domain of the fibroblast growth factor receptor-2 gene in Crouzon syndrome. *Hum. Mol. Gen.*, **4**, 1077–1082.

206. Wilkie, A.O., Slaney, S.F., Oldridge, M., Poole, M.D., Ashworth, G.J., Hockley, A.D., Hayward, R.D., David, D.J., Pulleyn, L.J. and Rutland, P. (1995) Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. *Nat. Genet.*, **9**, 165–172.

207. Bellus, G.A., Hefferon, T.W., Ortiz de Luna, R.I., Hecht,J.T., Horton, W.A., Machado, M., Kaitila, I., McIntosh, I. and Francomano, C.A. (1995) Achondroplasia is defined by recurrent G380R mutations of FGFR3. *Am. J. Hum. Genet.*, **56**, 368–373.

208. Wang, Y., Spatz, M.K., Kannan, K., Hayk, H., Avivi, A., Gorivodsky, M., Pines, M., Yayon, A., Lonai, P. and Givol, D. (1999) A mouse model for achondroplasia produced by targeting fibroblast growth factor receptor 3. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 4455–4460.

209. Segev, O., Chumakov, I., Nevo, Z., Givol, D., Madar-Shapiro, L., Sheinin, Y., Weinreb, M. and Yayon, A. (2000) Restrained chondrocyte proliferation and maturation with abnormal growth plate vascularization and ossification in human FGFR-3(G380R) transgenic mice. *Hum. Mol. Gen.*, **9**, 249–258.

210. Gartland, A., Mechler, J., Mason-Savas, A., MacKay, C.A., Mailhot, G., Marks, S.C. and Odgren, P.R. (2005) In vitro chondrocyte differentiation using costochondral chondrocytes as a source of primary rat chondrocyte cultures: an improved isolation and cryopreservation method. *Bone*, **37**, 530–544.

211. Ahrens, P.B., Solursh, M. and Reiter, R.S. (1977) Stage-related capacity for limb chondrogenesis in cell culture. *Dev. Biol.*, **60**, 69–82.

212. Böyum, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand. J. Clin. Lab. Invest. Suppl.*, **97**, 77–89.

213. Masuyama, R., Stockmans, I., Torrekens,S., Van Looveren, R., Maes, C., Carmeliet, P., Bouillon, R. and Carmeliet, G. (2006) Vitamin D receptor in chondrocytes promotes osteoclastogenesis and regulates FGF23 production in osteoblasts. *J. Clin. Invest.*, **116**, 3150–3159.

214. Du, X., Weng, T., Sun, Q., Su, N., Chen, Z., Qi, H., Jin, M., Yin, L., He, Q. and Chen, L. (2010) Dynamic morphological changes in the skulls of mice mimicking human Apert syndrome resulting from gain-of-function mutation of FGFR2 (P253R). *J. Anat.*, **217**, 97–105.

215. Lu, X., Su, N., Yang, J., Huang, W., Li, C., Zhao, L., He, Q., Du, X., Shen, Y., Chen, B., *et al.* (2009) Fibroblast growth factor receptor 1 regulates the differentiation and activation of osteoclasts through Erk1/2 pathway. *Biochem. Biophys. Res. Commun.*, **390**, 494–499.

216. Mangues, R., Corral, T., Lu, S., Symmans, W.F., Liu, L. and Pellicer, A. (1998) NF1 inactivation cooperates with N-ras in in vivo lymphogenesis activating Erk by a mechanism independent of its Ras-GTPase accelerating activity. *Oncogene*, **17**, 1705–1716.

217. Lau, N., Feldkamp, M.M., Roncari, L., Loehr, A.H., Shannon, P., Gutmann, D.H. and Guha, A. (2000) Loss of neurofibromin is associated with activation of RAS/MAPK and PI3-K/AKT signaling in a neurofibromatosis 1 astrocytoma. *J. Neuropathol. Exp. Neurol.*, **59**, 759–767.

218. Harrisingh, M.C. and Lloyd, A.C. (2004) Ras/Raf/ERK signalling and NF1: Implications for Neurofibroma Formation. *Cell Cycle*, **3**, 1255–1258.

219. Wang, Q., Green, R.P., Zhao, G. and Ornitz, D.M. (2001) Differential regulation of endochondral bone growth and joint development by FGFR1 and FGFR3 tyrosine kinase domains. *Development*, **128**, 3867–3876.

220. Sabbagh, Y., Carpenter, T.O. and Demay, M.B. (2005) Hypophosphatemia leads to rickets by impairing caspase-mediated apoptosis of hypertrophic chondrocytes. *Proc. Natl. Acad. Sci. U.S.A.*, **102**, 9637–9642.

221. Miedlich, S.U., Zalutskaya, A., Zhu, E.D. and Demay, M.B. (2010) Phosphate-induced apoptosis of hypertrophic chondrocytes is associated with a decrease in mitochondrial membrane potential and is dependent upon Erk1/2 phosphorylation. *J. Biol. Chem.*, **285**, 18270–18275.

222. Ballock, R.T. and Reddi, A.H. (1994) Thyroxine is the serum factor that regulates morphogenesis of columnar cartilage from isolated chondrocytes in chemically defined medium. *J. Cell Biol.*, **126**, 1311–1318.

223. Liu, Z., Xu, J., Colvin ,J.S. and Ornitz, D.M. (2002) Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. *Genes Dev.*, **16**, 859–869.

224. Weizmann, S., Tong, A., Reich, A., Genina, O., Yayon, A. and Monsonego-Ornan, E. (2005) FGF upregulates osteopontin in epiphyseal growth plate chondrocytes: implications for endochondral ossification. *Matrix Biol.*, **24**, 520–529.

225. Mugniery, E., Dacquin, R., Marty, C., Benoist-Lasselin, C., de Vernejoul, M.C., Jurdic, P., Munnich, A., Geoffroy, V. and Legeai-Mallet, L. (2012) An activating Fgfr3 mutation affects trabecular bone formation via a paracrine mechanism during growth. *Hum. Mol. Gen.*, **21**, 2503–2513.

226. Stickens, D., Behonick, D.J., Ortega, N., Heyer, B., Hartenstein, B., Yu, Y., Fosang, A.J., Schorpp-Kistner, M., Angel, P. and Werb, Z. (2004) Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. *Development*, **131**, 5883–5895.

227. Nishida, T., Kubota, S., Aoyama, E., Janune, D., Maeda, A. and Takigawa, M. (2011) Effect of CCN2 on FGF2-induced proliferation and MMP9 and MMP13 productions by chondrocytes. *Endocrinology*, **152**, 4232–4241.

228. Inada, M., Wang, Y., Byrne, M.H., Rahman, M.U., Miyaura, C., López-Otín, C. and Krane, S.M. (2004) Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 17192–17197.

229. Deckers, M.M.L., Van Beek, E.R., Van Der Pluijm, G., Wetterwald, A., Van Der Wee-Pals, L., Cecchini, M.G., Papapoulos, S.E. and Löwik, C.W.G.M. (2002) Dissociation of angiogenesis and osteoclastogenesis during endochondral bone formation in neonatal mice. *J. Bone Miner. Res.*, **17**, 998–1007.

230. Xiong, J., Onal, M., Jilka, R.L., Weinstein, R.S., Manolagas, S.C. and O'Brien, C.A. (2011) Matrix-embedded cells control osteoclast formation. *Nat. Med.*, **17**, 1235–1241.

231. Nakashima, T., Hayashi, M., Fukunaga, T., Kurata, K., Oh-hora, M., Feng, J.Q., Bonewald, L.F., Kodama, T., Wutz, A., Wagner, E.F., *et al.* (2011) Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat. Med.*, **17**, 1231–1234.

232. Matsushita, T., Chan, Y.Y., Kawanami, A., Balmes, G., Landreth, G.E. and Murakami, S. (2009) Extracellular Signal-Regulated Kinase 1 (ERK1) and ERK2 Play Essential Roles in Osteoblast Differentiation and in Supporting Osteoclastogenesis. *Mol. Cell. Biol.*, **29**, 5843–5857.

233. Ek-Rylander, B., Flores, M., Wendel, M., Heinegård, D. and Andersson, G. (1994) Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion in vitro. *J. Biol. Chem.*, **269**, 14853–14856.

234. Razzouk, S., Brunn, J.C., Qin, C., Tye, C.E., Goldberg, H.A., Butler, W.T. (2001) Osteopontin Posttranslational Modifications, Possibly Phosphorylation, Are Required for In Vitro Bone Resorption but Not Osteoclast Adhesion. *Bone*, **30**, 40-47.

235. Chrisman, T.D. and Garbers, D.L. (1999) Reciprocal antagonism coordinates C-type natriuretic peptide and mitogen-signaling pathways in fibroblasts. *J. Biol. Chem.*, **274**, 4293–4299.

236. Yamashita, Y., Takeshige, K., Inoue, A., Hirose, S., Takamori, A. and Hagiwara, H. (2000) Concentration of mRNA for the natriuretic peptide receptor-C in hypertrophic chondrocytes of the fetal mouse tibia. *J. Biochem.*, **127**, 177–179.

237. L'Hôte, C.G.M. and Knowles, M.A. (2005) Cell responses to FGFR3 signalling: growth, differentiation and apoptosis. *Exp. Cell Res.*, **304**, 417–431.

238. Iwata, T., Li, C.L., Deng, C.X. and Francomano, C.A. (2001) Highly activated Fgfr3 with the K644M mutation causes prolonged survival in severe dwarf mice. *Hum. Mol. Gen.*, **10**, 1255–1264.

239. Coffin, J.D., Florkiewicz, R.Z., Neumann, J., Mort-Hopkins, T., Dorn II, G.W., Lightfoot, P., German, R., Howles, P.N., Kier, A. and O'Toole ,B.A. (1995) Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. *Mol. Biol. Cell*, **6**, 1861–1873.

240. Garofalo, S., Kliger-Spatz, M., Cooke, J.L., Wolstin, O., Lunstrum, G.P., Moshkovitz, S.M., Horton, W.A. and Yayon, A. (1999) Skeletal dysplasia and defective chondrocyte differentiation by targeted overexpression of fibroblast growth factor 9 in transgenic mice. *J. Bone Miner. Res.*, **14**, 1909–1915.

241. St-Jacques, B., Hammerschmidt, M. and McMahon, A.P. (1999) Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev.*, **13**, 2072–2086.

242. Maeda, Y., Nakamura, E., Nguyen, M.-T., Suva, L.J., Swain, F.L., Razzaque, M.S., Mackem, S. and Lanske, B. (2007) Indian Hedgehog produced by postnatal chondrocytes is essential for maintaining a growth plate and trabecular bone. *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 6382–6387.

243. Koyama, E., Leatherman, J.L., Noji, S. and Pacifici, M. (1996) Early chick limb cartilaginous elements possess polarizing activity and express hedgehog-related morphogenetic factors. *Dev. Dyn.*, **207**, 344–354.

244. Wuelling, M. and Vortkamp, A. (2010) Transcriptional networks controlling chondrocyte proliferation and differentiation during endochondral ossification. *Pediatr. Nephrol.*, **25**, 625–631.

245. Beier, F., Ali, Z., Mok, D., Taylor, A.C., Leask, T., Albanese, C., Pestell, R.G. and LuValle, P. (2001) TGFbeta and PTHrP control chondrocyte proliferation by activating cyclin D1 expression. *Mol. Biol. Cell*, **12**, 3852–3863.

246. Dailey, L., Laplantine, E., Priore, R. and Basilico, C. (2003) A network of transcriptional and signaling events is activated by FGF to induce chondrocyte growth arrest and differentiation. *J. Cell Biol.*, **161**, 1053–1066.

247. Razzaque, M.S., Soegiarto, D.W., Chang, D., Long, F. and Lanske, B. (2005) Conditional deletion of Indian hedgehog from collagen type 2alpha1-expressing cells results in abnormal endochondral bone formation. *J. Pathol.*, **207**, 453–461.

248. Orr-Urtreger, A., Givol, D., Yayon, A., Yarden, Y. and Lonai, P. (1991) Developmental expression of two murine fibroblast growth factor receptors, flg and bek. *Development*, **113**, 1419–1434.

249. Su, N., Sun, Q., Li, C., Lu, X., Qi, H., Chen, S., Yang, ., Du, X., Zhao, L., He, Q., *et al.* (2010) Gain-of-function mutation in FGFR3 in mice leads to decreased bone mass by affecting both osteoblastogenesis and osteoclastogenesis. *Hum. Mol. Gen.*, **19**, 1199–1210.

250. Contractor, T., Babiarz, B., Kowalski, A.J., Rittling, S.R., Sørensen, E.S. and Denhardt, D.T. (2005) Osteoclasts resorb protein-free mineral (Osteologic discs) efficiently in the absence of osteopontin. *In Vivo*, **19**, 335–341.

251. Rittling, S.R., Matsumoto, H.N., McKee, M.D., Nanci, A., An, X.R., Novick, K.E., Kowalski, A.J., Noda, M. and Denhardt, D.T. (1998) Mice lacking osteopontin show normal development and bone structure but display altered osteoclast formation in vitro. *J. Bone Miner. Res.*, **13**, 1101–1111.

252. Chellaiah, M.A., Kizer, N., Biswas, R., Alvarez, U., Strauss-Schoenberger, J., Rifas, L., Rittling, S.R., Denhardt, D.T. and Hruska, K.A. (2003) Osteopontin deficiency produces osteoclast dysfunction due to reduced CD44 surface expression. *Mol. Biol. Cell*, **14**, 173–189.

253. Chellaiah, M.A. and Hruska, K.A. (2003) The integrin alpha(v)beta(3) and CD44 regulate the actions of osteopontin on osteoclast motility. *Calcif. Tissue Int.*, **72**, 197–205.

254. Chung, U.I., Schipani, E., McMahon, A.P. and Kronenberg, H.M. (2001) Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development. *J. Clin. Invest.*, **107**, 295–304.

255. Lanske, B., Amling, M., Neff, L., Guiducci, J., Baron, R. and Kronenberg, H.M. (1999) Ablation of the PTHrP gene or the PTH/PTHrP receptor gene leads to distinct abnormalities in bone development. *J. Clin. Invest.*, **104**, 399–407.

256. Wang, W., Lian, N., Ma, Y., Li, L., Gallant, R.C., Elefteriou, F. and Yang, X. (2012) Chondrocytic Atf4 regulates osteoblast differentiation and function via Ihh. *Development*, **139**, 601–611.

257. Abou-Khalil, R. and Colnot, C. (2014) Cellular and molecular bases of skeletal regeneration: What can we learn from genetic mouse models? *Bone*, **64**, 211–221.

258. Farrow, E.G., Davis, S.I., Mooney, S.D., Beighton, P., Mascarenhas, L., Gutierrez, Y.R., Pitukcheewanont, P. and White, K.E. (2006) Extended mutational analyses of FGFR1 in osteoglophonic dysplasia. *Am. J. Med. Genet.*, **140A**, 537–539.

259. Gebhard, S., Hattori, T., Bauer, E., Schlund, B., Bösl, M.R., de Crombrugghe, B. and von der Mark, K. (2008) Specific expression of Cre recombinase in hypertrophic cartilage under the control of a BAC-Col10a1 promoter. *Matrix Biol.*, **27**, 693–699.

260. Soriano, P. (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.*, **21**, 70–71.

261. Guagnano, V., Furet, P., Spanka, C., Bordas, V., Le Douget, M., Stamm, C., Brueggen, J., Jensen, M.R., Schnell, C., Schmid, H., *et al.* (2011) Discovery of 3-(2,6-Dichloro-3,5-dimethoxy-phenyl)-1-{6-[4-(4-ethyl-piperazin-1-yl)-phenylamino]-pyrimidin-4-yl}-1-methyl-urea (NVP-BGJ398), A Potent and Selective Inhibitor of the Fibroblast Growth Factor Receptor Family of Receptor Tyrosine Kinase. *J. Med. Chem.*, **54**, 7066–7083.

262. Takeda, S., Bonnamy, J.P., Owen, M.J., Ducy, P. and Karsenty, G. (2001) Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. *Genes Dev.*, **15**, 467–481.

263. Ducy, P., Zhang, R., Geoffroy, V., Ridall, A.L. and Karsenty, G. (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell*, **89**, 747–754.

264. Golovchenko, S., Hattori, T., Hartmann, C., Gebhardt, M., Gebhard, S., Hess, A., Pausch, F., Schlund, B. and von der Mark, K. (2013) Deletion of beta catenin in hypertrophic growth plate chondrocytes impairs trabecular bone formation. *Bone*, **55**, 102–112.

265. Eswarakumar, V.P., Lax, I. and Schlessinger, J. (2005) Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev.*, **16**, 139–149.

266. Lax, I., Wong, A., Lamothe, B., Lee, A., Frost, A., Hawes, J. and Schlessinger, J. (2002) The docking protein FRS2alpha controls a MAP kinase-mediated negative feedback mechanism for signaling by FGF receptors. *Mol. Cell*, **10**, 709–719.

267. Tavormina, P.L., Shiang, R., Thompson, L.M., Zhu, Y.Z., Wilkin, D.J., Lachman, R.S., Wilcox, W.R., Rimoin, D.L., Cohn, D.H. and Wasmuth, J.J. (1995) Thanatophoric dysplasia (types I and II) caused by distinct mutations in fibroblast growth factor receptor 3. *Nat. Genet.*, **9**, 321–328.

268. Provot, S., Nachtrab, G., Paruch, J., Chen, A.P., Silva, A. and Kronenberg, H.M. (2008) A-raf and B-raf are dispensable for normal endochondral bone development, and parathyroid hormone-related peptide suppresses extracellular signal-regulated kinase activation in hypertrophic chondrocytes. *Mol. Cell. Biol.*, **28**, 344–357.

269. Sebastian, A., Matsushita, T., Kawanami, A., Mackem, S., Landreth, G.E. and Murakami, S. (2011) Genetic inactivation of ERK1 and ERK2 in chondrocytes promotes bone growth and enlarges the spinal canal. *J. Orthop. Res.*, **29**, 375–379.

270. Yang, X., Harkins, L.K., Zubanova, O., Harrington, A., Kovalenko, D., Nadeau, R.J., Chen, P.Y., Toher, J.L., Lindner, V., Liaw, L., *et al.* (2008) Overexpression of Spry1 in chondrocytes causes attenuated FGFR ubiquitination and sustained ERK activation resulting in chondrodysplasia. *Dev. Biol.*, **321**, 64–76.

271. Valverde-Franco, G., Liu, H., Davidson, D., Chai, S., Valderrama-Carvajal, H., Goltzman, D., Ornitz, D.M., Henderson, J.E. (2003) Defective bone mineralization and osteopenia in young adult FGFR3-/- mice. *Hum. Mol. Gen.*, **13**, 271–284.

272. Su, N., Sun, Q., Li, C., Lu, X., Qi, H., Chen, S., Yang, J., Du, X., Zhao, L., He, Q., *et al.* (2010) Gain-of-function mutation in FGFR3 in mice leads to decreased bone mass by affecting both osteoblastogenesis and osteoclastogenesis. *Hum. Mol. Gen.*, **19**, 1199–1210.

273. Eswarakumar, V.P. and Schlessinger, J. (2007) Skeletal overgrowth is mediated by deficiency in a specific isoform of fibroblast growth factor receptor 3. *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 3937–3942.

274. Aldegheri, R., Trivella, G., Renzi-Brivio, L., Tessari, G., Agostini, S. and Lavini, F. (1988) Lengthening of the lower limbs in achondroplastic patients. A comparative study of four techniques. *J. Bone Joint Surg. Br.*, **70**, 69–73.

275. Aldegheri, R. (1999) Distraction osteogenesis for lengthening of the tibia in patients who have limb-length discrepancy or short stature. *J. Bone Joint Surg. Am.*, **81**, 624–634.

276. Kojimoto, H., Yasui, N., Goto, T., Matsuda, S. and Shimomura, Y. (1988) Bone lengthening in rabbits by callus distraction. The role of periosteum and endosteum. *J. Bone Joint Surg. Br.*, **70**, 543–549.

277. Yasui, N., Sato, M., Ochi, T., Kimura, T., Kawahata, H., Kitamura, Y. and Nomura, S. (1997) Three modes of ossification during distraction osteogenesis in the rat. *J. Bone Joint Surg. Br.*, **79**, 824–830.

278. Jazrawi, L.M., Majeska, R.J., Klein, M.L., Kagel, E., Stromberg, L. and Einhorn, T.A. (1998) Bone and cartilage formation in an experimental model of distraction osteogenesis. *J. Orthop. Trauma*, **12**, 111–116.

279. Fink, B., Pollnau, C., Vogel, M., Skripitz, R. and Enderle, A. (2003) Histomorphometry of distraction osteogenesis during experimental tibial lengthening. *J. Orthop. Trauma*, **17**, 113–118.

280. Aronson, J., Good, B., Stewart, C., Harrison, B. and Harp, J. (1990) Preliminary studies of mineralization during distraction osteogenesis. *Clin. Orthop. Relat. Res.*, **250**, 43–49.

281. Delloye, C., Delloye, C., Delefortrie, G., Delefortrie, G., Coutelier, L., Coutelier, L., Vincent, A. and Vincent, A. (1990) Bone regenerate formation in cortical bone during distraction lengthening. An experimental study. *Clin. Orthop. Relat. Res.*, **250**, 34–42.

282. Gerber, H.P., Vu, T.H., Ryan, A.M., Kowalski, J., Werb, Z. and Ferrara, N. (1999) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat. Med.*, **5**, 623–628.

283. Maes, C., Carmeliet, P., Moermans, K., Stockmans, I, Smets, N., Collen, D., Bouillon, R. and Carmeliet, G. (2002) Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Mech. Dev.*, **111**, 61–73.

284. Maes, C., Stockmans, I., Moermans, K., Van Looveren, Rno p., Smets, N., Carmeliet, P., Bouillon,R. and Carmeliet,G. (2004) Soluble VEGF isoforms are essential for establishingepiphyseal vascularization and regulating chondrocyte development and survival. *J. Clin. Invest.*, **113**, 188–199.

285. Kawanami, A., Matsushita, T., Chan, Y.Y. and Murakami, S. (2009) Mice expressing GFP and CreER in osteochondro progenitor cells in the periosteum. *Biochem. Biophys. Res. Commun.*, **386**, 477–482.

286. Logan, M., Martin, J.F., Nagy, A., Lobe, C., Olson, E.N. and Tabin, C.J. (2002) Expression of Cre recombinase in the developing mouse limb bud driven by a Prxl enhancer. *genesis*, **33**, 77–80.